Agricultural Biotechnology Research Results 2007-2019

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Ethiopian Institute of Agricultural Research

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Foreword

Agriculture plays a significant role in the Ethiopian economy; employing the majority of the workforce, supplying food for the ever-increasing human population and feed for animals and contributing for a significant portion of the Gross Domestic Product. The country is endowed with huge genetic resources of crops, livestock, microbial and other forms of fauna and flora that would serve as building blocks for propelling genetic improvement and advancement of the agriculture sector in general. Despite this enormous potential coupled with long tradition of crop production and animal husbandry and bounty of ecological diversity; Ethiopia is still grappling with the challenges of alleviating vulnerability of the agricultural, agro-pastoral and pastoral communities that represent the great majority of the population, to food and nutrition insecurity and degradation of natural resources. Ethiopian agriculture is epitomized as a predominantly low-input low-output system dominated by smallholder producers generating agricultural products that are far less surplus, competitive, diverse and sustainable than would be required for the sector and the country thereof to appease relentlessly spiking food and feed gaps and also to thrive in the domestic as well as world markets. Contrary to this, Ethiopia is now on the verge of completing the second phase of its five-year growth and transformation plan (GTP-II), which was launched in 2015/16. The core determination of the plan is to perpetuate the growth and development trajectory attained during the previous phase and thereby ensuring food security and sovereignty, sustainable supply of raw materials for agro-industries and import substitution, expanding the base for foreign earnings from agricultural exports, and increasing livelihood resilience and environmental sustainability.

One of the strategic directions envisaged for attaining the above overarching objectives is through generating or adopting modern research techniques that would enable solving agricultural challengs. The Ethiopian Institute of Agricultural Research (EIAR), guided by its vision and mission, is striving to contribute to the fulfillment of these fundamental national objectives by undertaking research and generating appropriate technologies, information, and knowledge in different research sectors. The agriculture sector is being challenged with various emerging problems. Some of these are associated with climate change and probably exchange of germplasms. Some of agricultural challenges have been addressed through biotechnological approaches mainly genetic engineering and recombinant rDNA technologies.

Inlined with this, there is agricultural research sector undertaking various research in plant, animal and microbial biotechnologies that address agricultural challenges. It is relatively recent or young sector structured in the aforementioned three research programs. However, there has been more than a decade since plant tissue culture studies has been started. There are many published and completed activities in all the programs so far. However, these research results have never been compiled and made easily available in EIAR. Hence, this is to present the research outputs and information from the research sector during the GTP II period.

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Optimization of In Vitro Protocol for Mass Propagation of V6-2-888 Sugarcane (*Saccharum Officinarum* L.) Variety Using Shoot-Tip as Explant

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Abstract

An efficient protocol for mass propagation of V6-2-888 sugarcane ***(Saccharum Officinarum L.)*** *variety was optimized under this study. The shoot-tips used as an explant were surface sterilized with 0.3% (w/v) Kocide for an hour, 70% ethanol for 30 seconds and 5% Sodium hypochlorite solution for 25 minutes. Then sterilized shoot-tips were cultured on MS medium supplemented with 2mg/l BAP and 0.5mg/l IBA, 3% sucrose and 7g/l agar-agar powder. 0.5mg/l BAP and 3 mg/l Kinetin with average shoot number of 12.04 were found best for multiple shoots formation. Best root induction was observed at 2 mg/l IBA and 1mg/l NAA with 13.44 roots per shoot. All experiments were laid in Completely Randomized Design (CRD) with five replications per treatment and five explants per jar under each replicate. Data collected from each experiment were analysed using the SAS statistical software and exposed to ANOVA in conjunction with multiple comparisons (Tukey's post hoc test). In vitro grown plantlets were then transferred to greenhouse conditions where they survived successfully. High survival rate (94.55%) was obtained in a mixture of garden soil, manure, and sand at 3:1:1after four week of transplantation in glass house.*

Introduction

Sugarcane (*Saccharum officinarum L.*) belongs to the genus *Saccharum* of the tribe Andropogonae, family *Poaceae*. It is a tall-growing monocotyledonous perennial grass, which is cultivated in the tropical and subtropical regions of the world. The origin of *S. officinarum* is intimately associated with the activities of humans, as *S. officinarum* is a purely cultivated or garden species, which is not found in the wild. Sugarcane *(Saccharumoffficinarum L.)* is one of the important industrial cash crops. It is economically important that accounts for 60% sugar production in the world. Sugarcane is an important industrial raw material for sugar industries and allied industries producing alcohol, acetic acid, butanol, paper, plywood, industrial enzymes, and animal feed. Another important use of sugarcane is the production of press mud, which is used as a source of organic matter and nutrients for crop production.

Recently, increasing use of sugar and its products have created a challenging situation for sugarcane researcher and growers. Conventionally, sugarcane is propagated by three budded setts. Lack of rapid multiplication and continuous contaminations by systemic diseases are the serious problems to multiply an elite genotype in the open field (Lal and Singh, 1994).

Micro-propagation is currently the only realistic means of achieving rapid, large-scale production of disease-free seed canes in order to speed up the breeding and commercialization process in sugarcane. Successful In vitro culture of sugarcane depends mainly on the formulation of appropriate nutrient medium, including its chemical composition and physical form,plant growth regulators and culture environment(Sharma, Chaudhary *et al.* 2018). Media composition is the most important governing factor of growth and morphogenesis of plant tissue culture. The most essential substances in the manipulation of explant growth and development *in vitro* are growth regulators. The concentration and ratio of growth regulator determine the pattern of development in culture. The most commonly used growth regulator for sugarcaneshoot-tip cultures are IAA, NAA, 2,4-D, IBA, zeatin, kinetin, BA, 2ip, and TDZ (thidiazuron). Of these, BAP is found to have great relevance in sugarcane micro propagation. Shoot-tip culture has been reported for sugarcane propagation by other researchers (Ali, Naz *et* al. 2008, Behera, Sahoo *et al*. 2009, Tolera, Diro *et al.* 2014, Tesfa, Admassu *et al*. 2016, Sharma, Chaudhary *et al.* 2018).

Success of sugarcane micro propagation, depending on genotypes, culture environment, culture medium, explant source, hormonal, plant growth and development stage (A 2015, Lal and Saxena 2018, Sharma, Chaudhary *et al.* 2018). Therefore, further studies are needed in the wide range of genotypes to develop optimum *in vitro* techniques for rapid propagation of sugarcane. Thus, the major objective of this experiment is to optimize in vitro protocols for rapid propagation of V6-2-888 sugarcane variety using shoot-tips as explants.

Materials and Methods

Preparation of mother plants

V6-2-888 cultivar selected by sugarcane research directorate, Ethiopia Sugar Corporation (ESC) was used in this experiment. Stem cuttings (setts) with 2-3 bud eyes prepared from long cane from field and treated with a fungicide solution were planted in a pot containing sterilized substrate media and kept under greenhouse conditions at Debre zeit Agricultural Research Centers. After ninety days, shoots regenerated from planted cuttings were used as mother plant for explants source to carry out laboratory experiments figure 1A.

Surface sterilization and explant excision

The excised shoot-tips were collected from three to four months old actively growing sugarcane shoot raised in the green house. The surrounding leaf sheaths were carefully removed one by one until the inner white sheaths exposed (Figure 1A) the cuttings were further trimmed at the two ends locating the growing point somewhere in the middle of the top (Figure1 B). Initially, these pieces were washed thoroughly under tap water using a local liquid detergent solution. The cuttings were soaked with 0.3% (w/v) Kocide for one hour under laminar flow cabinet (Figure 1C). Then the cuttings were rinsed three times with double distilled water. Then after, it was disinfected with 70% ethanol for 30 seconds and left for five minutes to evaporate the alcohol. Finally, the cuttings were treated with local bleach (with 5% active chlorine content) for 25 minutes and rinsed with sterile distilled water and left up side down for 10 minutes to dry the surface. Leaf sheaths damaged during sterilization were removed using sterile forceps. Finally, 2 cm long shoot-tips were excised and cultured on culture initiation media.

Culture initiation

Sterilized shoot-tips were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 2mg/l BAP and 0.5mg/l IBA, 3% sucrose, PH 5.78 and 7g/ agar. The cultures were kept in a controlled growth rooms with constant temperature of 26 ± 2^{o}C and a photoperiod of 16 h under a 2500 lux light intensity from cool white fluorescent lamps.

Shoot Multiplication

After 4-6 weeks of incubation, initiated cultures were transferred to the next multiplication medium. The multiplication medium was MS (Murashige and Skoog, 1962) supplemented with 28 combinations of BAP and kinetin, 30g/l sucrose, 7g/l agar. The cultures were maintained on the same culture condition as initiation culture.

Rooting

Plantlets were transferred to ½ MS strength medium supplemented with 28 combinations of NAA and IAA and hormone free as a control, 20g/l sucrose, and 7g/l agar-agar powder.

Acclimatization

Plantlets with well-developed root and 3-4 cm height were washed with water to remove the agar and transferred to a pot containing three different ratio of soil, sand and manure (2:1:1, 3:1:1 and 8:2). After planting, the plantlets were covered with polyethylene plastic bag in order to maintain the humidity. After two weeks, the plastic bags were removed.

Data collection and analysis

In this study, data on survival percentage, number of shoots, number of roots, length of roots and plant height were collected. All experiments were laid in Completely Randomized Design (CRD) with five replications per treatment and five explants per

jar under each replicate. Data collected from each experiment were analysed using the SAS statistical software.

Result and Discussion

Explants sterilization and culture initiation

Explant source is important factor which should be taken into consideration during sugarcanemicro propagation(Lal and Saxena 2018). Shoot-tip containing apical meristem of V6-2-888 cultivar was inoculated on a medium containing 2mg/l BAP and 0.5mg/l IBA for shoot-tip initiations and establishment. Biradar *et al.* 2008 reported axillary bud is the most suitable explant for initiation with MS medium containing BAP at 2 mg/l concentrations. Small shoots started emerging within 7-10 days. Initially, there was problem of contamination and browning due to release of phenols and shoot developmental stage (A 2015) which slightly hindered the shoot growth. Sub culturing was done every 10 days during the early stages of establishment to reduce browning of tissues and the release of pigments in the medium (Gosal *et al*. 1998). Keeping the explants under room temperature for three days before culture has minimized the contamination. Most of the cultured explants were responding on the given concentration.

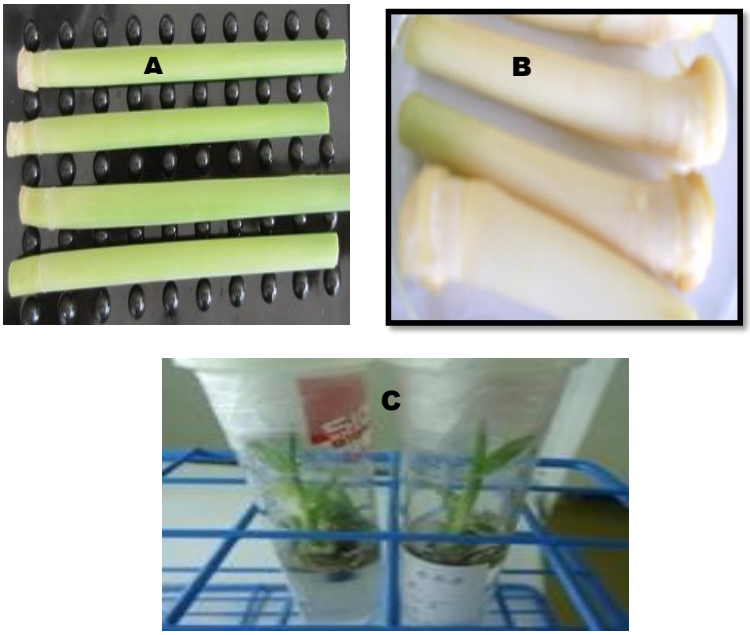


Figure 1.Surface sterilization and regeneration A. Cutting ready for sterilization, B. further trimmed Cutting, C. In vitro shoot induction of V6- 2-888 after four weeks.

Multiplication

The effect of two types of cytokinin BAP and Kinetin on different concentration were examined on shoot multiplication. 0.5mg/L BAP and 3 mg/l Kinetin with average shoot number of 12.04 were found best for multiple shoots formation (figure 2 and table 1). The impact of different hormones combinations on average shoot number and length was also highly significantly ($p<0.05$) different (Table 2). Significant impact of the BAP and Kinetin onshoot number and length has been also observed (Tolera, Diro *et al.* 2014, Tesfa, Admassu *et al.* 2016).

Figure 2. In vitro shoot multiplication of V6-2-888 after four weeks

PGR Conc.		Number of shoots/explant means ± SD	Shoot height/explants (cm) ± SD
Kinetin	BAP	V6-2-888	V6-2-888
0	0	5.00 ± 1.07	9.46 ± 0.76
0	0.5	12.00 ± 1.22	9.10 ± 0.88
0	1	10.80 ± 0.85	7.12 ± 0.76
0	1.5	10.40 ± 0.87	8.37 ± 0.37
0	2	11.92 ± 1.12	7.74 ± 0.63
0	2.5	8.68 ± 0.83	7.30 ± 0.83
0	3	3.92 ± 0.61	8.94 ± 0.63
0.1	0	4.44 ± 0.92	10.84 ± 0.47
0.1	0.5	9.12 ± 0.84	10.10 ± 0.14
0.1	1	10.24 ± 0.71	9.26 ± 0.84
0.1	1.5	10.16 ± 0.92	7.74 ± 0.40
0.1	2	7.80 ± 1.08	7.77 ± 0.28
0.1	2.5	6.12 ± 0.64	7.15 ± 0.62
0.1	3	7.36 ± 1.26	8.72 ± 0.51
0.5	0	6.52 ± 0.99	8.72 ± 0.51
0.5	0.5	8.48 ± 1.06	6.10 ± 3.45
0.5	1	10.12 ± 1.01	6.78 ± 0.64
0.5	1.5	10.12 ± 0.71	6.69 ± 0.66
0.5	2	10.68 ± 0.92	7.13 ± 0.53
0.5	2.5	9.68 ± 0.73	6.80 ± 0.36
0.5	3	12.04 ± 0.93	4.02 ± 0.59
1	0	6.44 ± 1.40	6.28 ± 0.61
1	0.5	9.20 ± 0.61	8.52 ± 0.80
1	1	9.88 ± 0.62	7.10 ± 0.55
1	1.5	9.60 ± 1.23	7.58 ± 0.21
1	2	9.00 ± 1.53	7.56 ± 0.82
1	2.5	8.40 ± 0.85	7.22 ± 0.51
1	3	8.76 ± 0.82	6.08 ± 0.40
CV		8.29	9.1

Table 1. Effect of different combination and concentration of bap and Kinetin on shoot Multiplication and shoot height.

Table 2. Analyses of variance for the effect of plant growth regulators and varieties on number of shoots and shoots height during multiplication.

Source of variation	DF	Parameter	SS	P-value
Variety	1	Number of shoots	1604.70	<.0001*
PGRs	27		1685.81	<.0001*
Variety × PGRs	6		1257.30	<.0001*
		Shoot height		
Variety	1		119.31	<.0001*
PGRs	27		937.33	<.0001*
Variety × PGRs	6		351.33	<.0001*

Rooting

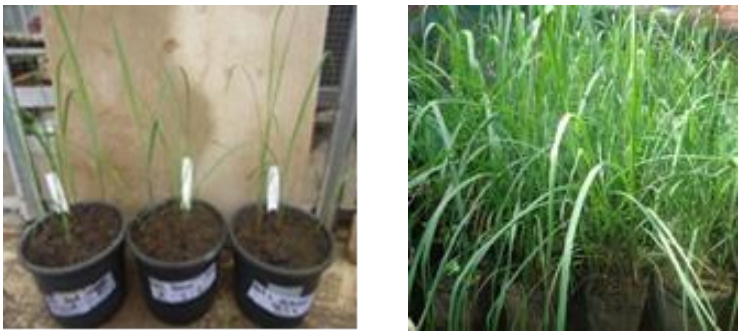
Auxin supplementary of In vitro grown plantlets are desirable for establishment of root system and for adaptation to open environment(Mustafa and Khan 2016). Half strength MS medium supplemented with 23 different auxin concentrations was used. Root formation was observed in all treatments including the control starting from tenth day. Best roots induction was observed at 2 mg/l IBA and 1mg/l NAA with 13.44 roots per shoot. This study is consistent with (Shimelis, Bantte *et al*. 2015). Yi *et al.* (2004) also favored the combination of NAA and IBA for rooting in *Phragmites communis.* Pruski *et al.* (2005) found the combination of IBA and NAA best for rooting.

Table 3. Effect of Iba and Naa on number of root and root length

PGR		Mean number of root	Mean number of root length
IBA	NAA	V6-2-888	V6-2-888
0	0	8.08 ± 0.51	4.24 ±0.2
	1	9.68 ± 0.54	5.85 ± 0.26
	2	9.48 ± 0.67	5.74 ± 0.48
	3	8.76 ± 0.60	4.90 ± 0.49
	4	10.16 ± 0.44	7.02 ± 0.27
	5	9.12 ± 0.64	3.32 ± 0.23
1	0	11.16 ± 0.56	10.54 ± 0.73
	1	11.48 ± 0.76	11.40 ± 0.48
	2	13.56 ±0.36	9.30 ± 0.39
	3	11.92 ± 0.52	6.56 ± 0.33
	4	8.2 ± 0.58	9.30 ± 0.51
	5	14.88 ± 0.33	6.46 ± 0.50
2	0	13.12 ± 0.41	11.44 ± 0.60
	1	13.44 ± 0.41	8.88 ± 0.24
	2	10.36 ± 0.47	4.74 ± 0.46
	3	8.04 ± 0.75	8.12 ± 0.58
	4	11.72 ± 0.59	6.87 ± 0.55
	5	9.52 ± 0.46	6.88 ± 0.28
3	0	8.56 ± 0.64	7.23 ± 0.59
	1	11.44 ± 0.62	5.86 ± 0.17
	2	10.4 ± 0.69	6.14 ± 0.24
	3	9.84 ± 1.04	4.62 ± 0.38
	4	10.92 ± 0.89	3.46 ± 0.43
	5	10.92 ± 0.67	6.76 ± 0.79

Acclimatization

For hardening In vitro raised plants were transferred in to pot containing three different compositions of soil,manure and sand in the ratio of 2:1:1,3:1:1and 8:1 respectively.The plantlets were covered with plastic bags for two weeks to maintain the humidity. High survival rate (94.55) was obtained in a mixture of garden soil, manure, and sandat 3:1:1after four week of transplantation in glass house (Figure 3 and Table 4).



A B

Figure 3. A acclimatized plantlets after four weeks, B. acclimatized plantlets after two months

Table 4. Survival rate of acclimatized plantlets

Treatment	Ratio	Survival rate/trt
Garden soil, Manure and Sand	3:01:01	94.55
Garden soil, Manure and Sand	2:01:01	82.00
Garden soil, Manure	8:02	85.70

Conclusion

To come up with good results an efficient protocol was important. Therefore, mass propagation of V6-2-888 sugarcane variety was optimized. The sterilized shoot-tips were cultured on MS medium supplemented with 2mg/l BAP and 0.5mg/l IBA, 3% sucrose and 7g/l agar-agar powder. 0.5mg/l BAP and 3 mg/l Kinetin with average shoot number of 12.04 were found best for multiple shoots formation. Best root induction was observed at 2 mg/l IBA and 1mg/l NAA with 13.44 roots per shoot. High survival rate (94.55%) was obtained in a mixture of garden soil, manure, and sand at 3:1:1after four week of transplantation in glass house.

Recommendations

For optimum production, from different varieties of sugarcane optimization of the condition is an important concern. The sterilized shoot-tips should culture on MS medium supplemented with 2mg/l BAP and 0.5mg/l IBA, 3% sucrose and 7g/l agar-agar powder. 0.5mg/l BAP and 3 mg/l Kinetin was found to be best for multiple shoots formation.

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In Vitro Regeneration Protocol through Direct and Indirect Organogenesis for Jatropha (*Jatropha curcas*) Accessions in Ethiopia

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Abstract

Jatropha curcas L*. is among the important tree crops in the world. It has a potential for biofuel production. Ethiopia has favorable environment for Jatropha production and there is a soaring investors' interest to produce Jatropha in the country for biodiesel production. However, insufficient good quality of propagation material is a major production constraint. In line with this, a study was undertaken to establish a protocol for in vitro mass propagation of Ethiopian Jatropha using three accessions, viz. Metema, Adami Tulu and Shewa Robit accession through direct and indirect organogenesis. The experiment was laid out in CRD with five replications in factorial arrangement. Nodal and leaf explants were used as explants for direct and indirect organogenesis, respectively. Local bleach (Berekina) at a concentration of 2.5% and 3% for 15min found to be effective for sterilization of leaf and nodal explants, respectively. For direct organogenesis, the highest percentage of shoot induction (86-90%) was achieved when on MS medium with BAP (1.5mg/l) and IBA (0.5 mg/l) for all the three accessions. The maximum (6) number of shoots was obtained for Metema when BAP (0.5mg/l) with Kn (0.5mg/l) was used. Whereas, the maximum (3.2cm) shoot length was recorded for Shewa Robit on media with 0.5mg/l Kn. The highest rooting percentage (84.8-88%) for all accessions and maximum root number (5.43) were recorded on media supplemented with 0.25mg/l IBA. The maximum root length was observed for both Shewa Robit (4.3cm) and Metema (4cm) on media with 0.25mg/l IBA. The medium supplemented with combination of 1mg/l BAP and 1mg/l 2,4-D resulted in maximum percentage of callus (100%) formed for all accessions. The maximum shoot regeneration (66.67%) from callus with 10.13 number of shoot was obtained from Shewa Robit in MS medium fortified with TDZ (0.5 mg/l) and IBA (0.1mg/l). In shoot multiplication, the maximum shoot number (3.5) was obtained from Shewa Robit on media with 0.5mg/l BAP. Whereas the maximum shoot length was recorded (2.1-2.26cm) for all accessions on media supplemented with combination 0.5 of mg/l BAP and 0.5mg/l Kn. However, the elongated shootlet, which transferred into half MS medium containing various concentrations of IBA and NAA, failed to induce root growth. On the other hand, micro shoots regenerated via direct organogenesis were well rooted and successfully established in green house environment with survival rate of 86.67% for Shewa Robit followed by 73.33% and 66.67% for Metema and Adami Tulu, respectively. This study provided optimal protocol for micro-propagation of Jatropha accessions through direct organogenesis to boost its production. In order to see further achievement in vitro propagation of Jatropha, it is imperative to include additional accessions and combinations of PGRs.*

Introduction

Jatropha (*Jatropha curcas L*.) is a succulent shrub or small tree, which belongs to the large Euphorbiaceae family. Among many other attributes and importance of Jatropha, in recent years it has gotten special attention for being a priority feed stock in production of biodiesel (Kumar and Sharma 2008). Biodiesel is an alternative diesel fuel made from different types of renewable sources such as plant oils and animal fats. It is environmentally friendly fuel with low emission profiles and also non-toxic and biodegradable (Abdulla *et al.* 2011). Among the plant species producing raw materials for biofuels, Jatropha is one of the plant species that stimulates the highest interest in tropical and subtropical regions. It has been identified as most suitable oil seed bearing plant due to its various favorable attributes like high oil content, hardy nature, adaptability in a wide range agro-climatic conditions, need for less irrigation and less agricultural inputs, pest resistance, short gestation periods and suitable traits for easy harvesting (Heller, 1996; Edrisi *et al.* 2015).

In Ethiopia, Jatropha grows in various parts of the country such as in Wolayita, Metekel, Southern wollo, Northern, and Eastern Shewa, Gamo Gofa Zones, Tigray and Gambella region and mainly used as living fences and for conserving soil and water (Getinet *et al*. 2009; Zufan, 2010). Now, in connection with green economy goals, the Ethiopian government has begun to promote supply of fuels from locally produced biofuel without affecting food self-sufficiency and by reducing environmental impacts (FDRE, 2007; FDRE, 2011). The strategy intends to make the country carbon neutral by 2030 (FDRE, 2011). To achieve these goals, biofuels that can be produced from non-edible oil like Jatropha, is the best solution.

Based on this strategy several local and foreign private investors have started growing plants for producing biodiesel. Eighty-five companies were registered in Ethiopia to invest in biofuels, mainly Jatropha (Mengistu, 2013). Most of these companies have the intention of going for large-scale commercial development (Abreham and Belay, 2015). However, several challenges remain before that plant biomass can be commercially exploited. Its supply on a large-scale requires massive production of phenotypically uniform plant material of a very high quality within a short time-frame that is adapted to the growth conditions of the plantation areas.

Traditionally, Jatropha is propagated through seed and vegetative cutting. However, these methods can be severely limited by several factors. A major bottleneck for seed propagation is significant variations inseed yield and oil content, low seed viability and germination rate. For vegetative propagation of Jatropha, stem cuttings has been achieved however the established plants are not deep rooted. Hence, they easily are uprooted when cultivated in lands with poor top soil (Openshaw, 2000). The In vitro multiplication would be a useful alternative method for mass production of plant. It offers an opportunity for large-scale production of uniform disease free planting material in a relatively short time and independent of the season (George, 2008).

One of the prospective and potential ways of In vitro plant culture of Jatropha is organogenesis. It refers to the process in which a unipolar structure can be derived either through differentiation of non-meristematic tissues or through pre-existing meristematic tissues (Hussain *et al*. 2012; Moniruzzaman *et al.* 2016). Different types of manipulation can achieve the regulation of organogenesis In vitro. These include appropriate choice of explant. (Sujatha *et al*. 2005; Feyissa *et al*. 2005; Deore and Johnson, 2008).

- Age of the explant;
- Orientation of explant;
- Proper choice of the culture medium;
- Plant growth regulators;
- Genotype;
- Source of carbohydrate;
- Gelling agent and other physical factors including light regime and
- Temperature and humidity.

Several authors have regenerated Jatropha through organogenesis using different explants (Sujatha and Muktra, 1996; Sujatha *et al.* 2005; Rajore and Batra, 2005; Deore and Johnson, 2008; Kumar *et al*. 2011). In Ethiopia, development and application of tissue culture techniques for propagation of Jatropha is at its early stage. Prior to this work, there are no documented studies on micropropagation of Ethiopian Jatropha accessions, which can be used for mass production. Keeping in view of the importance of the crop and its propagation methods, the present study was designed to optimize in vitro protocol for direct and indirect organogenesis of three Ethiopian Jatropha accessions using nodal and leaf explants.

Materials and Methods

Planting material

The seed of three Jatropha accessions were collected from Amhara and Oromia Region of Ethiopia and used for these tissue culture experiments (Table 1).

Table 1. Sources and growing altitudes of planting material of Jatropha accessions were used in this study.

Province (Region)	Place of collection	Altitude(m)	Collectors(Seed Source)
Oromia	Adami Tulu	1500	WGRC
Amhara	Metema	1000	SARC
Amhara	Shewa Robit	1250	SARC

Note: WGRC= Wondo Genet Research Center; SARC= Sirinka Agricultural Research Center

The seeds were germinated on growth trays containing sterilized combination of soil, sand, and manure in the ratio of 2:1:1, respectively and kept in the greenhouse condition of National Agricultural Biotechnology Research Center (NABRC). They were watered thrice a week using a spraying can. After three weeks, the seed that germinated was transplanted into pot containing sterilized soil and kept as mother stock plant. After three months of growth, very young, health and vigorous part of the plant (leaf and nodal) were collected andused as a source of explants. The overall experiment

(June 29 2017 to January 30 2018) was conducted at Plant Biotechnology Laboratory of Holetta Agricultural Research Center (HARC) 45 km West of Addis Ababa, Ethiopia.

Growth regulators stock preparation

The Plant Growth regulators (PGRs) used for the study were; the cytokinin, 6- benzyl amimopurine (BAP), Kinetin (Kn) and Thidiazuron (TDZ) the auxins, 2, 4- acid (2,4-D), indole-3- butyric acid (IBA) and α-naphthalene acetic acid (NAA), and gibberrellin, gibberrellic acid (GA3). All PGRs stock solutions were prepared by weighing and dissolving the powder in distilled water at the ratio of 1 mg/ml. To begin the dissolving process, the powdered crystal of the PGRs was first weighed and dissolved in 3-4 drops of 1N NaOH, 1N HCl and 99% ethanol based on the type of PGR (NaOH for auxins, HCl for cytokinin and ethanol for gibberellin). Then the volume was adjusted by adding distilled water. Finally, growth regulators' stock solutions were stored in a refrigerator at a specific temperature for each growth regulator.

Culture medium preparation

Culture medium was prepared by taking the proper amount of Murashige and Skoog (1962) stock solutions (mg/l). Full strength of MS with 30g/l of sucrose as carbohydrate source (w/v) for callus induction, shoot initiation, and multiplication were used.Whereas half- strength of MS with 15g/l sucrose were used for root induction. The pH of the medium was adjusted to 5.8 (using 1N NaOH and 1N HCl) after addition of the growth regulators. Gerlite (2.5g/l) was added as a gelling agent after the volume and pH of the medium was adjusted. The media was sterilized by autoclaving at a temperature of 121°C with a pressure of 15PSi for 15 min and stored at room temperature.

Explants and surface disinfection

Leaf and nodal (2^{nd} and 3^{rd} from the apex) explants were treated with commercial detergent for 5 minutes and were rinsed well with distilled water for three to five times. Under a clean Laminar flow hood, the explants were subjected to 70% (v/v) ethanol for one minute and rinsed with sterile distilled water three to four times. Further, the explant materials were then surface sterilized in four different concentration of local bleach (Berekina, Ethiopia) (2.0, 2.5, 3.0 and 3.5% active ingredient of chlorine) for 5, 10, 15 and 20min time of exposure. After that, the explants were rinsed with sterilized distilled water for three to four times to remove the residual effect of these sterilants.

To facilitate the reaction two drops of 1mg/1ml of Tween - 20 was added into all the sterilant solutions prior to treatment. After sterilization process was completed, individual leaf explant was trimmed aseptically into approximately $1cm^2$ leaf disc segments and placed with the adaxial side in contact with the MS basal medium. Moreover, the nodal segment was trimmed (l -l.5cm) with one node and inoculated by vertical orientation on the medium. Finally, the effect of both concentration of Berekina and length of time exposure were determined for both explants from the culture on PGR-free MS basal media between two weeks intervals.

Shoot and callus induction

For culture initiation, single nodal explant (1-1.5cm) was inoculated on full strength MS media supplemented with different combinations of BAP (0, 1, 1.5, 2.0 mg/l) and IBA (0, 0.5, 1.0mg/l) along with Ascorbic acid (10mg/l) for prevention of browning of cultures. Four nodal explants per culture jars and five replications for each treatment were used. The cultured explants were incubated in the light condition (1500 - 2000 lux) in growth room at 25 ± 2°C for three weeks. For callus induction, about $1cm^2$ leaf disc explant was transferred to callus induction media consisting of MS basal medium supplemented with various combinations of 2, 4-D (0, 0.5, 1.0, 1.5 mg/l) and BAP (0, 1.0, 1.5, 2.0 mg/l). Five leaf disc explants per jar were used and each treatment was replicated five times. All the cultures were incubated at 25 ± 2°C in darkness to promote callus formation and discourage greening of the callus. The percentage proportion of callus induction on leaf discs was evaluated at an interval of four weeks after inoculation of explant.

Shoot proliferation from callus

Well-established organogenic callus (4-week-old) grown on MS medium supplemented with combination of BAP (1.0 mg/l) and 2,4-D (1.0 mg/l) for all accessions were used. About 0.5gram of calli were transferred to shoot regeneration media, consisting of MS basal medium containing various combinations of cytokinins viz. BAP (0, 0.5, 1.0 mg/l) and TDZ (0, 0.25, 0.5, 1.0 mg/l) individually and in combination with different concentrations of IBA (0, 0.1, 0.2 mg/l). There were three callus clumps per jar and five replicates per treatment. Then the culture was maintained in a growth room at a temperature of 25 ± 2^0C and 16-hour photoperiod provided by white florescent lamps. The cultures were subcultured once transferring into fresh media after three weeks for further initiation of adventitious shoots. During subculture removal of dead, dark brown cells was done. The percentage of shoot organogenesis and number of adventitious shoot initiated from callus was recorded after six weeks of transferring the callus on shoot regeneration media.

Shoot multiplication and elongation

For shoot multiplication, shoots from best establishment (induction) medium were used to avoid the influence of different origin of media. Then highly aseptically initiated shoots were transferred to shoot multiplication MS fresh medium, which supplemented with various combinations of BAP (0, 0.5, 1.0, 1.5 mg/l) and Kn (0, 0.5, 1.0 mg/l). Five shoots per culture jars and five replications for each treatment were used.

The cultures were maintained at 25 ± 2°C with a 16-hour photoperiod at a light intensity of 1500 - 2000 lux from cool white florescent bulbs. After four weeks of culture, the growth response of the micro-shoots to different treatments was recorded. For shoot elongation, the multiplied shoots were transferred onto growth regulators free MS basal medium for two weeks. However, to achieve shoot elongation for shoot derived from callus, the highest multiple shoot clusters were dissected into smaller sizes and transferred onto fresh medium, which contains 0.5mg/l BAP and 1.0mg/l GA3 for two weeks according to the protocol of Jeevan *et al*. (2013). Then the

elongated shoots were transferred to PGRs free medium for two weeks to avoid the carry over effect of multiplication media on In vitro rooting (Tilahun *et al.* 2014).

Root induction

The elongated shoots with three to four leaves were excised and cultured on half strength MS media supplemented with different combinations of IBA (0, 0.25, 0.5 and 1.0 mg/l) and NAA (0, 0.25 and 0.5 mg/l) for root induction. There were five replicates with five shoots cultured for each jars. Then the culture was maintained in a growth room at a temperature of 25±20C and 16-hour photoperiod provided by white florescent lamps. Each root growth parameters were recorded within four weeks of the culture.

Acclimatization

After the well rooted In vitro propagated Jatropha plantlets are obtained, it was taken out gently from the culture vessels and the root system was washed under running tap water to remove traces of agar that prevent the absorption of nutrients from the acclimatization culture substrates by roots. Forty-five well-rooted shootlets (15 shootlets from each Jatropha accession) were transferred to plastic pots containing a mixture of sterilized sand, soil and compost in the ratio of 1:2:1, respectively and transferred to greenhouse for hardening. The potted plants were maintained in a greenhouse at a temperature of 25 ± 2°C with 60-75% relative humidity. The pots were covered with transparent plastic bags with random holes for air circulation and the underside of the pots was drilled for drainage. Then they were watered using sprayer every day. Plastic cover were removed partially after a week and completely removed after two weeks. Finally, after about one month, percent of plantlets successfully hardened were calculated.

Experimental design and statistical analysis

The experiment was laid out in Completely Randomized Design (CRD) for all the treatments. The experiment was comprised of different combination and concentrations of plant growth regulators combined with three accessions of Jatropha and explants taken from different parts of the plant. Growth regulators were one factor and accessions were another factor. Each treatment had five replicates of culture Jars and set as experimental unit. Data collected from each experiment was subjected to statistical analyses using the SAS statistical software (version 9.2) and ANOVA was constructed, followed by mean separation using Fisher's Least Significance Difference (LSD) at α=5%.

Result and Discussion

Surface sterilization of explants

Analysis of variance (ANOVA) revealed that the concentration of Berekina, duration of explants exposure to sterilant and interaction of Berekina with time had highly significant effect ($P<0.01$) on the level of contamination and plant tissue death. There was also highly significant effect ($P<0.01$) between the two explants. The highest

percentage of clean and healthy survived explants (86.16%) were recorded when the leaf explants were treated with 2.5% of Berekina for 15 minutes. In the case of nodal explants, the highest percentage of clean explants (80%) was observed when the explant treated with 3.0% of Berekina for 15 minutes. Whereas the lowest percentage of surviving of explants were recorded for leaf explants (18.16%) at 20 minutes exposure and 3.5% of Berekina and (19.89%) were obtained from nodal explant when it was surface sterilized with 2% of Berekina for 5 minutes (Table 2). In this study, the lowest concentrations (2%) of Berekina at 5 and 10 minutes resulted in low percentages of survived explants for both leaf and nodal explants. This might be due to the insufficiency of the concentration of Berekina and length of exposure time to kill culture contaminants. Similarly, among the given concentrations of Berekina the highest concentrations (3.5% of Berekina) at longer time (20 minutes) of exposure gave low percentages of survived plantlets for both explants. This might be due to the high bleaching activity of chlorine, which killed the cells. The current result is consistent with the findings of Oyebanji *et al.* (2009) and Tilahun *et al.* (2013) who reported that explants are surface sterilized only by treatment with disinfectant solution.This is done at suitable concentrations for a specified period. In this case, the plausible cause may be because different explants need different level of concentration for sterilization. The finding is in line with a study by Colgecen *et al.* (2011), which found that requirements on the concentration and time of exposure differ from one plant to another and for different parts of plants depending on their morphological characters like softness or hardness of the tissue.

Table 2. Different concentrations of surface sterilant of berekina (with 5% active ingredient of chlorine) and time exposure on survival of aseptic of leaf and nodal explants of Jatropha.

Berekina (%)	Time of exposure (minutes)	Survived aseptic explants (%)	
		Leaf	Nodal
0	0	0.00 ±0.00	0.00 ±0.00
2.0	5	23.89 ±0.67^{qr}	19.89 ±0.48^{rs}
2.0	10	36.66 ±1.66^{mn}	31.66 ±1.66^{op}
2.0	15	52.89 ±1.49^{ij}	42.53 ±1.44^{kl}
2.0	20	38.33 ±1.66^{lm}	41.16 ±1.96^{kl}
2.5	5	34.50 ±2.46^{mno}	25.00 ±0.00^{q}
2.5	10	72.33 ±1.45^{de}	52.66 ±2.33^{ij}
2.5	15	86.16 ±1.16^{a}	73.11 ±0.98^{cde}
2.5	20	50.00 ±0.00^{j}	58.33 ±1.66^{gh}
3.0	5	53.11 ±1.60^{ij}	44.50 ±2.46^{k}
3.0	10	75.00 ±0.00^{cd}	76.66 ±1.66^{bc}
3.0	15	56.83 ±1.58^{hi}	80.00 ±0.00^{b}
3.0	20	29.55 ±2.51^{p}	58.33 ±1.66^{gh}
3.5	5	61.60 ±0.00^{fg}	55.83 ±0.83^{hi}
3.5	10	41.66 ±1.66^{kl}	63.61 ±1.36^{f}
3.5	15	33.33 ±1.66^{nop}	69.11 ±0.89^{e}
3.5	20	18.16 ±1.16^{s}	21.16 ±1.96^{qrs}
CV (%)	5.31		
LSD (5%)	4.25		

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples

Means with different letter in a column and rows are statistically significant at p-values < 0.05. The values are mean ± se (where, n= 3).

Shoot regeneration through direct organogenesis

Effects of BAP and IBA on shoot induction

Analysis of variance showed that, there was highly significant difference ($P<0.01$) among growth regulator concentrations and combinations (BAP and IBA) and their interaction with the three Jatropha accession on percentage of shoot initiation. ANOVA also revealed that there was no significant difference ($P>0.05$) among the Jatropha accessions on percentage of shoot induction.

The highest percentage (90%) shoot induction was recorded for both Shewa Robit and Metema accessions on MS media supplemented with combination of 1mg/l BAP and 0.5 mg/l IBA followed by 86% for Adami Tulu accession on the same hormone combinations and concentrations. Whereas the lowest percentage (22-25%) induction was observed from the media containing combination of 1mg/l of BAP and 1mg/l IBA for all accessions (Table 3). Addition of IBA along with BAP has also been reported to regenerate shoot buds from the nodal explants in Jatropha (Shrivastava and Banerjee, 2008). This is mainly due to the reason that the hormone balance is apparently more important than the absolute concentration of any one hormone since plant hormones do not function in isolation within the plant body but instead, function in relation to each other (Deore and Johnson, 2008). Both cell division and cell expansion occur in actively dividing tissue, therefore, cytokinin and auxin balance plays a role in the overall growth of plant tissue (Jha *et al*. 2007; Shrivastava and Banerjee, 2008; Purkayastha *et al*. 2010).

However, in this study, good results also obtained on MS medium, which contains only BAP (2mg/l). This could be due to the endogenous auxin, which balances with cytokinin releasing the shoot buds from apical dominance. Several studies have reported that BAP is the most suitable cytokinin for shoot induction and multiplication in Jatropha. Maharana *et al.* (2012) has also reported that MS medium fortified with 8.0μ*M* BAP regenerated shoot buds (6.2 shoots) from nodal explants of Jatropha.In contrast, In vitro growing shoots of Jatropha had revealed a tendency of callusing when cultured on a medium fortified with sole IBA, at a concentration of 1mg/l. Hence, we can say that an optimum ratio of cytokinin to auxin is essential for proper shoot bud formation. The study by Xiansong (2010) indicated that the sole effect of BAP, IBA and their interactions had significant effects on plant regeneration and type of regeneration in sweet potato. This is supported by many researches implying that the interaction between auxin and cytokinin is important for the regulation and guiding developmental processes, such as the formation and maintenance of the meristem and formation of callus, which are crucial mechanisms for the establishment explant (Kalimuthu *et al*. 2007).

Table 3. Percentage of shoot initiation from nodal explant cultures of three Jatropha accession (Metema, Adami Tulu and Shewa Robit) at different concentrations and combinations of BAP and IBA after 30 days of culture.

PGRs (mg/l)		Shoot induction (%)		
BAP	IBA	Metema	Adami Tulu	Shewa Robit
0	0	-	-	-
0	0.5	34.80 ±0.20^{op}	35.20 ±0.0.20^{o}	33.00 ±1.22^{p}
0	1.0	-	-	-
1.0	0	70.00 ±0.00^{g}	68.00 ±1.22^{h}	69.80 ±0.20^{gh}
1.0	0.5	90.00 ±0.31^{a}	86.00 ±1.00^{b}	90.00 ±0.00^{a}
1.0	1.0	24.00 ±0.00^{q}	23.14 ±0.00^{q}	25.00 ±0.5^{q}
1.5	0	74.00 ±1.00^{ef}	73.00 ±1.22^{f}	75.00 ±0.00^{e}
1.5	0.5	80.00 ±0.00^{d}	80.00 ±0.00^{d}	82.00 ±1.22^{c}
1.5	1.0	55.00 ±0.00^{k}	52.00 ±1.22^{l}	53.00 ±1.22^{l}
2.0	0	85.00 ±0.00^{b}	85.00 ±0.00^{b}	85.00 ±0.00^{b}
2.0	0.5	63.00 ±1.22^{j}	65.00 ±0.00^{i}	64.00 ±1.00^{ij}
2.0	1.0	41.00 ±1.00^{n}	43.00 ±1.22^{m}	40.00 ±0.00^{n}
CV (%)		2.92		
LSD (5%)		1.86		

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n= number of samples, '-' indicates no response

Efects of BAP and Kinetin on shoot multiplication

ANOVA showed that genotype(*Jatropha accessions*), kinetin(Kn), BAP and their interactions had very high significant ($p<0.01$) effects on average shoots number, average shoot length and average number of leaves per shoot. The maximum number of shoots was recorded for Metema (6 shoots) on MS media supplemented with combination of 0.5mg/l BAP and 0.5 mg/l Kn followed by 5.70 and 5.56 shoots for Shewa Robit and Adami Tulu accessions, respectively, on the same growth regulator combination and concentration as for Metema. Whereas the lowest mean number (1.88-1.96) of shoots of the experiment was recorded for all the three accession shoots on growth regulator free MS media (Table 4). In this study, the optimum combination of BAP and Kn which was 0.5mg/l BAP and 0.5 mg/l Kn showed better response on number of shoot per explants for all accession than the other treatments. This is in line with the earlier studies where in media containing kinetin in conjunction with BAP induced higher frequency of shoot multiplication and greater number of shoots in some perennial plants (Figueiredo *et al*. 2001; Baskaran and Jayabalan, 2005). Weaker effect on axillary shoot regeneration for all the three accessions was observed in hormone free treatments. Thus, results confirmed with Thepsamran *et al.* (2008) who reported that exogenous application of cytokinins has become obligatory for induction of multiple shoot in Jatropha. In case of shoot length, the maximum shoot length was recorded for Shewa Robit (3.2cm) on media supplemented with 0.5 of mg/l Kinetin. Whereas, the lowest mean shoot length (1.8-1.84cm) was recorded for all the three accession shoots developed on media supplemented with 1.5 mg/l BAP (Table 4). On other hand, the highest mean shoot length for Metema and Adami Tulu accession were obtained from both MS medium containing 0.5 mg/l Kn and PGRs free MS medium with no significant difference to the accessions type as well as hormone concentrations from which the shoot buds were raised. This result also confirmed with Jeevan *et al.* (2013) who reported that 1.0 mg/l kinetin gave the highest shoot length than media which containing BAP alone during in vitro culture of Jatropha. Kaminek (1992) also

reported that variation in the activity of different cytokinins can be explained by their different uptake rate in different genomes, translocation rates to meristematic regions and metabolic processes in which cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds.

Table 4. Effect of different concentration and combination of BAP and Kn on number of shoots and shoot length of three Jatropha accessions.

Jatropha Accessions	Cytokinin (mg/l)		Number of shoot per explant	Length of shoot(cm)
Metema	BAP	Kn		
	0	0	1.96 ±0.04^{r}	3.00±0.00 ^{b}
	0	0.5	2.10 ±0.03^{pqr}	3.04 ±0.02^{b}
	0	1.0	3.00 ±0.00^{lm}	2.90 ±0.00^{c}
	0.5	0	4.60 ±0.10^{de}	2.48 ±0.02^{i}
	0.5	0.5	6.00 ±0.31^{a}	2.80 ±0.03^{e}
	0.5	1.0	5.50 ±0.16^{b}	2.60 ±0.00^{f}
	1.0	0	4.10 ±0.10^{fg}	2.18 ±0.02^{kl}
	1.0	0.5	3.72 ±0.09^{hi}	2.34 ±0.02^{j}
	1.0	1.0	3.40 ±0.03^{jk}	2.06 ±0.02^{mn}
	1.5	0	3.50 ±0.00^{ijk}	1.80 ±0.00^{q}
	1.5	0.5	2.78 ±0.02^{mn}	2.10 ±0.03^{m}
	1.5	1.0	2.40 ±0.00^{op}	1.92 ±0.05^{p}
Adami Tulu	0	0	1.88 ±0.05^{r}	3.00 ±0.00^{b}
	0	0.5	2.06 ±0.06^{qr}	3.02 ±0.02^{b}
	0	1.0	2.84 ±0.10^{m}	2.87 ±0.02^{cd}
	0.5	0	4.00 ±0.00^{fgh}	2.50 ±0.00^{hi}
	0.5	0.5	5.56 ±0.23^{b}	2.82 ±0.02^{de}
	0.5	1.0	4.70 ±0.20^{cd}	2.56 ±0.02^{fgh}
	1.0	0	3.80 ±0.12^{ghi}	2.20 ±0.03^{k}
	1.0	0.5	3.68 ±0.07^{ij}	2.34 ±0.02^{j}
	1.0	1.0	3.33 ±0.00^{k}	2.10 ±0.00^{m}
	1.5	0	3.30 ±0.12^{kl}	1.80 ±0.00^{q}
	1.5	0.5	2.80 ±0.00^{mn}	2.06 ±0.02^{mn}
	1.5	1.0	2.34 ±0.04^{opq}	1.96 ±0.04^{op}
Shewa Robit	0	0	1.92 ±0.05^{r}	3.00 ±0.00^{b}
	0	0.5	2.02 ±0.08r	3.20 ±0.00a
	0	1.0	2.96 ±0.04^{m}	2.92 ±0.02^{c}
	0.5	0	4.30 ±0.12^{ef}	2.52 ±0.02^{ghi}
	0.5	0.5	5.70 ±0.12^{ab}	2.80 ±0.00^{e}
	0.5	1.0	5.00 ±0.00^{c}	2.58 ±0.02^{fg}
	1.0	0	4.00 ±0.16^{fgh}	2.22 ±0.02^{k}
	1.0	0.5	3.80 ±0.00^{ghi}	2.36 ±0.04^{j}
	1.0	1.0	3.53 ±0.12^{ijk}	2.12 ±0.02^{lm}
	1.5	0	3.70 ±0.12^{hij}	1.84 ±0.04^{q}
	1.5	0.5	2.84 ±0.04^{m}	2.10 ±0.00 ^{m}
	1.5	1.0	2.50 ±0.16^{no}	2.00 ±0.00^{no}
CV (%)			6.98	2.03
LSD (5%)			0.30	0.06

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).

The maximum mean number of leaf (7 per shoot) was recorded on MS media supplemented with 0.5mg/l of BAP followed by (6.43 per shoot) on media supplemented with combination of 0.5mg/l of BAP and 0.5mg/l of Kn. Whereas the lowest mean number of leaf (2.97 per shoot) was recorded for shoots induced on MS media supplemented with 1.5mg/l of BAP (Table 5). In this study, a significant influence of the type of cytokinins and their concentrations on the number of leaves per shoot was observed. As the concentration of Kn increased the number of leaf per shoot also increased. However, the cultures growing on the media containing higher BAP produced the smallest number of leaves. Kozak and Salata (2011) reported that the lower concentration of BAP (0.5mg/l) produced maximum number of leaves (16.8 leaves) than other concentration of Kinetin, 2iP and TDZ in *Rheum rhaponticum*. Similarly, Behera *et al.* (2014) obtained highest number of leaf per shoot (7.1) on MS medium supplemented with 1mg/l BAP and 1mg/l IAA in Jatropha. More recently, Rady *et al.* (2016) also reported that MS supplemented with combination of 0.5 mg/l BA and 0.05 mg/l IBA gave the highest number (14.67) of leaves per proliferated shoot bud of Jatropha after one month of cultivation in growth room.

Table 5. Main effect of the different concentrations and combinations of cytokinins (BAP and Kn) on number of leaves per shoot of Jatropha accessions.

PGR concentration (mg/l)		
BAP	Kinetin(Kn)	Number of leaf per shoot
0	0	3.43±0.08^{hi}
0	0.5	4.00±0.05^{g}
0	1.0	4.50±0.09^{f}
0.5	0	7.00±0.09^{a}
0.5	0.5	6.43±0.09^{b}
0.5	1.0	5.00±0.05^{e}
1.0	0	5.53±0.10^{d}
1.0	0.5	6.00±0.17^{c}
1.0	1.0	4.13±0.06^{g}
1.5	0	2.97±0.03^{j}
1.5	0.5	3.67±0.11^{h}
1.5	1.0	3.30±0.05^{i}
LSD (5%)		0.27
CV (%)		7.93

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples
Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=15).

Effects of IBA and NAA on rooting induction

ANOVA showed very highly significant ($p<0.01$) effectof all main and interaction effect of accession, IBA andNAA on rooting percentage and root length of In vitro root induction of the three Jatropha accessions. The result showed also that number of roots induced per shoot was highly significantly ($P<0.01$) affected due to the main effect of PGRs concentrations and combinations. However, the effect of accessions and interaction of the two factors affected the number of roots non-significantly ($P>0.05$).

The highest rooting percentage (88%) was recorded for Shewa Robit accession on media supplemented with 0.25mg/l IBA followed by 86% and 84.8% for Metema and Adami Tulu accessions, respectively, on the same IBA concentration as for Shewa Robit. Whereas the lowest rooting percentage (38.6-40.2%) were recorded for all accession shoots induced on media supplemented with combination of 1mg/l IBA and 0.5mg/l NAA (Table 7). On the other hand, there is no root formed on the media supplemented with NAA alone. On this medium, NAA often induces the formation of callus at the base of the shoot and rooting response was almost negligible (Figure 1f). Shrivastava and Banerjee (2008) and Maharana *et al*. (2012) obtained similar results to the present study. These authors observed that well-developed shoots of Jatropha when transferred to half MS medium fortified with NAA intermittent callus formation takes place and no roots were observed. The reason for low performance of NAA treatments may be due to the reason that NAA is more persistent than IBA, remains present in the tissue and may block further development of root meristemoids (Nanda *et al*. 2004). Studies also showed that, NAA was more consistent in stimulating cell divisions, which favors callus formation (Kim *et al*.2003). The promotory effect of IBA on in vitro rooting of shoots has also reported in Jatropha (Rajore and Batra, 2005; Kochhar *et al*. 2005) and they concluded that IBA alone was effective in the rooting of Jatropha.

The maximum root number (5.43) was recorded on MS media supplemented with 0.25mg/l of IBA followed by (4.8) on media supplemented with combination of 0.5mg/l of IBA and 0.25mg/l of NAA (Table 6). Whereas the lowest mean number (1.8) of roots per shoot was recorded for roots induced on PGR free MS media.In contrast, there is no root formed on the media supplemented with NAA alone due to callus formation at the base of shoot. In this study, the root number decreased with higher concentration of IBA. These results are in line with Datta *et al*. (2007), who reported 0.2mg/l IBA for best rooting in Jatropha. However, there are researchers who recommended 3mg/l IBA addition to half MS medium for best rooting in Jatropha (Shrevastava and Banerjee, 2008). This deviation is owed the variation to the underlining genetic differences of the genotypes in response to rooting media composition and affecting rooting and other associated developments.

Besides, the decline in root number beyond the optimum level in this study might be due to the toxic effect of IBA beyond certain level, which affects root growth and development. This observation is in agreement with the report of Thomas (2007) in *Curculigoorchioides* where a higher level of IBA produced a negative effect resulting in lower root number. Moreover, high levels of IBA can result in ethylene accumulation in the tissue culture vessel, which also inhibits the induction of root primordia (De Klerk, 2002; Hartman *et al*. 2009).

The maximum mean root length was obtained for Shewa Robit (4.3cm) on the media supplemented with 0.25mg/l IBA followed by (4cm) and (3.8cm) for Metema and Adami Tulu accession, respectively on the same hormone concentration as for Shewa Robit. Meanwhile, the lowest root length (1.88-1.92) of the experiment was recorded for all the three accession shoots developed on hormone free half MS media (Table 7).

In this study, the optimum level of IBA concentration is 0.25mg/l for all the three accessions. Datta *et al.* (2007) have also reported that half MS medium fortified with 0.2mg/l regenerated better root length (8.7cm) from nodal explants of Jatropha. The results also revealed that root length tend to reduce with higher than optimum concentration of IBA. Kollmeier *et al*. (2000) reported that root elongation phase is very sensitive to auxin concentration and it is inhibited by high concentration of auxin in the rooting medium. It is possible that supra-optimal concentration of auxins inhibit root elongation through enhancement of ethylene biosynthesis, which is root growth inhibitor (Hartman *et al*. 2009).

Table 6. Number roots per shoots as affected by different concentration and combination of auxins (Iba and Naa).

PGR concentration (mg/l)		Number of roots per shoot
IBA	NAA	
0	0	1.80±0.14^{i}
0	0.25	-
0	0.5	-
0.25	0	5.43±0.08^{a}
0.25	0.25	4.07±0.12^{d}
0.25	0.5	3.31±0.06^{e}
0.5	0	4.53±0.08^{c}
0.5	0.25	4.80±0.11^{b}
0.5	0.5	2.87±0.13^{f}
1.0	0	3.50±0.05^{e}
1.0	0.25	2.47±0.06^{g}
1.0	0.5	2.07±0.12^{h}
LSD (5%)		0.25
CV (%)		12.28

LSD=Least Significant Difference, CV= Coefficient of variation, SD= Standard Error and n=number of samples, '-' indicates no response. Different letters (Within columns) indicate significant differences at P-Values < 0.05. The values are Mean ± Se (Where, N=15).

Table 7.Effects of different concentration and combination of IBA and NAA on rooting percentage and root length of three Jatropha accessions.

Jatropha Accessions	Auxin (mg/l)		Rooting response (%)	Root length (cm)
Metema	IBA	NAA		
	0	0	51.20 ±0.66^{mn}	1.92 ±0.05^{n}
	0	0.25	-	-
	0	0.5	-	-
	0.25	0	86.00 ±0.63^{b}	4.00 ±0.00^{b}
	0.25	0.25	76.200 ±0.20^{f}	2.74 ±0.10^{gh}
	0.25	0.5	64.00 ±0.55^{ij}	2.58 ±0.05^{hi}
	0.5	0	78.80 ±0.37^{e}	3.20 ±0.12^{e}
	0.5	0.25	84.20±0.20^{cd}	3.62 ±0.07^{cd}
	0.5	0.5	59.20 ±0.58^{k}	2.30 ±0.12^{jkl}
	1.0	0	71.60 ±0.51^{h}	3.00 ±0.00^{ef}
	1.0	0.25	54.40 ±0.68^{l}	2.26 ±0.06^{jklm}
	1.0	0.5	40.20 ±0.49^{o}	2.08 ±0.02^{lmn}
Adami Tulu	0	0	50.40 ±0.60^{n}	1.88 ±0.05^{n}
	0	0.25	-	-
	0	0.5	-	-
	0.25	0	84.80 ±0.86^{bc}	3.80 ±0.00^{bc}
	0.25	0.25	74.80 ±0.73^{g}	2.87 ±0.03^{fg}
	0.25	0.5	63.60 ±0.24^{j}	2.48 ±0.13^{ij}
	0.5	0	80.00 ±0.00^{e}	3.50 ±0.16^{d}
	0.5	0.25	83.20 ±0.37^{d}	3.60 ±0.24^{cd}
	0.5	0.5	59.40 ±0.40^{k}	2.36 ±0.10^{ijk}
	1.0	0	72.00 ± 0.00^{h}	2.80 ±0.12^{fgh}
	1.0	0.25	55.00 ±0.45^{l}	2.22 ±0.09^{klm}
	1.0	0.5	38.60 ±0.81^{p}	2.06 ±0.02^{mn}
Shewa Robit	0	0	52.20 ±0.20^{m}	1.88 ±0.05^{n}
	0	0.25	-	-
	0	0.5	-	-
	0.25	0	88.00 ±0.00^{a}	4.30 ±0.00^{a}
	0.25	0.25	76.00 ±0.32^{fg}	2.79 ±0.09^{fgh}
	0.25	0.5	65.00 ±0.45^{i}	2.74 ±0.02^{gh}
	0.5	0	76.60 ±0.98^{f}	3.60 ±0.10^{cd}
	0.5	0.25	83.60 ±0.68^{cd}	3.68 ±0.07^{cd}
	0.5	0.5	60.00 ±0.00^{k}	2.42 ±0.05^{ijk}
	1.0	0	71.60 ±0.51^{h}	3.10 ±0.10^{e}
	1.0	0.25	55.60 ±0.24^{l}	2.40 ±0.00^{ijk}
	1.0	0.5	40.00 ±0.55^{o}	2.04 ±0.02^{mn}
CV(%)			1.89	7.63
LSD (5%			1.29	0.22

CV= Coefficient of variation, LSD=Least Significant Difference, SD= Standard Error Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).

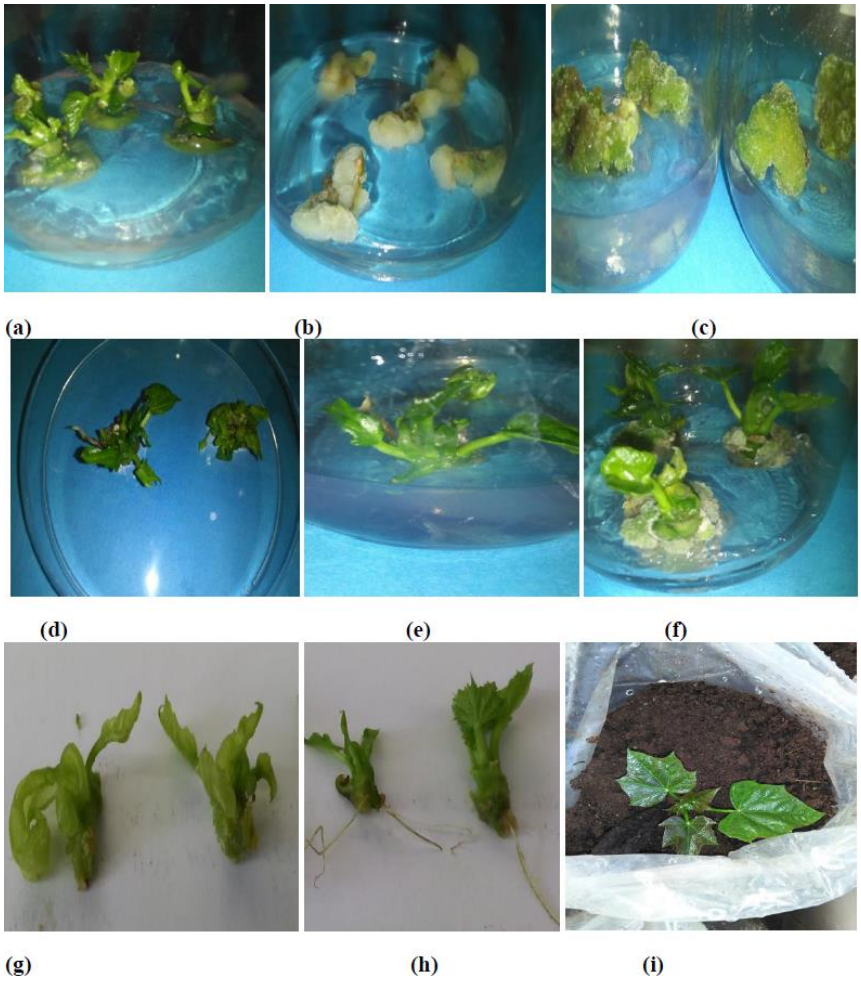

Figure 1.Plant regeneration in jatropha through direct and indirect organogenesis.

(a) *Shoot induction on 1.5mg/l of BAP from nodal explant,* ***(b)*** *callus induction on leaf discs at 1mg/l BAP and 1mg/l 2,4-D,* ***(c)*** *Calli failed to differentiate shoots,* ***(d)*** *Adventitious shoot formation on 0.5mg/l TDZ and 0.1mg/l IBA,* ***(e)*** *Shoot multiplication on 0.5mg/l BAP,* ***(f)*** *Callus formation on rooting MS media with 0.5mg/l NAA,* ***(g)*** *Shoots showed moderate hyperhydricity on rooting medium,* ***(h)*** *Rooting on half - strength medium with 0.25mg/l IBA,* ***(i)*** *plantlet undergoing acclimatization.*

Shoot regeneration through indirect organogenesis

Effects of BAP and 2,4-D on callus induction

The analysis of variance (ANOVA) showed that there is highly significant ($P<0.01$) difference due to the main effect of PGRs combinations and concentrations on mean number of days taken to form calli formation, Percentage of callus formationand callus fresh weight. However, the percentage of callus induction was not significantly ($P>0.05$) different among the accessions.

And there were no significant (P>0.05) effects recorded due to difference in accession and the interaction effect of PGRs and accessions on days taken to form calli formation and callus fresh weight.

The minimum number of days (13.6 days) to callus induction was recorded for explants cultured on MS media supplemented with combination of 1mg/l BAP and 1mg/l 2,4-D followed by (14 days) on MS media supplemented with 1.5mg/l BAP and 1mg/l 2,4-D. Whereas the longest time (18.87 days) taken to form callus was observed when callus induction medium was supplemented with combination of 2mg/l BAP and 0.5mg/l of 2,4-D (Table 9). From these results, increasing BAP levels in media within constant rate of 2,4-D led to an increase in the mean days taken to form calli. The media, which supplemented with BAP alone did not induce a callus. This indicated that presence of auxins in the media play a crucial role in the callus formation of Jatropha explants In vitro (Rajore and Batra, 2007; Kumar *et al*. 2008; Kumar *et al.* 2015). The result of this study also revealed that the number of days to callus emergence decreased with increased in concentration of 2,4-D (0.5-1mg/l) in the treatments. Kumar *et al.* (2015) also reported that leaf explants Jatropha started callusing within two weeks on the media containing 2, 4-D (10μM). However, further increasing 2, 4-D levels in media led to an increase in the mean days taken to form calli. Radhakrishnan *et al*. (2001) reported that the cells used up 2,4-D as required and any excess began to actively show the herbicidal effects that therefore slowed down the callus induction process.

The best callus formation (100%) was observed when MS medium was supplemented with combination of BAP (1.0 mg/l) and 2, 4-D (1.0 mg/l) for all accessions. Whereas the lowest percentage (47.8-48.6%) of callus formation was recorded for all the three accessions explants cultured on media supplemented with 2.0mg/l BAP and 1.5mg/l 2,4-D (Table 8). This indicated that callus induction frequency decreases with further increasing the concentration of both 2,4-D and BAP. Several authors reported that appropriate concentrations and combinations of cytokinins and auxins are important to produce Jatropha callus (Costa *et al.*, 2015; Kumar *et al*. 2015). On the other hand, the MS media containing only 2,4-D, even at a low concentration resulted in a better callus formation. Similar observation was also made by Thao *et al*. (2003) and Soomro and Memon (2007) concluding that 2,4-D was prerequisite for callus formation in many of plant species. Meanwhile, no callus formation was observed and the explants only showed leaf expansion on medium containing BAP alone. Kaewpoo and Techato (2009), who used different concentration of BAP (1mg/l, 2mg/l and 3mg/l) to induce callus from embryo cultures of Jatropha support these results. The authors finding report showed that BAP alone has induced shoot rather than callus. It has long been suggested that strong auxins such as 2,4-D are mainly efficient in promoting cell clumping and further developing of the callus.

The highest calli weight (2.23g) was recorded on MS media supplemented with combination of 1.0mg/l of BAP and 1.0mg/l 2,4-D followed by (2.06g) on media with 1.5mg/l of BAP and 1.0mg/l 2,4-D (Table 9). Whereas, the lowest overall mean calli

weight (0.98g) were observed for explant induced on MS media containing high levels of BAP (2.0mg/l) and 2,4-D (1.5mg/l) were used. This results confirmed that despite 2,4-D being an effective auxin in producing callus in Jatropha, it was active for callus induction when used in small amounts (Soomro and Memon,2007). Radhakrishnan *et al*. (2001) also reported that high 2,4-D concentration has been shown to have herbicidal effects on plants causing cell growth inhibition and at highest cell death.

Table 8. Interaction effect of PGRs (BAP and 2,4-D) combination with different jatropha accessions on callus formation percentage of explants excised from leaf after 30 days of culturing.

PGRs Concentration (mg/l)		Callus formation (%)		
BAP	2,4-D	Metema	Adami Tulu	Shewa Robit
0	0	-	-	-
1.0	0	-	-	-
1.5	0	-	-	-
2.0	0	-	-	-
0	0.5	82.00 ±0.00^{fg}	84.00 ±0.00 ^{f}	84.00 ±0.00^{f}
1.0	0.5	52.20 ±0.80^{kl}	52.00 ±0.00^{kl}	53.20 ±0.20^{k}
1.5	0.5	60.00 ±0.00^{i}	61.20 ±0.20^{i}	60.20 ±0.20^{i}
2.0	0.5	48.80 ±0.49^{mn}	49.20 ±0.49^{mn}	50.00 ±0.00^{m}
0	1.0	82.60 ±0.60^{f}	80.60 ±0.20^{g}	81.00 ±0.00^{g}
1.0	1.0	100.00 ±0.00^{a}	100.00±0.00^{a}	100.00 ±0.00^{a}
1.5	1.0	95.60 ±0.40^{b}	93.20 ±0.49^{c}	94.80 ±0.8^{b}
2.0	1.0	75.20 ±0.20^{h}	75.00 ±0.00^{h}	75.60 ±0.60^{h}
0	1.5	58.00 ±1.22^{j}	57.00 ±1.22^{j}	57.60 ±1.12^{j}
1.0	1.5	87.20 ±0.49^{e}	86.40 ±0.40^{e}	88.80 ±0.49^{d}
1.5	1.5	52.00 ±1.22^{kl}	52.00 ±1.22^{kl}	51.60 ±0.40^{l}
2.0	1.5	48.60±0.97^{mn}	48.00 ±0.00^{n}	47.80 ±0.80^{n}
CV (%)		2.19		
LSD (5%)		1.43		

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples, '-' indicates no response

Different letters (within columns and rows) indicate significant differences at P-values <0.05. The values are mean ± SE (where,n=5).

Table 9. Main effect of PGRs combinations and concentrations on days to callus formation and callus fresh weight of leaf explants of Jatropha.

PGRs concentration (mg/l)		Days to callus formation	Fresh weight of callus(g)
BAP	2,4-D		
0	0	-	-
1.0	0	-	-
1.5	0	-	-
2.0	0	-	-
0	0.5	15.67±0.13^{f}	1.44±0.004^{h}
1.0	0.5	17.00±0.00^{d}	1.27±0.003^{j}
1.5	0.5	17.53±0.13^{c}	1.43±0.015^{h}
2.0	0.5	18.87±0.13^{a}	1.32±0.008^{i}
0	1.0	15.03±0.12^{g}	1.97±0.001^{c}
1.0	1.0	13.60±0.16^{i}	2.23±0.004^{a}
1.5	1.0	14.07±0.07^{h}	2.06±0.005^{b}
2.0	1.0	16.47±0.19^{e}	1.55±0.003^{f}
0	1.5	16.87±0.09^{d}	1.48±0.002^{g}
1.0	1.5	14.93±0.12^{g}	1.75±0.009^{d}
1.5	1.5	17.93±0.12^{b}	1.71±0.004^{e}
2.0	1.5	18.00±0.00^{b}	0.98±0.001^{k}
LSD (5%)		0.29	0.015
CV (%)		3.32	1.75

CV= Coefficient of variation, LSD= Least Significant Difference, SE= Standard Error, n=number of samples, '-' indicates no response
Different letters (within columns) indicate significant differences at P-values <0.05. The values are mean ± SE (where, n=15).

Effect of cytokinins (TDZ or BAP) and auxin (IBA) on adventitious shoot proliferation from callus

The analysis of variance (ANOVA) showed that the percentage of adventitious hoot regeneration and mean number of adventitious shoots per callus were significantly ($P<0.01$) affected by the main effects of PGRs, accessions and the interaction effect of the two factors.

The highest percentage of shoot regeneration from callus (66.67%) was observed for Shewa Robit in MS medium supplemented with combination of 0.5mg/l TDZ and 0.1 mg/l IBA followed by 64%(Metema) and for and Adami Tulu (61.33%). On the same PGRs concentrations as for Shewa Robit.Meanwhile, the lowest percentage (39.2-40.8%) of shoot regenerations were recorded for all accession when the callus was cultured on media supplemented with 0.25mg/l TDZ (Table 10a). However, MS supplemented with BAP alone and combination with IBA did not induce shoot regeneration from callus culture. Calli that were cultured on this medium(BAP supplemented) was proliferated large quantities of healthy, green callus, but failed to differentiate shoots on all accessions type (Figure 1c).Even on subculturing to respective media it continued toform massive callus rather than formation of organogenesis.Furthermore, on the control medium the shoot regeneration was not noticed. However, the addition of TDZ to the medium improved the shoot induction potential of the calli as many shoot primordia were observed after 45 days of culture (Figure 1d). Khurana-Kaul *et al.* (2010) who also showed that the combination of TDZ and IBA was more effective than the combination of BAP and IBA in Jatropha shoot regeneration using leaf segments as explants.

Aishwariya *et al.* (2015) also reported that Thidiazuron (TDZ) is among the most active cytokinin like substances and it induces greater In vitro shoot proliferation than many other cytokinins in many plant species. Variation in the activity of different cytokinins can be explained by their differential uptake rate in different genomes, translocation rates to meristematic regions and metabolic processes in which the cytokininmay be degraded or conjugated with sugars or amino acidsto form biologically inert compounds also reported (Kaminek, 1992;Kumar *et al*. 2011). The highest adventitious shoot number (11shoot) was recorded for Shewa Robit callus cultured on MS media supplemented with 0.5mg/l TDZ and 0.1mg/l IBA followed by (10.4) and (9 shoot) shootlet per callus for Adami Tulu and Metema accessions, respectively. Whereas the lowest shoot number (3.8-4) was recorded for all accessions, when the callus subcultured on MS media supplemented with 0.25mg/l TDZ (Table 10b). In this study, the number of shoots per callus was increased as the concentration of TDZ was increased from 0.25 to 1mg/l. These results suggest that TDZ plays a very important role in the formation of adventitious shoot buds of Jatropha. These effects may be involved in stimulating *de novo* synthesis of auxins by increasing the levels of IAA and its precursor, tryptophan, as well as increase in contents of endogenous cytokinin (Murthy *et al*. 1995; Murthy and Saxena, 1998). Besides, the ability of TDZ to induce high shoot regeneration efficiency in plant tissue has been reported for a number of species (Feyissa *et al*. 2005; Landi and Mezzetti, 2006).Interaction effects of either BAP or TDZ incombination with IBA on percentage of shoot regeneration from callus derived from leaf of three Jatropha accessions.

Table 10. Effect of different concentrations of either BAP or TDZ in combination with IBA on percentage and number of shoot regenerated from leaf-derived calli of Jatropha accessions after 45 days of culture growth.

PGRs conc. (mg/l)			Adventitious shoot induction (%)		
BAP	TDZ	IBA	Metema	Adami Tulu	Shewa Robit
0	0	0	-	-	-
0.5		0	-	-	-
0.5		0.1	-	-	-
0.5		0.2	-	-	-
1.0		0	-	-	-
1.0		0.1	-	-	-
1.0		0.2	-	-	-
	0.25	0	40.00 ±0.54^{jk}	39.20 ±0.49^{k}	40.80 ±0.34^{j}
	0.25	0.1	46.38 ±0.22^{h}	44.60 ±0.24^{i}	46.60 ±0.00^{h}
	0.25	0.2	50.00 ±0.00^{fg}	47.20 ±0.49^{h}	51.20 ±0.49^{f}
	0.5	0	43.36 ±0.16^{i}	43.20 ±0.00^{h}	43.52 ±0.19^{i}
	0.5	0.1	64.00 ±1.63^{b}	61.33 ±1.33^{c}	66.67 ±0.00^{a}
	0.5	0.2	53.30 ±1.35^{e}	54.40 ±1.1^{de}	55.50 ±0.16^{d}
	1.0	0	48.8 ±0.00^{g}	48.80 ±0.00^{g}	49.04 ±0.24^{g}
	1.0	0.1	31.20 ±0.80^{lm}	29.90 ±1.35^{mn}	32.40 ±0.40^{l}
	1.0	0.2	28.20 ±0.92^{o}	27.80 ±0.80^{o}	28.80 ±0.49^{no}
CV (%)			4.63		
LSD (5%)			1.45		

CV= Coefficient of variation, LSD=Least Significant Difference, SE= Standard Error, n=number of samples, Conc. =concentration, '-' indicates no response.
Different letters (within rows and columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).

Effect of BAP and Kinetin(Kn) on multiplication of shoot derived from Callus

The ANOVA revealed that there is significant difference on number of shoots proliferated per explants, length of shootlet per explant due to the main effects of PGRs combinations and concentrations, accessionsand interaction effect of the two factors. Number of leaf per shootlet was also highly significantly affected ($P<0.01$) by the main effects of PGRs combinations and concentrations. However, the main effect of accessions and the interaction effect of the PGRs and accessions were non-significant.

The highest shoot number (3.5 shoots) was recorded for Shewa Robit on MS media supplemented with 0.5mg/l BAP whereas the highest shoot number (3.3 shoots) for both Metema and Adami Tulu were recorded on MS media supplemented with combination of 0.5mg/l BAP and 0.5mg/l Kn. The lowest shoot number (1.32-1.52) was recorded for all accession shoots cultured on hormone free MS media (Table 11). There are no conclusive reports on effect of growth regulators on number of shoot per shoot induced from calli for Jatropha but reports on other crops like sweet potato (Tasew, 2011) showed that the highest (2.4 shoots) number of shoots produced per shoots regenerated from leaf calli, which cultured on MS media supplemented with 1mg/l BAP. The result of the present study is partially agreed with this study. In similar pattern, the number of shoots formed in both direct organogenesis and indirect organogenesis affected by variation in concentrations of different cytokinins (BAP and Kn). It is well established that, cytokinins (BAP and Kn) stimulate protein synthesis and participate in cell cycle control and if added into shoot culture media,stimulate lateral bud growth and thus causing multipleshoot formation by breaking shoot apical dominance (George *et al.* 2008).

However, the number of shoot obtained through direct organogenesis (6 shoot per explant) is almost twice in rate as compared with indirect regeneration (3.5 shoot per explant). This shows that direct organogenesis is more efficient and easier to multiply shoots. In line with present study, Adiyecha *et al.* (2013) also reported that number of shoots formed were higher (10.3 shoots) in plantlets developed through direct organogenesis as compared toindirect organogenesis (8.1 shoots) via calli in *Curculigoorchioides.* The maximum shoot length was recorded for Shewa Robit (2.26cm) on media supplemented with combination 0.5 of mg/l BAP and 0.5mg/l Kn followed by 2.24cm and 2.1cm for Adami Tulu and Metema accession, respectively, on the same PGRs concentrations.

Meanwhile, the lowest shoot length (0.8-0.84cm) was recorded for all the three accession shoots developed on media supplemented with higher concentration of BAP (1.5 mg/l) (Table 11).In this experiment, maximum shoot length for all the three accessions was recorded on the media containing both BAP and Kn. This may be due to synergetic effects of BAP to Kn. However, the shoots produced showed stunted growth when the concentration of BAP increases for all accessions. These results are in line with those of Sujatha *et al.* (2005) and Imtiaz *et al.* (2014) who found that the length of the shoot decreased with increasing concentration of BAP. This is mainly

because when the concentrations are beyond optimum level absorbed and used by cultured explants, which causes accumulation in the tissues and lead to toxicity to the plant. This most possibly leads to ceasing cell growth and sometimes cell death (Deore and Johnson, 2008; Imtiaz *et al.* 2014). When indirect organogenesis and direct organogenesis are compared, the lower shoot length (2.26cm) was obtained from indirect organogenesis than direct organogenesis (3.2 cm). Likewise, Adiyecha *et al.* (2013) also found that shoot length was higher (9.3cm) in plantlets developed from direct organogenesis than indirect organogenesis, which is 7.03cm in *Curculigoorchioides.*

Table 11.Effect of different concentrations of cytokinins on multiplication of shoot from callus of three Jatropha accessions after 30 days of culturing.

Jatropha accession	Cytokinin (mg/l)		Number of shoot per explant	Length of shootlets (cm)
Metema	BAP	Kn		
	0	0	1.48 ±0.16^{op}	1.02 ±0.02^{q}
	0	0.5	2.36 ±0.16^{jkl}	1.39 ±0.02^{jkl}
	0	1.0	2.70 ±0.08^{fgh}	1.60 ±0.03^{g}
	0.5	0	3.10 ±0.10^{cd}	1.76 ±0.04^{ef}
	0.5	0.5	3.30 ±0.03^{b}	2.10 ±0.00^{b}
	0.5	1.0	2.76 ±0.05^{efgh}	1.90 ±0.00^{c}
	1.0	0	3.24 ±0.06^{bc}	1.26 ±0.02^{nop}
	1.0	0.5	2.86 ±0.02^{efg}	1.48 ±0.04^{hi}
	1.0	1.0	2.14 ±0.04^{mn}	1.24 ±0.02^{op}
	1.5	0	2.44 ±0.02^{ijk}	0.80 ±0.03^{r}
	1.5	0.5	2.24 ±0.04^{lm}	1.34 ±0.04^{klmn}
	1.5	1.0	2.00 ±0.00^{n}	1.20 ±0.00^{p}
Adami Tulu	0	0	1.32 ±0.08^{p}	1.10 ±0.03^{q}
	0	0.5	2.40 ±0.10^{jkl}	1.40 ±0.00^{ijkl}
	0	1.0	2.68 ±0.02^{gh}	1.58 ±0.02^{g}
	0.5	0	3.10 ±0.10^{cd}	1.80 ±0.00^{de}
	0.5	0.5	3.30 ±0.00^{b}	2.24 ±0.02^{a}
	0.5	1.0	2.76 ±0.08^{efgh}	2.04 ±0.06^{b}
	1.0	0	3.14 ±0.04^{bc}	1.28 ±0.05^{mnop}
	1.0	0.5	2.88 ±0.02^{ef}	1.46 ±0.02^{hij}
	1.0	1.0	2.12 ±0.05^{mn}	1.24 ±0.04^{op}
	1.5	0	2.40 ±0.00^{jkl}	0.82 ±0.02^{r}
	1.5	0.5	2.26 ±0.04^{klm}	1.32 ±0.02^{lmno}
	1.5	1.0	2.00 ±0.00^{n}	1.24 ±0.04^{op}
Shewa Robit	0	0	1.52 ±0.12^{o}	1.20 ±0.00^{p}
	0	0.5	2.60 ±0.06^{hi}	1.42 ±0.02^{ijk}
	0	1.0	2.70 ±0.00^{fgh}	1.70 ±0.00^{f}
	0.5	0	3.50 ±0.08^{a}	1.86 ±0.02^{cd}
	0.5	0.5	3.12 ±0.07^{bcd}	2.26 ±0.02^{a}
	0.5	1.0	2.80 ±0.05^{efg}	2.08 ±0.02^{b}
	1.0	0	3.26 ±0.04^{bc}	1.32 ±0.02^{lmno}
	1.0	0.5	2.94 ±0.02^{de}	1.52 ±0.05^{gh}
	1.0	1.0	2.22 ±0.02^{lm}	1.22 ±0.04^{p}
	1.5	0	2.48 ±0.02^{ij}	0.84 ±0.02^{r}
	1.5	0.5	2.28 ±0.02^{klm}	1.36 ±0.04^{klm}
	1.5	1.0	2.00 ±0.00^{n}	1.22 ±0.02^{p}
CV (%)			5.72	4.58
LSD (5%)			0.18	0.083

CV= Coefficient of variation, LSD=Least Significant Difference, SE=Standard Error

Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=5).

Table 12. Main effects of BAP and Kn in combinations and independently on the number of leaf per shootlet recorded 30 days after culture growth.

PGR concentration (mg/l)		Number of leaf per shootlet
BAP	Kn	
0	0	2.00 ± 0.05^{j}
0	0.5	3.00 ± 0.00^{g}
0	1.0	3.33 ± 0.02^{ef}
0.5	0	4.47 ± 0.13^{a}
0.5	0.5	4.01 ± 0.01^{b}
0.5	1.0	3.63 ± 0.10^{cd}
1.0	0	3.43 ± 0.13^{de}
1.0	0.5	3.80 ± 0.07^{bc}
1.0	1.0	3.13 ± 0.06^{fg}
1.5	0	2.43 ± 0.05^{i}
1.5	0.5	2.70 ± 0.07^{h}
1.5	1.0	2.17 ± 0.06^{j}
LSD (5%)		
CV (%)		

CV= Coefficient of variation, LSD= Least Significant Difference, SE=Standard Error, n=number of samples.
Different letters (within columns) indicate significant differences at P-values < 0.05. The values are mean ± SE (where, n=15).

Induction of Root for Callus-Derived Shoot

Elongated shoots of at least 1.5cm were also cultured on media containing various concentrations of IBA and NAA to induce adventitious root formation. However, unlike the in vitro shoots derived via direct organogenesis experiments, no adventitious roots were observed on any of the medium after four weeks.

Many shoots showed moderate hyperhydricity as indicated by callus formation at the base of shootlet and necrosis ofthe cut ends was observed (Figure 8, E and F). As alternative we also tried another protocol such as adding of activated charcoal, since as per literature shootlet is expected to form adventitious roots on MS medium supplemented with various concentrations of IBA and 0.2% activated charcoal (Rajore and Batra, 20005). Activated charcoal is a common additive used in plant tissue culture primarily for its adsorbent properties scavenging phenols, reactive oxygen species and toxic accumulations from the PGRs (Thomas, 2008). These properties have led to improvements in shoot multiplication, elongation and rooting along with many other positive effects for In vitro growth (Thomas, 2008). Nonetheless, in the present study none of these treatments enables the shoots to form roots (data not shown) and however, only occurrence of unwanted callus was reduced. In general, this might be due to the longer time of the cultured on cytokinins especially TDZ and BAP could be inhibit root formation. This is in agreement with findings by Naik *et al*. (2000) and Mohamed *et al*. (2006), who have shown that long exposure of the shoots to high concentration of either TDZ or BAP, suppressed the capacity of shoots to form

adventitious roots. In addition, it has been recorded that extra TDZ in medium might have the effectiveness of suppression of the growth of roots of Jatropha (Liu *et al*. 2015). Mundhara and Rashid (2006) has attributed triple role to TDZ in the cultures of Linum, where it was involved in the inhibition of roots formation, elongation, shortening and swelling of hypocotyls and tightening of cotyledons towards apex. Besides, the authors claim that the carryover of the TDZ to next stage of micropropagation is high and it is seen to cause the typical growth mishaps.

Acclimatization

In vitro induced shoots are very delicate and prone to sudden environmental changes that may damage the plantlets unless it is gradually adapted to the new environment. Thus, acclimatization is essential to enable the rooted plantlets to adapt the natural environment in Ex vitro conditions at temperature of 25±2^{o}c and 60-75% of relative humidity at greenhouse conditions.Therefore, plantlets from direct organogenesis experiments were transferred to greenhouse compartments at Holetta Agricultural Research Center for acclimatization.

Accordingly, the highest percentage of survival rate of shoots (86.67%) was recorded for Shewa Robit plantlets. whereas 73.33% and 66.67% recorded for Adami Tulu and Metema respectively after 30 days of acclimatization (Table 13 and Figure. 1i). Loss of some plantlets might be due to differences in the genotype in adaptation to the new environment (Ex vitro).The less development of cuticle under In vitro condition and the drop in relative humidity from near 100% in the culture vessels to much lower values in the greenhouse might result in excessive water loss and death (Biradar *et al.* 2009).The current result is in agreement with the report of Jeevan *etal*.(2013) who declared 87% greenhouse acclimatization potential of In vitro generated Jatropha cultures. There were no observable variations with respect to morphological and growth characteristics between Ex vitro sown parent plants and In vitro raised plants in pots.

Table 13. Survival rate of plantlets derived from *in vitro* regeneration through direct organogenesis of three Jatropha accessions during acclimatization.

Accession	Total number of plants acclimatized	Number of plants survived	Number of died plants	% of Survived Plants	% of died plants
Adami Tulu	15	10	5	66.67	33.33
Metema	15	11	4	73.33	26.67
Shewa Robit	15	13	2	86.67	13.33

Conclusion

Jatropha ***(****Jatropha curcas L.)* is a drought resistant multipurpose perennial plant. Due to its enormous potentials of substituting fossil fuels and saving ecological system from further deterioration, its production and propagation has paramount importance. However, conventional propagation methods are not adequate for efficient production

of this crop. Only the application of plant tissue culture and transformation techniques can expedite this process. The prerequisite for this is the establishment of efficient regeneration protocols. Generally, in this study, an efficient direct organogenesis protocol was developed for large-scale multiplication, genetic transformation and conservation of Jatropha using nodal explant sources. Besides, this study also gives useful insight of the protocols for the indirect regeneration of the Jatropha.

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In Vitro Propagation of Ethiopian Lowland Bamboo (*Oxthenanthera abyysinica* (A.Rich. Munro) from Seed Culture

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Abstract

Ethiopian low land bamboo ***(Oxthenanthera abyysinica (A.Rich. Munro)*** *belongs to the family Poaceae and is one of the abundant resources in Ethiopia with a diversified economical value. Conventional propagation methods of bamboo are generally inefficient due to their low multiplication rate, time consuming, labor intensive and too costly. Now a day, application of biotechnology including In vitro culture is becoming a promising tool for conserving and mass propagation of different bamboo species. However, there is no report on micropropagation of LLB using seeds in Ethiopia. Therefore, the objective of this study was to develop a protocol for micropropagation of LLB. Using seeds as explants source, sterilization, shoot initiation, multiplication, rooting and acclimatization were carried out using CRD design at Holetta Agricultural Biotechnology Laboratory. Sodium hypochlorite (NaOCl) of 5.0% of active chlorine (3, 4, and 5%) was used for surface sterilization and each concentration was tested against three different exposure time (15, 20, and 25 min). MS medium augmented with different types of cytokinins (BAP and KN) at different concentrations (0.5, 1.0, 2.0, 4.0 and 8.0 mg/l) were used separately for shoot initiation and multiplication. For rooting, ½MS medium supplemented with different types of auxins (IBA and NAA) at different concentrations (0.5, 1.0, 2.0, 4.0 and 8.0 mg/l) were used separately. PGRs-free MS or ½MS medium was used as control treatment for shoot initiation and multiplication or for rooting experiment respectively. Data were subjected to ANOVA and significantly different means were separated using Fisher's LSD test. Seeds disinfected in 4.0% NaOCl for 25 minutes gave 71.6% clean survived and 23.45% germinated explants. In shoot initiation experiment, all viable seeds were able to proliferate in 5-7 days of culturing in all treatments. And MS + 4.0 mg/l BAP was found better in maximum shoot initiation percent (86.67) and mean number of shoots/explants (4.8) while PGRs-free MS medium showed longest mean shoot length (13.40 cm) and highest mean number of leaf (2.90) per shoot. Similarly, in shoot multiplication MS + 4.0 mg/l BAP was effective in highest mean number of shoot (11.33) and multiplication rate (3.77) while PGRs-free MS medium gave highest leaf number/shoot (1.86) and longest shoot length (3.92 cm). The maximum rooting percent (93.33) and maximum root number/clump (9.42) were found at MS + 8.0 mg/l IBA and longest (8.90 cm) root was observed at PGRs-free ½MS medium. Finally, the survival rate of plantlets in greenhouse condition was found to be 91.67% after 30 day of acclimatization.*

Introduction

Bamboo is the strongest and fastest-growing perennial grass species (BPG, 2012) and is unique with complex branching patterns; woody culms and gregarious, monocarpic flowering plant (Singh *et al*. 2013) belonging to the sub family Bambusoideae under the family Poaceae (GPWG, 2001). Forty-three species of bamboo distributed in 11 genera found in Africa (Bystriakova *et al*. 2004), covering an estimated area of 3.6 million ha (FAO, 2010). Out of these Africa bamboo species, Ethiopia has only two endemic species namely, the highland or African alpine bamboo (*Yushania alpine* K. Schumach.) and lowland bamboo *(Oxytenanthera abyssinica A. Rich. Munro)* (Melaku, 2006; Seyoum, 2008). These species are found in some other African countries, confined to the sub Saharan region, but nowhere else outside the African continent (Bystriakova *et al*. 2004). The coverage area of lowland bamboo is estimated to be 850,000 ha (Seyoum, 2008), while the highland bamboo is estimated over 120,000 ha (Kigomo, 2007). The lowland bamboo grows mainly in the western part of Ethiopia along major river valleys and in the lowlands bordering Sudan (Phillips, 1995).

Throughout history, bamboo and its properties have been repeatedly used by different cultures to provide the goods and services needed for their lives. Today, it remains highly important as a basic livelihood grass and material for rural people living in Asia, Latin America and Africa, as well as higher income people who purchase green bamboo products throughout the world (Yiping *et al*. 2010). It is used for housing, handicrafts, pulp and paper industries, energy source, food (FAO, 2010). In addition, its woody stem has great agricultural potential to contribute to a sustainable development in reducing the risk of erosion (Benton *et al*. 2011; Yenesew *et al*. 2014). Furthermore, bamboo has great value in carbon sequestration and reducing global warming by holding higher carbon content (Zhou *et al*. 2005; Yiping *et al*. 2010; Benton *et al*. 2011).

Conventionally, bamboos are propagated through seeds, clump division, rhizome and culm cuttings (Banik, 1994; 1995). However, gregarious flowering at long intervals followed by the death of clumps (Austin and Marchesini, 2012), short viability of seeds (Bereket, 2008), presence of diseases and some pests (Demelash *et al*.2012; Singh *et al*. 2013) are limiting factors to use seeds as valuable source of propagation. Vegetative propagation methods have limitation for mass propagation *i.e.* propagules are difficult to extract, bulky to, transport and planting materials are insufficient in number for large-scale plantation (Kassahun, 2003; Mudoi *et al*. 2013). Seasonal dependence, low survival rate and limited rooting of the propagules are other limitations (Singh *et al*. 2013).

Considering problems encountered in both sexual and asexual conventional propagation of the lowland bamboo species and growing interest of the country on the economic and ecological benefit of bamboo, a method that brings about rapid large-scale production of bamboo is highly desirable.

In this regard, different scholars recommended micropropagation as an excellent means to achieve this aim. Micropropagated plants are grown In vitro in sterile media, unlike plants grown from cuttings at field. Typically, the media comprises agar, with the addition of various compounds such as nutrients, inorganic salts, growth regulators, sugars, vitamins and other compounds (Burr *et al*. 2013).

A benefit to micropropagation of plants with tissue culture technique is that the plants can be disease free. Multiplying large numbers of plants in a small space by starting from a few explants, reduced water and nutrient needs for micropropagation of plants, rapid multiplication of tissues that can in turn be used to yield more tissue culture material, ease of transport and delivery of plantlets (Samora, 1994; Burr *et al*. 2013). All these advantages bring both quantitative and qualitative gain of micropropagated planting materials. With the advancement of other tissue culture techniques, such as somatic hybridization and induction of *In vitro* flowering, even high-yielding bamboo hybrids may soon be a reality (Saxena and Dhawan, 1995).

The first tissue culture study on bamboo (*Dendrocalamus strictus*) was conducted by Alexander and Rao (1968) who germinated embryos In vitro. Since then a large number of studies have been undertaken on micropropagation of bamboos using seeds, somatic embryogenesis, nodal culture, and In vitro flowering. Different researchers have been publishing scientific articles on successful micropropagation protocol through seed culture in different bamboo species like:

- Arya *et al*. (1999) on *D. asper* ;
- Bag *et al*. (2000) on *T. spathiflorus* ;
- Arya *et al*. (2012) on *D. hamiltonii* and
- Devi *et al*. (2012) on *D. giganteus*.

Nevertheless, their result shows there is interaction of species with hormonal types and levels included in the culture medium, which necessitate the development or optimization of micropropagation protocols for every species under different conditions. Incorporation of 6-Benzylamino-purine (BAP) into the medium improved the shoot proliferation (Tuan *et al*. 2012; Arya *et al*. 1999; 2012), while Kinetin (KN) alone was found to be less effective (Sharma and Sarma, 2011). In addition, the combined effect of the two cytokinins BA and KN was reported best for shoot multiplication (Parthiban *et al*. 2013; Waikhom and Louis, 2014). To achieve a successful In vitro propagation, rooting of plantlets is a crucial stage prior to hardening. Therefore, Indole-3-butyric acid (IBA) alone (Diab and Mohamed, 2008) or in combination with 1-naphthaleneacetic acid (NAA) (Nurul Islam and Rahman, 2005) or BAP (Devi and Sharma, 2009) were used to regenerate a well-developed roots depending on the genotype and on cultural methods.

So far, there is no report available, at least to the author, on an efficient and reproducible protocol that can enable the In vitro rapid multiplication of Ethiopian low land bamboo species from seed. Therefore, this study was undertaken to develop an In vitro propagation of *O. abyssinica* species from seed.

Materials and Methods

Experimental site

The study was conducted at the Plant Tissue Culture Laboratory, National Agricultural Biotechnology Laboratory, Holetta Agricultural Research Center from February 2014 to June 2014. Holetta is located 34 Km West of Addis Ababa in the Finfine Zuria of Oromia Liyu Zone of the Oromia Region.It has a latitude and longitude of 9O 3'N and 38O 30'E, respectively with an altitude of 2391masl. Average maximum and minimum temperatures of 22.2oC and 6.13oC, respectively and average annual rainfall of 1100 mm characterize it.

Source of experimental material

As the species had not produced seed during the experimental period due to its gregarious flowering characteristic, the seeds were obtained from Asossa Agricultural Research Center Department of Forestry. Healthy seeds were selected carefully and used as explants for this study. To maintain the seeds viability they were stored in plastic bag in refrigerator at +4 0C.

Media preparation

To prepare a ready to use of one litter MS media, 50 ml of MS1 and 5 ml of MS2, MS3, MS4, MS5 and MS6 stocks were poured into one litter beaker (Appendix Table 1). The stock solutions were mixed along with 3% sucrose (Tuan *et al*. 2012) and plant growth regulators. Magnetic stirrer was used to mix the solution well. Then the volume was adjusted by adding DDW. The pH of the medium was adjusted to 5.80 (Arya *et al*. 2012) using 0.1N NaOH or 0.1N HCl before adding 0.4% agar (Agar-Agar, Type 1), and heated till the agar melts properly. About 30ml of the medium were dispensed in each culture jar (150 ml) for sterilization and initiation experiment and 50ml of the medium were dispensed in each culture jar (250 ml) for multiplication and rooting experiments. The culture jars containing the medium were plugged tightly with autoclavable lids prior to autoclaving at 121OC with 0.15KPa pressure for 20 minute. Then it was allowed to cool at room temperature and stored in culture room until used.

Explants surface sterilization

Before seeds are inoculated on a medium, it must be sterilized to get rid of all micro- organisms. Seeds were washed with tap water to remove debris and dehusked. The dehusked seeds were rinsed in distilled water and soaked for 2 h. Then the imbibed seeds were washed by DDW with liquid soap plus 2-3 drops of Tween-20 for 25 minutes with agitation to physically remove most microorganisms and to remove some debris followed by antifungal treatment with Curzate® R WP 0.25% (w/v) for 20 minute with gentle shaking. Finally, the seeds were washed with DDW (3×) to remove traces of Curzate® R WP.

Then the seeds were treated with 70% ethanol for 30 seconds under laminar airflow cabinet. After pretreatment with ethanol, the explants were rinsed with autoclaved

distilled water three times, to lower the toxic effect of ethanol. They were then treated with three concentration levels (3%, 4% and 5% (w/v) active ingredient of chlorine) of locally produced bleach, NaOCl with 5% (w/v) active ingredient of chlorine for varying exposure times (15, 20 and 25 minutes) (Table 1). To increase the efficiency of NaOCl, 2-3 drop of Tween-20 per 200 ml solution was added as wetting agent. After decanting the sterilizing solutions under safe condition, the seeds were washed three times each for five minutes with autoclaved distilled water to remove traces of NaOCl.

The sterilized seeds were inoculated horizontally on jars, containing 30 ml of MS medium fortified with 3% sucrose (Arya *et al*. 2012; Tuan *et al*. 2012) and 0.4% agar (Agar Agar, Type 1). The jars with cultured seeds were properly sealed with parafilm and labeled. Thereafter, the cultures were transferred and randomly placed on the growth room shelf with a photoperiod of 16/8h light/dark using cool-white fluorescent lamps (photon flux density, 40µmol m-2s-1 irradiance) at 25 ± 20C and relative humidity (RH) of 70-80%. For each sterilization treatments, three jars, each with three seeds or explants,were placed randomly in completely randomized design (CRD). Data recorded from the sterilization experiment include the number of germinated, contaminated, dead and survived (clean) cultures after 15 days of culturing. Then the data were converted into percentage.

Table 1.Treatment combinations for sterilization experiment

Treatment code	NaOCl (Cl%) (local bleach)	Time of exposure (min)
T01	3	15
T02	3	20
T03	3	25
T04	4	15
T05	4	20
T06	4	25
T07	5	15
T08	5	20
T09	5	25

Establishment of culture shoots

Sterilized seeds' were cultured using similar procedures described above in jars containing 30 ml of MS (Murashige and Skoog 1962) medium fortified with 3% sucrose (Arya *et al*. 2012; Tuan *et al*. 2012), 0.4% agar (Agar-Agar, Type 1) and varying level of BAP (0.5, 1.0, 2.0, 4.0 and 8.0 mg/l). A separate experiment was set out with KN with 0.5, 1.0, 2.0, 4.0 and 8.0 mg/1 concentration for seed germination and shoot initiation study. MS medium without PGRs was used as control. Eleven treatments were employed and for each treatment five jars, each with three seeds, were used and distributed randomly to form CRD arrangement. All jars were placed on the growth room shelf with a photoperiod of 16/8h light/dark using cool – white fluorescent lamps (photon flux density, 40µmol m-2 s-1 irradiance) at 25 ± 20C and RH of 70- 80%. Number of days to shoot initiation and number of initiated seeds were recorded. Length of shoots, number of shoots/seed and shoot proliferation percentage were computed after 30 days.

Shoot multiplication

To avoid the carry over effect of shoot initiation media during shoot multiplication, initiated propagules consisting of three shoots each were excised from seeds and sub-cultured on PGRs free MS medium for two weeks. Each propagule was placed vertically and lightly pressed into the medium in 200 ml jars each with 50 ml MS medium supplemented with 3% sucrose (Arya*et al*. 2012; Tuan *et al*. 2012), 0.4% agar (Agar-Agar, Type 1) and varying level of BAP (0.5, 1.0, 2.0, 4.0 and 8.0 mg/l). A separate experiment was set out with KN concentration of 0.5, 1.0, 2.0, 4.0 and 8.0 mg/1 for shoot multiplication study. MS medium without PGRs was used as control. Eleven treatments were employed and for each treatment five jars, each with three clumps were used and distributed randomly to form CRD arrangement. All jars were placed on the growth room shelf with a photoperiod of 16/8h light/dark using cool -white fluorescent lamps (photon flux density, 40µmol m-2 s-1 irradiance) at 25 ± 20C and RH of 70- 80%. Data on number of shoot per explant, number of leaves per shoot, and shoots length (cm) were recorded after 6 weeks.

Table 2. Treatments for establishment of shoot cultures' and shoot multiplication experiments

	Treatment code	Cytokinins	
Initiation	Multiplication	BAP(mg/l)	KN(mg/l)
IT01	MT01	0	0
IT02	MT02	0.5	0
IT03	MT03	1	0
IT04	MT04	2	0
IT05	MT05	4	0
IT06	MT06	8	0
IT07	MT07	0	0.5
IT08	MT08	0	1
IT09	MT09	0	2
IT10	MT10	0	4
IT11	MT11	0	8

Rooting of shoots

The In vitro regenerated shoots, three shoots in a bunch, were excised from multiple shoot bunches and transferred on conditioning media for 2 weeks were used for rooting studies. The rooting response of these shoots was studied on half MS medium supplemented with 3% sucrose (Arya *et al*. 2012; Tuan *et al*. 2012), 0.4% agar and different concentrations of IBA (0.5, 1.0, 2.0 and 4.0 and 8.0 mg/l) and NAA (0.5, 1.0, 2.0, 4.0 and 8.0 mg/l).The MS medium without PGRs was used as control. For each treatment five jars, each with three clumps, were used and distributed randomly to form CRD arrangement. All shoots were incubated on rooting medium for 4 weeks at 25+ 2 0C with a photoperiod of 16/8h light/dark using cool-white fluorescent lamps (photon flux density, 40µmol m-2s-1 irradiance) at 25 ±

20C and RH of 70-80%. Number of rooted clumps, number of roots per clumps, and average root length (cm) were recorded after 30 days.

Acclimatization

For hardening the In vitro well-rooted plantlets, they were first washed with distilled water to remove adhered agar/medium that harbors microorganism growth to prevent contamination. The washed plantlets then were planted into plastic bags filled with mixture of autoclaved soil (forest soil: sand: manure in 2:1:1 ratio). Moreover, they transferred to greenhouse for hardening with an average temperature of (24±20c) and relative humidity of (60-70%). The transplanted plantlets were kept in greenhouse under shade of polyethylene sheets and red cheese cloth, to reduce light intensity and maintain moisture, for ten days and were sprayed with water two to three times every day. For the next five days the plantlets were acclimatized in an open greenhouse environmental condition with in the same room by removing the polyethylene sheet and red cheese cloth and watered once per a day. Subsequently, those plants that appeared strong and healthy were shifted to a warmer (temperature of 30±20c) and drier (humidity of 25-30%) open greenhouse room. In this room, the plantlets were allowed to grow for fifteen days with a watering frequency of once per two day. After 15 and 30 days of transplanting, percentage of plantlets that were successfully acclimatized was recorded. Finally, the plants were further transferred to bigger plastic bags after 30 days of hardening in greenhouse.

Data collection and analysis

Number of contaminated explants, average number of days for shoot initiation, number of initiated shoots; number of shoots per seed; shoot length, number of rooted clumps, number of roots, root length and Acclimatization percentages were collected data. The data was subjected to one-way analysis of variance (ANOVA) using the SAS software packages (version 9.1) and significant differences among mean values were compared using Least Significant Difference (LSD) at a 5% of probability level.

Results and Discussion

The results and discussion are presented in five parts *viz*., sterilization technique, In vitro shoot proliferation, In vitro shoot multiplication, In vitro rooting of shoots and acclimatization of In vitro propagated plantlets.

Sterilization of the explants

Analysis of variance (ANOVA) revealed that concentration of local bleach, exposure time, and their interaction had highly significant difference ($P<0.0001$) on overcoming contamination of growth media and improving survival and germination level of *O. abyssinica* In vitro seed culture.

Among the different disinfectant treatments investigated, 3.0 % NaOCl for 15 min duration recorded the highest contamination (100%) and by default the lowest

clean explants percentages (0.00%). The lowest contamination (1.23%) and maximum clean explant percentages (98.76%) was recorded from 5.00% NaOCl for 25 min duration. In addition, the contamination percentage of treatment three (3% NaOCl concentration for 25 min duration) was lower than treatment four (4% NaOCl concentration for 15 min duration); and the same was true between treatment six and seven. This result indicated that the concentration of active chlorinated local bleach and exposure time used for disinfectant were interrelated factors to obtain microorganism free explants.

The highest seed germination percentage (23.45%), the third minimum seed contamination percentage (4.93%), and 71.60% clean explants were recorded from treatment six (4.00% active chlorinated local bleach for 25 min). No and poor germination was recorded from treatment one, two, three, four, seven, eight, and nine. Although treatment five (4.0% active chlorinated local bleach for 20 min) showed statically equivalent germination percentage (22.22%) it was less in disinfection efficiency (46.91%) and minimum in occurrence of clean explants (30.86%) availability than treatment six. These results indicated that the chemical concentration and the duration at which the bamboo seeds exposed to the disinfectant time affected the seed germination percentage significantly.

The data also revealed that the contamination percentage was dramatically decreased as the exposure time increased within the same level of NaOCl solution. However, disinfection with higher concentration (5.00%) of local bleaching solution for 25 minute resulted in least contamination and highest clean explants but no seed does germinated (Appendix Figure 1). This may be due to phytotoxic ability of local bleach at longer time exposure.

Comparing the interaction effect posed by the chemical and the duration of disinfection against the aim of sterilization, disinfection bamboo seed with 4% NaOCl solution for 25 min was the most effective sterilization treatment, which gave highest germination percentage, lowest contamination and moderately clean explants. This sterilization method is easy and the material is locally available, less costly, and less toxic to human compared to other disinfectant (eg. $HgCl_2$). It does not also require special handling and waste removal.

Effect of BAP and KN on establishment of culture shoots

Analysis of variance revealed that concentration of BAP and KN had highly significant effect ($P < 0.0001$) on shoot proliferation, days to proliferation, the ability of the seeds to form multiple shoots, shoot length, and leaf number. All viable seeds were able to multiply shoots after 5-7 days of culturing in both control and cytokinin fortified MS medium. However, the initiation percentage, the days for initiation, number of shoots initiated, length of shoots, and leaves number were found to vary in the different concentrations of cytokinins and control treatment. The maximum shoot initiation percentage (86.67%) was recorded from 4.0 mg/l BAP supplemented MS medium; followed by 73.33%, which was obtained from 2.0 mg/l

KN augmented MS medium which were statistically equivalent. However, initiation percentage of 2.0mg/l KN is also not statically different from other treatments (IT03, IT04, IT08 and IT10). The minimum shoot initiation percentage was 33.33%, which was observed on seeds cultured at MS medium containing hormone free and 0.5 mg/l KN and 39.99%, which was, recorded seeds cultured at MS medium containing 0.5 mg/l BAP and 8.0 mg/l KN. This showed that the shoot inanition percentage from seed was greatly influenced by type and concentrations of cytokinin. Arya *et al.* (1999; 2012) has already confirmed that, the capacity of BAP on effectively induction of shoot from *D. asper* and *D. hamiltonii* seeds respectively.

Due to their ability enhancing in seed germination (Miransaria and Smith, 2014) and shoot initiation (Ashraf *et al*. 2014), investigations have been revealed that cytokinins were a key factor for bamboo species seed germination and multiple shoot proliferation (Nadgri *et al*. 1984; Arya *et al*. 1999; Devi *et al*. 2012). The present study also found that, the ability to germinate and form multiple shoots of *O. abyysinica* seeds was dependent on the concentration of cytokinins (BAP and KN). Culturing of seeds for more than 30 days in a medium resulted browning of shoots and consequently died up the whole plantlet Seeds those cultured in PGRs-free MS medium were the least in proliferation with a mean shoot number of 1.0±0.00 /seed (Figure 1 IT01) than seeds those cultured in MS medium with cytokinins.

This indicates application of PGRs was necessary to induce the rate of proliferation in *O. abyssinica* tissue culture. Of the various concentrations of cytokinins (BAP and KN) tried, highest shoot number was obtained at 4.0mg/l BAP; Where 4.8±0.27 shoots/explant (Figure 2 IT05) was formed after six weeks of culturing followed by 3.46±0.02 shoots/seed and 3.00±0.00 shoots/seed obtained from 8.0 mg/l BAP and 4.0 mg/l KN fortified MS medium respectively (IT06 and IT10). Generally, the present study indicated that the effect of BAP was better than KN in shoot proliferation percentage and multiple shoot induction. The present result is in agreement with the findings of other workers who have noted the effectiveness of BAP for the induction of multiple shoofrom seeds in different bamboo species (Nadgir *et al*. 1984; Kapoor and Rao, 2006; Tuan *et al*. 2012).

The longest (13.40 cm) and shortest (3.82 cm) shoots were recorded from PGRs-free and 8.0 mg/l BAP fortified MS medium respectively (IT01 and IT06). In both BAP and KN mean, shoot length was negatively correlated with those cytokinins concentration level (Appendix Figure 2). While increasing BAP and KN concentration from 0.5 to 8.0mg/l in the MS medium, shoot length was decreased from an average of 5.90 cm to 3.82 cm and 7.56 to 4.52 cm, respectively (Table 5). This result might be due to seeds cultured on those medium gave less number of shoots (1-2 shoots), thus the nutritional competition was not happened strongly or due to the toxic effects of ethylene, which can be produced at higher cytokinin concentration (Woeste *et al*. 1999). Generally, shoot length was better on KN supplemented MS medium than BAP supplemented MS medium. This is consistent with report of Arya *et al*. (1999) on decreasing of shoots height developed from seeds

as cytokinin concentration increased. The maximum and minimum number of leaves (2.90 and 1.64) per shoot was recorded on control and 8.0 mg/L KN treatment respectively. The number of leaves per shoot increases as the shoot length increases (Appendix Figure 2). This is in harmony with the findings of Tuan *et al*. (2012) on *D. asper* and Modui *et al*. (2014) on *B. nutans* bamboo species. They noticed a negative correlation between leaf number and shoot length; leaf number increases as the shoots length increases.

Seeds cultured on MS medium without or with lower (like 0.5 mg/l) cytokinins developed 1-2 shoots/culture with roots (Figure 1, IT01 and IT07), indicating that the seeds have enough optimum endogenous auxins to cytokinins level necessary to induce limited number of shoots and roots upon culturing. Seeds cultured at MS medium supplemented with higher BAP and KN concentration induce greater number of shoots but without roots. This is due to the imbalance between cytokinins to auxin ratio in which the increased level of cytokinins favors only shoot regeneration in the absence of equivalent auxin levels inside the plant.

IT01 **IT05** **IT06**

Figure1. Effect of BAP and KN on seed germination and shoot formation from seed of *O. abyssinica* after 30 days

***Note**: IT01= PGRs-free (control); IT05 = MS + 4.0 mg/l BAP; IT06 = MS + 8.0 mg/l BAP; IT07 = MS + 0.5 mg/l KN; IT10 = MS + 4.0 mg/l*

Effect of BAP and KN on shoot multiplication

Analysis of variance revealed that the various concentrations of BAP and KN tested had highly significant effect (P< 0.0001) on number of shoot, shoot length, and number of leaves (Table 3) in In vitro developed explants of *O. abyssinica*. Cytokinins were known to promote the function of other growth regulators like 2ip,

zeatin (Gaspar *et al*. 1996). In this study too, the addition of either BAP or KN on most microshoots of *O. abyssinica* resulted increased multiplication rate and higher mean shoot number over PGRs-free MS medium (Table 3). Among the different concentration of BAP and KN investigated, MS + 4.0 mg/l BAP showed maximum multiplication rate of 3.77±0.20, followed by MS + 8.0 mg/l BAP (2.41±0.35). Lower rate of multiplication was recorded from MS + BAP with 0.5 mg/l, 1.0 mg/l, and 2.0 mg/l and MS + KN with 1.0 mg/l, 2.0 mg/l, and 4.0mg/l but better than MS + 0.5 mg/l KN, 8.0 mg/l KN and PGRs – free MS medium (Table 3).

With regard to multiple shoot proliferation, MS + 4.0 mg/l BAP displayed superiority over the other treatments with a mean of 11.33±0.62 mean number of shoot per propagule; followed by 8.0 mg/l and 2.0 mg/l BAP with 7.26±1.08 and 5.8±0.20, respectively (Figure 2 MT05, MT06 and MT04). Treatments such as MS + 0.5 mg/l, MS + 1.0 mg/l, and MS + 2.0 mg/l BAP and MS + 1.0 mg/l, MS + 2.0 mg/l, and MS + 4.0 mg/l KN showed less mean number of shoot, but significantly better than the control. The lowest and not significantly different from control treatment mean number of shoot was obtained from 0.5 mg/l and 8.0 mg/l KN treatments (Table 3). The average superiority of BAP over KN in shoot induction may due to its ability to induce production of natural hormones such as zeatin within the tissues than other synthetic cytokinins (Zaerr and Mapes, 1982). Accordingly, the ability of plant tissues to metabolize the natural hormones is faster than artificial growth regulators.

The effect of BAP in inducing multiple shoots has already been reported in bamboo species like *A. callosa* (Devi and Sharma, 2009), *D. hamiltonii* (Sood *et al*. 2002), *G. angustifolia* (Jimenez *et al*. 2006), and *B. oldhamii* (Thiruvengadam *et al*. 2011). Interestingly, the synergistic effect of BAP and KN for increased shoot multiplication rate and proliferation was also reported on *B. tulda* and *M. baccifera* (Waikhom and Louis, 2014).

In both cytokinins (BAP and KN) the shoot length is inversely related to their respective concentration (Appendix Figure 3). The longest shoot length (3.92±0.30) was recorded from PGRs-free MS medium, followed by 3.66±0.20 and 3.4±0.14 from MS+ 0.5 mg/l BAP and MS + 0.5 mg/l KN, respectively (Table 3). The shortest shoot length 2.62±0.20, 2.64±0.32, and 2.65±0.19 were recorded from higher concentration of cytokinins: 8.0 mg BAP, 8.0 mg/l KN and 4.0 mg/l KN, respectively (Table 3). This finding is in line with Arya *et al*. (1999), Shorti *et al*. (2012), and Singh *et al*. (2012) who reported reduction in shoot length in different bamboo species as the cytokinins concentration increases. According to Woeste *et al*. (1999), the reduction in shoot length at higher concentration of BAP and KN might be due to the toxic effects of ethylene produced at higher cytokinin concentration.
The maximum leaf number per shoot (1.86) was recorded on PGRs-free media followed by 1.41 and 1.34 in 0.5 mg/l BAP and 0.5 mg/l KN supplemented MS medium, respectively. On decreasing the concentration of cytokinins, the number of shoots decreased but the leaf number and shoot length were increased (Appendix Figure 3). These findings are in agreement with what was reported by Tuan *et al*. (2012) on *D. asper* and Modui *et al*. (2014) on *B. nutans In vitro* propagation.

Table 3. Effect of bap and kinetin concentration in Ms medium on In Vitro shoot propagation of *O. Abyssinica*

Treatment code	Con. Of PGRs(mg/l) BAP	Number of mean shoots KIN	Number of mean shoots length(cm)	Number of mean leafs	Mean of leafs multiplication	Shoot on rate
MT01	0	0	0.50.86±0.08^{g}	4.4±0.29^{e}	1.46±0.09^{e}	09^{e}2.6±0.24^{g}
MT02	0	0	3.66±0.20^{ab}	3.92±0.30^{a}	1.86±0.05^{a}	1.60±0.13^{b}
MT03	1	0	5.2±0.12^{d}	3.08±0.08^{c}	1.34±0.09^{c}	1.73±0.03^{d}
MT04	2	0	5.8±0.20^{c}	2.96±0.13^{c}	0.99±0.05^{e}	1.93±0.06^{c}
MT05	4	0	11.33±0.62^{a}	2.92±0.13^{c}	0.99±0.11^{e}	3.77±0.20^{a}
MT06	8	0	7.26±1.08^{b}	2.62±0.20^{d}	0.93±0.10^{e}	2.41±0.35^{b}
MT07	0	0.5	2.73±0.27^{g}	3.4±0.14^{b}	1.41±0.11^{c}	0.91±0.09^{g}
MT08	0	1	3.40±0.36^{f}	3.09±0.21^{c}	1.29±0.08^{cd}	1.13±0.12^{f}
MT09	0	2	5.0±0.23^{d}	3.02±0.24^{c}	1.16±0.07^{d}	1.66±0.07^{d}
MT10	0	4	4.13±0.29^{e}	2.65±0.19^{d}	0.76±0.14^{f}	1.37±0.09^{e}
MT11	0	8	2.19±0.18	2.64±0.32	0.64±0.12	0.73±0.05^{g}
CV		9.03	9.03	6.77	8.89	8.99
LSD			0.56	0.26	0.13	0.18

Note: Means in the same column that are followed by different letters are significantly different at P≤0.05 using Fisher's LSD test, LSD=Least Significant Difference CV= coefficient of variation (%), ± = Standard Deviation.

The occurrence of phenol exudation at the cut ends of explants was the main problem faced during the multiplication study (Figure 2 A). This phenolic exudation delayed the time required for sub-culturing accompanied with gradual browning of the shoots leaf and medium that eventually ends up in death of the explants without any response. However, proper removal of brown leaf sheath and recurrent transfer of explants to micro-propagation overcame this problem. Central European journal of Biology, 6(3):352-358.

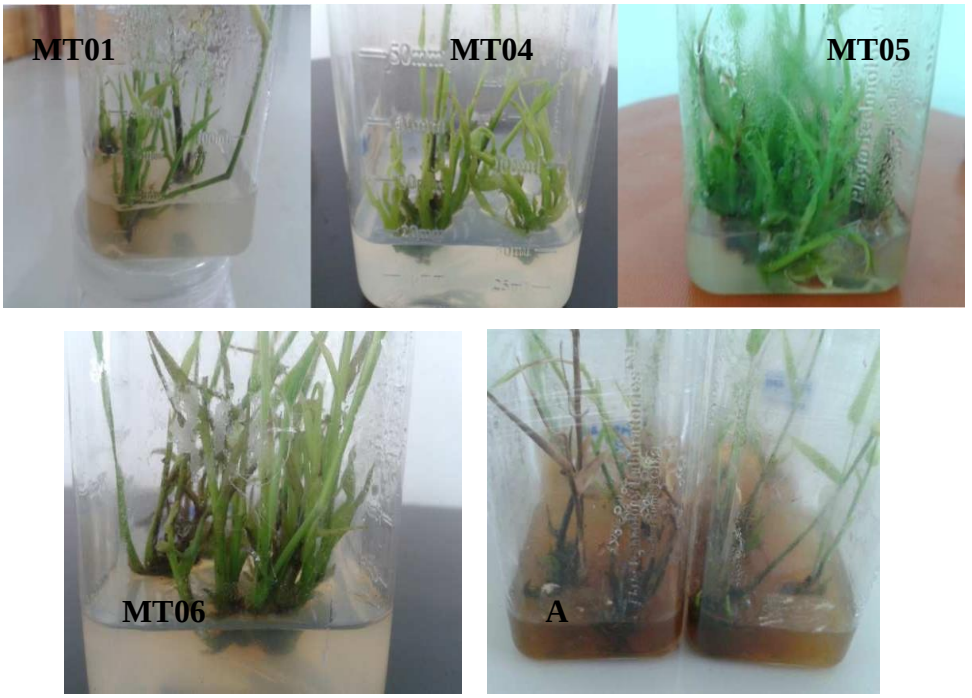

Figure 2. Effect of BAP and KN on *in vitro* multiplication of *O. abyssinica* microshoots after four weeks Note: MT01 = PGRs-free (control); MT04 = MS + 2.0 mg/l BAP; MT05 = MS + 4.0 mg/l BAP; MT06 = MS + 8.0 mg/l BAP; A = Phenol exuded from *in vitro* explants.

Effect of IBA and NAA on root induction

The development of healthy root system is required for the successful establishment of *In vitro* regenerated shoots to adapt the external environments. Therefore, in the present study the potential of IBA and NAA on rooting of *O .abyssinica* microshoots separately was investigated for drawing possible recommendation in future large scale use. For these studies bunch (three shoots per bunch) of microshoots were used. Accordingly, the ANOVA (Appendix Table 3) showed that type and concentration of auxins had highly significant effect ($P< 0.001$) on rooting percentage, root number and root length of *O. abyssinica in vitro* multiplication. In most of the treatments, rooting induction was started after 10 day culturing but in higher auxins concentration (8.0 mg/l) it was delayed up to 15 day culturing.

Table 4. Effect of Iba and Naa on *In Vitro* Rooting Of *O. Abyssinica* Microshoots at ½ Strength Ms Mediu

Treatment code	Con. Of PGRs (mg/l) BAP	M rooting percentage	mean shoots length(cm)	mean of roots length	Mean of roots length (cm)
IBA	NAA				
RT01	0	0	33.33±0.00^{c}	1.20±0.44^{g}	8.90±0.49^{a}
RT02	0.5	0	39.99±14.9^{c}	1.80±0.44^{f}	7.48±0.21^{b}
RT03	1	0	46.66±18.2^{c}	2.18±0.32^{f}	7.02±0.23^{c}
RT04	2	0	66.67±0.00^{b}	3.80±0.44^{de}	5.73±0.09^{d}
RT05	4	0	93.33±14.9^{a}	4.72±0.15^{b}	5.69±0.16^{d}
RT06	8	0	93.33±14.9^{a}	9.42±0.08^{a}	4.26±0.07^{e}
RT07	0	0.5	39.99±14.9^{c}	4.10±0.22^{cd}	3.34±0.12^{f}
RT08	0	1	53.33±18.2^{bc}	34.20±0.27^{cd}	3.12±0.11^{f}
RT09	0	2	66.67±0.00^{b}	4.50±0.00^{bc}	2.31±0.20^{g}
RT10	0	4	46.66±18.26^{c}	3.40±0.41^{e}	2.18±0.06^{g}
RT11	0	8	39.99±14.91^{c}	2.10± 0.22^{f}	1.21±0.21^{h}
CV		9.03	24.58	8.34	4.55
LSD			17.66	0.40	0.26

Note. Means in the same column that are followed by different letters are significantly different at P≤0.05 using Fisher's LSD test, LSD=Least Significant Difference, CV= Coefficient of Variation (%), ± = Standard Deviation.

With regard to average number of roots per clump, ½ strength MS + 8.0 mg/l IBA registered its superiority over the other treatments by exhibiting 9.42±0.08 root number per clump (Figure 3 RT06). The 2nd (4.72±0.15) (Figure 3 RT05) and 3rd (4.50±0.00) (Figure 3 RT09) highest number of roots per clump, which were not significantly different from each other, were obtained by fortifying ½ strength MS medium with 4.0 mg/l IBA and 2.0 mg/l NAA, respectively. The other level of concentrations showed lower mean number of root but significantly different from the control (Table 4). Parthiban *et al*. (2013) reported that the best root number for *B. Balcooa* was obtained from higher IBA concentration supplemented MS medium. In present study, the treatment MS + 8.0 mg/l IBA was found to be superior in both rooting percent and number of roots produced, which is in agreement with reports of Parthiban *et al*. (2013) and Diab and Mohamed (2008). According to the preliminary observation (data not included) from this study, further increase in IBA concentration (12 mg/l) causes poor root induction and condensed root length.

Perusal of Table 4 reveals that the longest root of 8.90±0.49 cm was obtained from PGRs-free ½ strength MS medium, followed by 0.5 and 1.0 mg/l IBA fortified ½ strength medium with respective mean length of 7.48±0.21 and 7.02±0.23 cm. The shortest mean root length (1.21±0.21) was obtained from 8.0 mg/l NAA fortified ½ strength MS medium. Generally, the mean root length decreased as auxins concentration increased. Comparing the two auxins, IBA supplemented ½ strength MS medium showed better efficiency in favoring root growth than NAA. The positive effect of IBA alone on rooting of In vitro propagated plantlets had been already established in different bamboo species *viz*. *D. giganteus* (Devi *et al*.

2012), *D. hamiltonii* (Agnihotri *et al*. 2009; Arya *et al*. 2012), *B. bambos* (Anand and Brar, 2013), and *D. asper* (Banerjee *et al*. 2011).

Differential effectiveness among auxins might be attributed to the concentration of free auxin that reached the target competent cells, and the metabolic stability of the auxins. Caboche *et al*. (1987) observed an inhibitory and toxic effect of NAA at above optimum concentration. IBA, because of its longer side chain (Nordström *et al*. 1991; Strader and Bartel, 2011), is more stable than IAA, which rapidly oxidizes and metabolize in plant tissues. IBA is not only stable than IAA but also is also convertible to IAA (Strader and Bartel, 2011). Therefore, its stability on one hand and convertibility to IAA on the other hand enable IBA to sustain auxin longer and enhances rooting in the culture media than NAA.

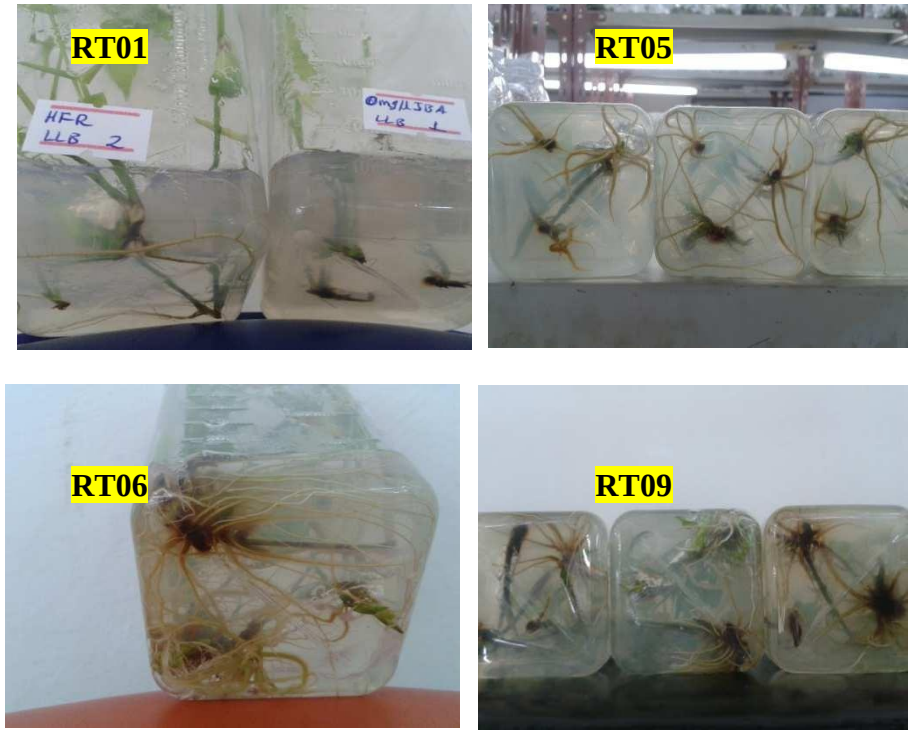

Figure 3. Effect of IBA and NAA on *in vitro* rooting of *O. abyssinica* microshoots after four weeks.

Note: RT01= PGR-free medium (control); RT05 = ½MS + 4.0 mg/l IBA; RT06 = ½MS + 8.0 mg/l IBA; RT07 = ½MS + 0.5 mg/l NAA; RT09 = ½MS + 2.0 mg/l NAA.

Acclimatization

In vitro developed plantlets have morphology and physiology abnormalities due to the In vitro culture conditions (Pospišilova *et al*. 1999). Direct transfer of In vitro plantlets to Ex vitro environment may result in rapid wilt and death (Lesar *et al*. 2012).

Therefore, acclimatization is essential for the survival and successful establishment of plantlets (Deb and Imchen, 2010). For this study, sixty In vitro well rooted *O. abyssinica* plantlets (Figure 3 A) were used and they were successfully acclimatized in the mixture of autoclaved forest soil, sand, and manure (2:1:1). The survival rate was 95% and 91.67% after 15 and 30 day of hardening, respectively.In the present study, during hardening stage some plantlets were found wilting in the first three days of transferring (Figure 3 B) and some leaves were dried up and subsequently detached from the shoots. This may be due to unrestricted loss of water from their leaves or low hydraulic conductivity of roots and root-stem connections (Pospišilova *et al*. 1999; Kumar and Rao, 2012). However, after ten day of hardening two-three new leaves were developed from each shoots. Gradually, the plantlets started growing and the leaf number increased as the plant height increases. Progressively, as the acclimatization process continue, color of the leaves turned to deep green and size of the leave get increased with the size of the plant (Figure 3C, E). Furthermore, proliferations of new tillers were observed after 30 days of hardening.

Summary and Conclusion

While tissue culture based rapid multiplication proved to be promising in different species of plants including bamboo, optimizing efficient and reproducible In vitro rapid multiplication protocol for Ethiopian low land bamboo species from seed would increase the social, environmental and economic benefit derived from the species. Therefore, this work was undertaken with the following objectives:

- To develop effective explants-surface sterilization procedure;
- To asses effects of different types and concentrations of cytokinin on shoot proliferation and multiplication;
- To asses effects of different types and concentrations of auxins on root induction and
- ToDevelop method to acclimatize *In vitro* seedling derived plantlets of *O. abyssinica*.

The studies was comprised of five experiments *viz.* explant sterilization, shoot induction, shoot multiplication, root induction and acclimatization, and were conducted in Holetta Plant Biotechnology Lab. The experimental design used was CRD with five replications except, for sterilization experiment where three replications were used. In the sterilization experiment, seeds/explants were washed with DDW water, liquid detergent, Curzate® R WP (0.25% (w/v)), and 70% alcohol. Then the dehusked seeds were treated with different concentration of NaOCl solution (3.0%, 4.0% and 5.0%) at different exposure time (15, 20, and 25 minutes).

The establishment of culture shoot from disinfected seed was carried out in full strength MS augmented by different concentration (0.5, 1.0, 2.0, 4.0, and 8.0 mg/l) for each of BAP and KN to induce shoots. Aseptically initiated propagules (three shoot per propagule) were ransferred in to full-strength MS medium supplemented by different BAP and KN concentrations (0.5, 1.0, 2.0, 4.0, and 8.0 mg/l) for shoot multiplication experiment.

Root induction experiment was carried out in half-strength MS medium fortified by different IBA and NAA concentration (0.5, 1.0, 2.0, 4.0 and 8.0 mg/l). Plantlets with well-developed shoots and roots were transplanted on a plastic bag containing mixture of autoclaved forest soil: sand: manure in 2:1:1 ratio for acclimatization in green house at different light intensity and humidity condition.

Among the different disinfectant treatments investigated, 3.0 % active chlorinated local bleach for 15 min duration recorded the highest contamination (100%) and by default the lowest clean explants percentages (0.00%). The lowest contamination (1.23%) and maximum clean explant percentages (98.76%) was recorded from 5.00% NaOCl for 25 min duration. The highest seed germination percentage (23.45%), the third minimum seed contamination percentage (4.93%) and 71.60% clean explants were recorded from treatment six (4.0% active chlorinated local bleach for 25 min). With regard to shoot induction and multiplication, type and concentration of cytokinins had significantly affected the duration of shoot induction, rate of shoot proliferation, number and growth of shoot and leaf number. Accordingly, 4.0 mg/l BAP supplemented MS medium and 2.0 mg/l KN supplemented MS medium showed statistically equivalent and the highest shoot proliferation percentage (86.67% and 73.33%) respectively. However, 4.0 mg/l BAP supplemented MS medium showed the highest shoot number (4.8), and requires 5.4 day to induce shoot. The leaf numbers were increased as the shoots length increased and the same was true as the cytokinins concentration decreased. Similarly, for the shoot multiplication experiment, among the tested cytokinin BAP at 4.0 mg/l gave the highest shoot number (11.33) and shoot multiplication rate (3.77%). Shoot length was decreased dramatically as concentration of cytokinins increased in both shoot induction and shoot multiplication experiments.

Similar to the shoot induction and multiplication experiment, in the root induction too, IBA was superior to NAA. The highest percent of root induction, 93.33%, was recorded from two concentrations of the same auxin, IBA: 4.0 mg/l and 8.0 mg/l IBA fortified half-strength MS medium. Nevertheless, the 8.0 mg/l IBA supplemented half-strength MS medium gave the maximum root number, 9.42 roots per clump.

Regardless of the significance of both types of auxins, IBA and NAA, in enhancing root induction and multiplication, the longest root (8.90 cm) was obtained without auxin treatment, PGRs-free half-strength MS medium induced longest roots Ultimately, hardening well rooted plantlets grown on pots filled with sterilized forest soil under a reduced light intensity by covering with polyethylene sheet and red cheesecloth and high humidity condition for 10 days assured a survival rate of as much as 92 % after 30 days in the open greenhouse environments. It can be also recommended that *In vitro* rooting of lowland bamboo seedlings using auxins especially IBA at 8.0 mg/l was the best for root induction and development. Hardening of *In vitro* propagated plantlets grown on pots filled autoclaved forest soil: sand: manure in 2:1:1 ratio at a reduced light and higher humidity condition in greenhouse condition was fruitful and effective method. This study can serve as baseline for germplasm conservation and further studies on this bamboo species.

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Micropropagation of peach *(Prunus persica (L.) Batsch)*

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Abstract

*Peach (**Prunus persica L. (Batsch)** belongs to Rosaceae family grow in temperate regions f or their edible fruits and ornamental values..Conventional vegettive propagation methods of peach are generally time consuming and seaso bound with low multiplication rate.This result a limited availability of quality planting materials. Recently, breeding p ractices in **Prunus** have been advanced by the development and application of biotechnolo gy including in vitro culture. To date, there is no report on micropropagation of peach i n Ethiopia. Hence, the objective of this study was to develop a protocol for micropro pagation of peach **(cv.Garnem).** Using nodal explants as explant source, steri lization, shoot initiation, multiplication, and rooting and acclimatization were carried out in randomized complete design with three replication at National Agricultural Biot echnology Laboratory. Sodium hypochlorite (NaOCl) of 5.0% of active chlorine (0.15, 0.25, a nd 0.5%) was used for surface sterilization and each concentration was tested against three diffe rent exposure time (10, 15, and 20 minutes). The effects of types of cytokinins (BAP and Kn)with different concentration(0.5, 1.0, 2.0, and 4.0 mg/L) alone and along with consistent suppl mentati on of 0.01 mg/LIBA and 0.5 mg/L GA3 were used for shoot initiation and multiplication. For roo ting half strength MS medium supplemented with different types of auxins (IBA and NAA) wi th different concentrations (0.75, 1.5, 3.0 and 6.0 mg/L) alone were used. Data were subjec ted to analysis of variance (ANOVA) and significant means were separated using Dunke n Multiple Range Test. Explants sterilized in 0.25% NaOCl for 15 minutes gave 85 .71% clean survived explants. The two cytokinins were significantly different on all p arameters considered for shoot initiation and shoot multiplication. Of the two cytokinin s BAP was better than Kn in early shoot initiation, shoot initiation and multiplication whi le Kn showed better shoot length and leaf number per shoot. BAP (1.0 mg/L) with IBA (0.01mg/L) and GA3 (0.5 mg/L) was found to be an optimum concentration for shoot initi ation (100%). BAP (2.0 mg/L) with IBA (0.01mg/L) and GA3 (0.5 mg/L) was found to be a n optimum concentration yielding maximum shoots number per explant (7.67).The m aximum rooting (42.86%),maximum root number/shoot (6.33) and longest rooting (7. 17 cm) were found at 1.5 mg/L IBA. NAA was found to be not effective. The plantlets were acclimatized in the glasshouse and survival percentage was 73.3%.*

Introduction

Prunus persica L. Batsch (peach) belongs to the Prunoideae, a sub family of Rosaceae with 8 basic and 16 somatic chromosome numbers (2n=16) (Hesse, 1975). Prunus include several approximately 400 species adapted primarily to the temperate regions of the northern hemisphere (krussmann,1986).Many species and cultivars are grown for their edible fruits.

while others are planted for their ornamental value. Peach is often called a stone fruit and one of the third most produced temperate fruit tree after apple and pear (Byrne *etal*.,2012). Due to its genetic and biological characterstics(small genome size:5.8 $x10^8$ Bp or 0.60+0.03 pg/diploid nucleus,taxonomic proximity to the other important species and short juvenile period 2-3 years (Baird *et al.* 1994)), it has been selected as a model species for studying genomics in the rosaceae (Abbot *et al.*; Aranzana *et al*. 2010).

China is the native home for peach and was domesticated there 4000-5000 years ago. (Aranzana *et al*. 2010). It is still possible to find considerable genetic variations among these species mainly in the mounainous areas of Central Asia from the Tian shan region in china to kurdistan, including Turkmensitan,Afghanistan,Iran (Martinez-Gomez *et al*. 2003).Peach cultivation was extended to Central Asia and later to Europe where it is known to have been cultivated by the Romans.Introduction of temperate fruits especially peach of to Ethiopia and North Africa is during the era of exploration and colonization of europeans in sixteenth centuries (Scorza Sherman,1996).Peach plants grow very well in a fairly limited range range, since they have a chilling requirement that tropical areas cannot satisfy and they are not very cold hardy.

Peaches are now commercially grown arounf the world between 30º and 45º latitude above and below the equator (Hesse , 1975); but this genus can be cultivated in any latitude with similar climatic conditions like in cool highland ecologies (the lower the latitude the higher the altitude). The total world production of peaches during the year 2012 was 18.1 million tons (Burke and Change, 2013).

Peaches are considered to be very delicious and attractive in flavor and aroma (Annua l report of ARI Tarnab,2008). It is widely popular for its sweet,juicy and beautiful blossoms.It has often been called the queen of fruits. Its beauty is surpassed only by its delightful flavor and texture. It is recommended for low-cholestrol,low fat,low and weight reduction or diabetic diets. Peaches are a good source of vitamin A, calcium and potassium. A medium size peach contains about calories. Recently,breeding practices in *Prunus* have been advanced by the development and application of biotechnology including micropropagation molecular marker, gene transfer, and and genome mapping (Martinez-Gomez *et al*. 2005).

The cloning of fruit tree species In vitro is a process that is introduced all over the world with the aim to obtain virus-free identical plant material,Micropropagation to propagation methods (Martinez-Gomez *et al.* 2005). Stylianides *et al.* (1988) reported that conventional vegetative propagation methods like budding, grafting or cutting,are generally cumbersome,time consuming and highly season bound with low multiplication rate and also quite difficult in particular on peach.

The challenges of temperate fruit production including peach are shortage of high yielding resistance variety, disease and pest and shortage of planting material. To increase productivity of peach fruit, yield potential,disease and insect pest reaction has been done at selected highland areas of Ethiopia. The production potential of peach cultivar,McRed, is 46 ton/ha which exceeds other temperate fruit production potential like apple and plum (Endale and Kerssa, 2006).The growth, productivity and longevity of a peach tree are influenced greatly by the slection of an appropriate rootstock. The cultivar Garnem*(Prunus persica var.garnum(G×N)* is highly immune to rootstock-knot nemtode to rootstock-knot nematode (*Meloidogyne javanica (Treub)* Cjotwood (Pinochet *,et al*.1999), the most vigorous and iron chlorosis resistant rootstocks (Jimenez et al. n.d.). It is a Spanish rootstock and ranks third following GF677 and seedling (Reighard, 2011.)

Jimenez *et al.* (n.d.) noted that Garnem affect tree size as measured by Trunk cross sectional area (TCSA). In 2009, a scion 'Calrico' showed the higher TCSA values on tissue culture, an important area of biotechnology can be used to improve the productivity of planting material through enhanced availability of identified planting stock with desired traits. Tissue culture technique results in large production in short period. There has been no report on micropropagation of peach in Ethiopia.

Materials and Methods

The study was conducted at the Plant Tissue Culture Laboratory, National Agricultural Biotechnology Laboratory, Holetta Agricultural Research Center from October 2013 to January 2014. Holetta is located 34 Km West of Addis Ababa in the West shewa Zone of Oromia region.

It has a latitude and longitude of of 9◦ 3'N 38◦ 30'E and an altitude of 2391 masl. It is characterized by average maximum and minimum temperature 22.2 degree centigrade, and 6.13-degree centigrage and average rainfall of 1100mm.

Source and Choice of Plant Materials

The growth, productivity, and longevity of a peach tree are influenced greatly by the se lection of an appropriate rootstock. The cultivar Garnem (prunus persica var.garnum (GxN) is highly immune to root-knot nematode (Meloidogyne javanica (Treub.) Cjotwood) (Pinochet *et al.* 1999), the most vigorous, and iron chlorosis resistant rootstocks (jimenez *etal*.nd.). It is a Spanish rootstock and ranks Young and healthy shoots (4-6cm long) containing axillary buds (third, fourth and fifth

nodes; form shoot apex, were excised and collected from three years old Garnem fruit crop by cutting with sterile scissor and used as explant. Actively growing shoots (juvenile plants) were taken as they are more responsive to shoot regeneration and proliferation than shoot explants from adult forms as reported by (Naghmouchi *et al.* 2008).

Explant surface sterilization

Before explants were placed on a medium (inoculated), it must be sterilized to make f ree of all micro- organisms. The leaves were removed from the explants and soaked in tap water and brought to laboratory.The explants were then thoroughly washed with tap water 3-5 times followed by liquid soap for 30 minutes with agitation to physically remove most microorganisms. Then the explants were treated with 70% ethanol for 30 seconds under laminar airflow cabinet. After pretreatment with ethanol, the explants were rinsed with distilled water three times, to lower the toxic effect of ethanol. They were then treated with three concentration levels (0.25%,0.5% and 1.0% (w/v) active ingredient of chlorine) of locally produced bleach,sodium hypochlorite (NaOCl) with 5% active (0.25%, 0.5% and 1.0% (w/v) active ingredient of chlorine) of ingredient of chlorine for varying exposure times (10, 15 and 20 minutes) (Table 1).

To increase the efficiency of NaOCl, a drop of Tween-20 per 50 ml solution was added as wetting agent. After decanting the sterilizing solutions under safe condition, the explants were washed three times each for five minutes with sterile distilled water to remove traces of NaOCl. Both ends of the sterilized explants were trimmed under aseptic condition to provide a cut surface and to remove any cells damaged by sterilant. Then the dorsal portion (1 cm long) nodal explants containing a single node were trimmed in a 'V-shape' to expose the xylem and increase MS(Murashige and Skoog 1962) medium fortified with 1 mg-1 BAP, 3% sucrose and 0.4% agar (Agar-Agar,Type 1)

The test tubes with cultured explants were properly sealed with parafilm and labeled.After wards, the cultures were transferred and randomly placed on the growth room shelf with a photoperiod of 16/8h light dark using cool white fluorescent lamps.(photon flux density, 40μmo m^{-2} s^{-1} irradiance) at 25± 2 degree centigrade and relative humidity (RH) of 70-80 %.

For each sterilization treatments seven test tubes were line up randomly in completely in randomized design (CRD) with three replications. The sterilization experiments data recorded include the number of contaminated ,dead and survived (clean) cultures after 10 days of culturing. The data were converted into percentage.

Shoot initiation

Sterilized explants' were cultured using similar procedures described above in test tubes containing 10 ml of MS (Murashige and Skoog 1962) medium fortified with 3% sucrose, 0.4 % agar (Agar-Agar,Type 1) and varying level of BAP (0.5,1.0,2 and 4mg 1-1) alone and in combination with 0.01 mg 1-1 IBA and 0.5 mg 1-1 GA3. In addition, Kn (0.5, 1.0, 2 and 4 mg l-1) alone and in combination with 0.01 mg l-1 IBA and 0.5 mg l-1 GA3 (Table. 2) was the other treatment seven test tubes were

line up randomly in CRD with three replications. All test tubes were placed on the growth room shelf with a photoperiod of 16/8h light/dark using of cool white fluorescent lamps (photon flux density,40µmol m-2s-1 irradiance) at 25 ± 20 C and RH of 70-80 %.

Shoot multiplication

To avoid the carry over effect of initiation media on shoot multiplication, initiated shoots were maintained on PGRs free MS medium with 1 g-1 activated charcoal for two weeks.Aseptically initiated 1.0-1.5 cm long shoots with 7-10 nodes were selected for shoot multiplication. Each shoot was trimmed at both sides, placed horizontally, and lightly pressed into the medium with agar, type 1). Furthermore, varying levels of BAP (0.5,1.0,2 and 4 mg 1-1) alone and in combination with 0.01mg 1-1 IBA,0.5mg 1-1 GA3.The other treatment combination was Kn (0.5,1.0,2 and 4 mg 1-1) alone and in combination with 0.01 mg 1-1 IBA 0.5 mg 1-1 GA3 (Table 1).

Table 1.Treatment combination for initiation and shoot multiplication experiment

Cytokinin BAP	Iba	GA3	Treatment code initiation	Multiplication
0	0	0	It01	Mt01
0.5	0	0	It02	Mt02
1	0	0	It03	Mt03
2	0	0	It04	Mt04
4	0	0	It05	Mt05
0.5	0.01	0.05	It06	Mt06
1	0.01	0.05	It07	Mt07
2	0.01	0.05	It08	Mt08
4	0.01	0.05	It09	Mt09
kn				
0.5	0	0	It10	Mt10
1	0	0	It11	Mt11
2	0	0	It12	Mt12
4	0	0	It13	Mt13
0.5	0.01	0.05	It14	Mt14
1	0.01	0.05	It15	Mt15
2	0.01	0.05	It16	Mt16
4	0.01	0.05	It17	Mt17

MS medium without PGRs was used as control. Seventeen treatments were employed and for each treatment four jars line up randomly in CRD with three applications.All shoots were incubated on multiplication medium for 6 weeks at 25+2^{o}C with a photoperiod of 16/8h light/dark using cool- white fluorescent lamps (photon flux density, 40µmol m-2 s-1 irradiance) and RH of 70-80%.

Rooting of shoots

Microshoot that were 1.5 cm long or above derived from the multiplication experiment, cultured In vitro for 6 weeks and then for 2 weeks on conditioning media with 1g/l acti vatedcharcoal, were used for rooting studies. The rooting response of these shoots was studied on half MS medium supplemented with 3% sucrose, 0.4% agar,1mg/1 activated charcoal and different concentrations of IBA (0.75, 1.5, 3.0 and 6.0 mg l-1) and NAA (0.75, 1.5, 3.0 and 6.0 mg l-1) (Table 3). The MS medium without PGRS was used as control.

For each treatment, seven test tubes, each with one plantlet, were lined up randomly in CRD with replications. All shoots were incubated on rooting medium for 4 weeks at 25+2degree centigrade with a photoperiod of16/8h light/dark using cool-white fluorescent lamps (photon flux density 40µmol m^{-2} s^{-1} irradiance) at 25 ± 2 degree centigrade and RH of 70-80%. Numbers of rooted microshoots, number of per micro-shoots and average root length (cm) were recorded after 30 days of culture on rooting media.

Table 2.Treatment combination for the rooting experiment

Treatment code	IBA
RT1	½ MS + 0
RT2	½ MS + 0.75
RT3	½ MS + 1.5
RT4	½ MS + 3.0
RT5	½ MS + 6.0
RT6	NAA
RT7	½ MS + 0.75
RT8	1/2MS+0.15
RT9	1/2MS +3.0

Acclimatization

The transplanted plantlets were kept in greenhouse under shade of polyethylene sheets and red cheese cloth, to reduce light intensity and maintain moisture, for one week and were sprayed with water two to three times every day. After 15 days, percentage of plantlets that were successfully acclimatized was recorded. Well-rooted plantlets were carefully removed from culture vessels. Besides, they were gently washed indistilled water to remove the remnants of agar. These plantlets with well-developed shoots and roots were transplanted on a tray containing a mixture of autoclaved river sand, forest soil and manure in a 2:1:1 (v/v/v) ratio and transferred to greenhouse for hardening. To reduce soil born contamination, the soil mixture was autoclaved before transferring plantlets.

Data collection and analsis

Shoot length, root number and root length were transferred into square root value for statistical analysis. Rooting percentage data was also transferred into arc sine value for

SAS computer software (version 9.1). Difference between means were assessed by Duncan's Multiple Range Test (DMRT) (P=0.05) (Gomez and Gomez, 1984).

Results and Discussion

Explant sterilization

Analysis of variance (ANOVA) (Appendix Table 3) revealed that concentration oflocal bleach and exposure time, and the interaction effect had highly difference (P< 0.01) on the contamination of growth media, death and survival level of explants. These findings agree with the result of Ahmed *et al.(*2003); the application of NaOCL at 0.25% (w/v) for 10 minutes gave minimum death (5%) and survival (55%). The highest explant contamination T (100 %) and least explant survival (0%) were recorded when explants were treated with 0.15% active chlorinated local bleach for 10 minute (Table 3).

This might be due to the insufficiency of the the concentration of active chlorine in local bleach and short exposure time to take life of microorganism from cultured explants. The least culture contamination and minimum tissue death of 9.51% and 4.75%, respectively, and the highest culture survival (86.1%) were recorded when explants were disinfected with 0.25% active chlorinated local bleach local bleach for 15 minutes.

Mahmood *et al.* (2009) also noted that treatment with ampicillin after surface sterilization with 0.3% NaOCl was very effective in reducing conamination to 10%. The data revealed that as the exposure time increased from 10 to 20 minute for all level of local bleach, the contamination decreased,and the same was true when contamination of local bleach time (appendix Figure 1). However, surface sterilization with higher concentration (0.5% w/v) of local bleach at and beyond 15 minute resulted in no contamination but high rate (71.43%) of explants mortality (Table 3).

This could be due to phytotoxic effect of 0.5% chlorinated local bleach at longer exposure time. Ervin and Wetzel (2002) had also noticed that high concentration of sterilant causing plant tissue death.Surface sterilization should not kill or break off the biological activity of explants , but the contaminants. Explants must be surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period (Oyebanji *et al.*2009). Therefore, 0.15%% (w/v) local bleach for 15 minute exposure time was found to be the most effective one for explants taken from peach shoots.

Table 3.Effect of different concentrations of local bleach and length of exposure time on contamination, mortality and survival of explants

NaOCL(local bleach) level(%)	Exposure time (minute)	Contamination (%)	Mortality (%)	Clean survived explant (%)
0.15	10	100[a]	0[f]	0g
0.15	15	71.43[b]	0[f]	28.57[f]
0.15	20	57.14[c]	0[f]	42.86[e]
0.25	10	28.57d[e]	0[f]	71.43[c]
0.25	15	9.51[e]	4.75[e]	85.71[a]
0.25	20	0[f]	28.57[c]	71.43[c]
0.5	10	4.75ef	14.29[d]	80.95[b]
0.5	15	0[f]	42.86[b]	57.14[d]
0.5	20	0[f]	71.43[a]	28.57[f]
CV (%)		12.87	15.28	53

Means in column with the same letter are not significantly different by DMR test at α=0 .05 significant level.

Shoot initiation

Analysis of variance revealed that concentration of BAP and Kn alone and incombination with IBA and GA3 had highly significant effect ($P< 0.0001$) on days for initiation ,percent shoot intiation and percent usable shoot (Appendix Table 4). The nodal explants started to initiate after a week of culturing on most of the media. Shoot initiation was observed in all treatments including the control, hormone free MS medium (81 % initiation) (Table), indicating that the cultivar has enough endogenous cytokinin and auxin combination for initiation.

Treatment	% of Usable							
BAP IBA	GAS Shoot initiation			% Shoot initiation	Days for initiation	Shoot number of explant	Shoot length	Leaf number of shoot
0	0	0	81+8.24^{cde}	0 ± 0^{e}	11.3+0.6^{e}	1.3+0.6^{e}	0.50 ± 0^{i}	4.7+0.6^{f}
0.5	0	0	81+8.24^{cde}	14.26+0^{d}	12.3+0.6^{d}	4.3+0.6^{c}	0.73+0.06^{fg}	7.7+0.58^{d}
-1	0	0	85.7+ 0^{bcd}	28.6 ± 0^{c}	10.3+0.6^{f}	6.0 ± 0^{b}	0.87+0.06^{cde}	8.0 ± 0^{cd}
-2	0	0	85.7+ 0^{bcd}	52.4+8.2^{b}	11.0+0^{ef} (A)	7.3+0.6^{a}	0.67 +0.06^{gh}	8.0 ± 0^{cd}
4	0	0	76.2+8.2^{def}	0 + 0^{e}	9.0 + 0^{g}	2.7+0.6^{d}	0.53+ 0.06^{i}	5.3+0.58f
0.5	0	0	76.2+8.2^{a}	13.3+0.6^{bc}	4.7+0.6^{c}	1.0 ± 0^{ab}	1.0 ± 0^{ab}	9.3+0.6^{ab}
1	0.5	0.01	100+ 0^{a}	81+8.24^{a}	13.3+0.6^{bc}	5.7+0.6^{b}	0.93+0.12^{bc}	8.7+0.6^{bc}
2	0.5	0.01	85.7 ± 0^{bcd}	57.14+0^{b}	14.3+0.6^{a}	7.7+0.6^{a}	0.80 ± 0^{def}	8.3+0.6^{cd}
4	0.5	0.01	71.43+ 0^{efg}	28.57+0^{c}	13.3+0.6^{bc}	.3+0.6^{b}	0.83+0.06^{cdef}	0.8+0
Kn	GA3	IBA	66.7+8.3^{fg}	9.5+8.2^{de}	13.3+0.6^{bc}	1.7+0.6^{e}	0.77+0.06^{efg}	8.7+0.6^{bc}
0.5	0	0	81+8.24^{cde}	14.26+0d	13.00+0cd	1.3+0.6e	0.90+0.1bcd	7.7+0.6d
1	0	0	90.5+8.3abc	28.6 ± 0c	13.00 +0cd	1.0 ± 0e	1.10 ± 0.2a	9.7+0.6a
2	0	0	81+8.2cde	14.26+0d	13.3+0.6bc	1.0 ± 0e	0.57+0.06ih	6.7+0.6e
4	0	0	52.4+ 8.2h	14.26+0d	1.0 ± 0e	0.50 ± 0.06	5.3+0.6f	76.2+8.2def

0.5	0.5	0.01	76.2+8.2def	9.5+8.2de	13.00+0cd	1.3+0.6e	0.73+0.06fg	6.7+0.6e
1	0.5	0.01	76.2+8.2def	9.5+8.2de	14.0+ 0ab	1.3+0.6e	0.73+0.06fg	6.3+0.6e
2	0.5	0.01	66.7+8.3gh	4.8+8.2de	14.0+ 0ab	0 ± 0f	0 ± 0j	0 ± 0g
4	0.5	0.01	76.2+8.2def	13.00+0cd	.73+0.06fg	6.3+0.6e	28.6 ± 0c	0.57+0.06ih
CV(%)			8.74	20.3	3.51	6.82	2.64	3.55

Table 4.The effect of bap and Kn alone and in combination Ga3 and Iba on mean of shoot Initiation, Shoot number per epxplant, shoot length and leaf number pershoot

For each parameter, values followed by the different letter are significantly different according to Duncan Multiple Range Taste at α=0.05 significant level ;CV=coefficient of variation.The maximum intiation percentage was 100% mg, with 81% usable shoot(Figure 2,IT07) on MS medium containing 1.0 mg/l BAP + 0.5 mg/l GA3 and 0.01 mg/IBA.

The maximum shoot initiation on a culture medium containing Kn alone was 90.5% with 28.6% usable shoots. Shoot initiation declined when Kn concentrations was lower lower and higher (Appendix Figure 2).The addition 0.5 mg/l GA3 and 0.01 mg/l IBA on MS medium containing Kn did not produce better shoot initiation than Kn alone. The best shoot initiation on culture medium fortified with Kn in combination with GA3 and IBA was 76.2% and 14.3 % usable shoots.

Shoot initiation of 81% was recorded on hormone free culture medium although their length were too short (<0.5cm) (Figure 2, IT01) and these shoots dried when sub-cultured. Most of the initiated shoots from a culture medium fortified with BAP in combination with GA3 and IBA were longer than those shoots regenerated on a culture medium fortified with BAP and Kn alone and Kn in combination with GA3 and IBA Table.

Figure 1. The effect of Bap and Kn alone nd in combination with Ga3 and Iba on shoot initiation of nodal explants.

Note: IT01 = hormone free (control); and IT07 = 1.0 + 0.5 + 0.01 mg/L BAP + GA3 + IBA.

Shoot multiplication

The result of the experiments conducted to study the effect of different concentrations (0.5, 1.0, 2.0, 4.0 mg/l) of BAP and Kn alone and in combination with 0.5mg/l GA3 and0.01mg/l IBA, and also the control effect on the microshoot multiplic ation of *Prunus persica* is shown in (Table 5). Analysis of Variance (Appendix Table 4) revealed that the treatment had highly significant effect on mean number of shoots, length of shoot, and mean number of leaves.

The combination of IBA, GA3 and BAP was more effective in shoot multiplication co mpared to BAP alone (Appendix Figure 3). Data indicated that the maximum mean number of shoots(7.3 - 7.7) per explant was found on MS medium containing 2 mg/l BAP alone, and a combination of 2 mg/l BAP, 0.5 mg/l GA3 and 0.01 mg/l IBA (Table 5). There was no significance difference regarding in shoot number between the two treatments.

However, 2mg/1 BAP,0.5mg/GA3 and 0.01 mg1/IBA showed better number of shoots(7.7) and shoot length (8.0 cm) as compared to 2mg/l BAP (Table 5). This is consistent with the findings of Demsachew (2011) on Malus domestic acv.MM106 and Anna. Hammat and Grant (1997) also reported the the hormonal balance; BAP(2mg/l), IBA 0.1 mg and GA3 0.1 mg/1 with 1mm phloroglucinol showed positive influence in Prunus avium multiplication.

There was no sign of shoot multiplication on media supplemented with 4.0 mg/1 kn,0.5 mg/l GA3 and 0.01 mg/l IBA. All samples cultured on which were dried (Figure 2,MT17). The addition of IBA and GA3 in MS medium fortified with Kn did not produce better shoot number per explants as compared to those explants cultured on MS medium fortified with Kn alone. Shoot multiplication on Kn supplemented MS medium did not differ with shoot multiplication on control MS medium. This indicates that the use of Kn for *P.persica cv.*Garnem micropropation is not advisable.

Further increase in the concentration of BAP beyond the optimal level reduced the number of shoots indicating an upper limit in concentration (Appendix Figure 3). When the concentration of BAP increased to 2-4 mg/l on multiplication medium,the shoots turned very dwarf and bushy.They also callused and became to red color (Figure 2, MT04). The type and concentration of cytokinin influenced shoot multiplication.

Among the two cytokltinins (BAP/Kn) in combination with GA3 and IBA,BAP was better and more effective than Kn,for shoot multiplication. This result agreed with previous reports of Kalinina and Brown (2007) and Mansseri-Lamrioui *et al.*(2011) that found multiple shoot formation in nine ornamental *Prunus* species using 1 mg/l BAP, among the studied cytokinins like 2ip,Kn and BAP.

The maximum shoot length (1.1+0.2 cm) was found at 2.0 mg/l Kn and the minimum (0.5cm) was at the control treatment (Table 5). The maximum and minimum number of leaves 9.7 + 0.6,4.7+0.6) (per shoot was recorded on 2.0 mg/l Kn and control treatment respectively (Table 5). The number of leaves per shoot increased as the shoot length increased. Shoot length was better on Kn supplemented MS medium than BAP supplemented MS medium. Hormone needed to power the shoot multiplication. Hence, 2.0 mg/l BAP in combination with 0.5mg/l GA3 and 0.01 mg/l IBA is recommended for shoot multiplication of *P,persica cv.* Garnem, since it produces the higher number of shoot per explants as well as comparable elongated shoots than other treatments(Figure 2, MT08).

Figure 2. The effect of BAP and Kn alone and in combination with GA3 and IBA on shoot number per explant, shoot length and leaf number per shoot

Note: MT04 = 2.0 mg/L BAP; MT05 = 4.0 mg/L BAP; MT08 = 2.0 + 0.5 + 0.01 mg/L BAP + GA3 + IBA; and MT17 = 4.0 + 0.5 + 0.01 mg/L Kn + GA3 + IBA

Root induction

In order to produce complete plantlets, micr-oshoot derived from multiplication satage were separated and transferred to half-strength of MS rooting medium fortified with four concentrations (0.75,1.5,3.0 and 6.0 mg/l) of IBA and NAA. Half –strength MS medium without PGRs was used as a control. Analysis of variance revealed that concentration of IBA and NAA had highly significant effect (P<0.0001) on rooting %,root number per shoot and root length (Appendix Table).

The results presented in Table 5 showed that the addition of IBA (1.5 mg/l) with 1g/l AC was the only treatment, which initiated roots. The percentage of rooting (42.86%), number of root per shoot (6.3) and root length (7.2 cm) were recorded (Table 5 and Figure 3 A). Earlier studies on peach by Alanagh *et al.*(2010), reported that the highest rate of rooting (up to 40% and the number of roots per shootlet(2.62 +0.56) was obtained on the induction medium supplemented with 2mg/l IBA.

Table 5. The effect of Iba and naa on rooting %, root number per shoot and root leng

Root length (cm) IBA	NAA	Rooting (%)	Number of Root	Number of Shoots
0	0	0^{b}	-	-
0.75	0	0^{b}	-	-
1.5	0	42.86a	6.3	7.2
0	0	0^{b}	-	-
3	0	0^{b}	-	-
6	0	0^{b}	-	-
0	0.75	0^{b}	-	-
0	1.5	0^{b}	-	-
0	3	0^{b}	-	-
0	6	0^{b}	-	-
CV (%)		2.5	3.96	6.54

The application of NAA resulted in no rooting of Garnem shoots. This might be explained by the NAA resistance of degradation by the auxin-oxidase enzyme (Smulders *et al.* 1990). Nissen and Sutter (1990) have shown that in tissue culture, media IAA is rapidly photo-oxidized (50% in 24h), while the IBA is oxidized slowly(10%) and NAA is very stable. Slow movement and delayed degradation of IBA may be the reason for better performance as compared to IAA and NAA may also enhance rooting via increased internal free IBA or may synergistically modify the action of endogenous synthesis of IAA(Krieken *et al.* 1993).

Figure 3. Root formation after 30 days from cultivation on rooting medium containing 1.5 mg/L IBA

(A), Polythelene sheet and cheese close covered shoots (B), shoots at a time of transplantation (C), and shoots after 15 days (D)

Acclimatization

Developed rooted plantlets were gently removed from the culture tubes,washed initially to ac adhered agar and traces of the medium to avoid contamination . Then they were transferred to plastic planting tray containing a mixture of autoclaved river sand, forest soil sheet and cheese cloth (Figure 3B) were covered over the plantlets to ensure high humidity and reduce light intensity for one week and watered regularly.

The acclimatized plantlets of In vitro regenerated plants under controlled atmosphere and humidity. After 15 days they look healthy plants (Figure 3 D).

Summary and Conclusion

Peach belongs to the genus *prunus* of the family rosaceace, with 8 basic and 16 stomac chromosome numbers. It is grown for its edible fruits. Peach is often called a stone fruit and the third most produced temperate fruite tree species after apple and pear.

Peaches are now commercially grown around world between the world between 30 degree centigrage and 45 degreecentigrage latitude above and below te equator;but this genus can be cultivated in any latitude with similar climatic conditions like in cool highland ecologies (the lower the latitude,the higher the altitude.

Conventional vegetative propagation methods: budding, grafting or cutting are generally cumbersome, time consuming and highly season bound with low multiplication rate and difficult in particular on peach. Tissue culture can be used to improve the productivity of planting material with desired traits. However, there has been no report on micro-propagation of peach in Ethiopia. This work was aimed to find out the following objects: To assess effective explants surface sterilization; to assess effect of different types and concentrations of auxin on root induction and to acclimatize In vitro seedling.

The study was comprised of five series experiments (explants sterilization, shoot induction, shoot multiplication, root induction and acclimatization). The experimental design used was CRD with three replications. Two months old and healthy Garnem shoots were excised and collected by using sterile scissor and used as explant.

The explants were washed with tap water liquid detergent and 70% alcohol. Then they were treated with different concentration levels of NaOCl for varying exposure time. Aseptically initiated 1.0-1.5 cm long shoots were placed horizontally and lightly pressed into the multiplication medium fortified with of (0.5.1.0.2.0 and 4.0 mg/l) BAP and Kn in combination with IBA (0.01 mg/l and GA3 (0.5 mg/l). Micro-shoot that were 1.5 cm long or above were cultured on half strength MS rooting medium supplemented with 3% sucrose 0.4%,1mg/l activated charcoal and different concentrations of IBA(0.75, 1.5,3.0 and 6.0 mg1-1) and NAA (0.75,1.5,3.0 and 6.0 mg11). Plantlets with well-developed shoots and roots were transplanted on a tray containing potting mixture in a 2:1:1 ratio and transferred to greenhouse for hardening.

The frequency of bacterial or fungal contamination was reduced to a very low level (9.51%). Maximum aseptic culture was obtained from the nodal explants taken from two month old peach shoots (4-6 cm long top ,to down) and treated in 70% ethanol for 30 seconds and then in NaOCl (0.25% w/v) containing one drop of 'Tween 20' per 50ml solution for 15 minutes.

From the comparisons between treatments of two cytokinins with control, it was determined that the culture medium should be augmented with exogenous PGRs, GA3 and IBA, respectively) for new shoot regeneration and PGRs(2.0+0.5+0.01 mg/l BAP,GA3 and IBA, respectively shoot multiplication. The best rooting (42.86%) was obtained at 1.5 mg/l IBA and it is proved to be better rooting PRG for each in terms of rooting percentage, number of roots per shoot and root length as compared to NAA. The maximum acclimatized plantlets (73.33 %) were achieved under controlled atmosphere and humidity.

Use of the developed micro-propagation protocol for large-scale production of peach and distributing them to farmers to increase peach fruit production in Ethiopia should be given due attention. Sterilant for surface sterilization other than sodium hypochlorite and different exposure time duration should be tried. Shoot explants may able to tolerate other sterilant and low exposure time explants other than nodal explants should be used for further development of the protocol for better result. To achieve an efficient protocol for the mass propagation of Garnem, further experiments especially in the rooting stage need to be done by using auxins combinations and/or changing mineral concentration of culture medium before any commercialization.

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Amylase Production from Bacterial Isolates of Forest Soil, Selected Agricultural Waste and Kitchen Waste

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Abstract

Amylases are starch hydrolyzing enzymes that have a wide spectrum application in industrial and non-industrial sectors. In Ethiopia and other east African countries, where the production of starch rich cereals and tubers is high, the application of amylases in the afore-mentioned sectors is believed to have a greater socio-economic benefit. The aim of the current study was to isolate amylase producing bacteria from samples collected from forest soil, selected agricultural waste and kitchen waste within the premises of Holetta Agricultural Research Center (HARC) and to optimize their cultivation conditions for maximum amylase production. One hundred fifty-seven bacterial colonies were isolated and screened for amylase activity, out of which 50 (31.8%) were positive for the desired activity. Three isolates (FS11, M5 and S5) were selected for the study as they displayed relatively high activities and were subsequently identified as members of Bacillus based on morphological, physiological and biochemical tests. The effects of incubation period, temperature, initial pH of the media, carbon and nitrogen sources, and size of inoculum in amylase production were studied using laboratory- scale submerged fermentation processes. Bacillus sp.M5 produced maximum amylase (0.107U/ml) after 36 hrs of incubation whereas Bacillus sp. F11 and S5 yielded maximum amylase activities, 0.109 U/ml and 0.065U/ml, respectively, after 48 hrs of incubation. Media adjusted with an initial pH of 7 was found to support maximum amylase production in all isolates. An incubation temperature of 45°C was optimum (0.091U/ml) for FS11 whereas 40 and 37°C were the optima for M5 (0.101U/ml) and S5 (0.064U/ml), respectively. Non-defined potato starch was found to induce maximum amylase production in FS11 (0.096U/ml) and M5 (0.112 U/ml), while maltose gave the highest amylase production in S5 (0.072U/ml). The production medium containing 0.5% yeast extract was found to be optimum for FS11 (0.085U/ml). A 0.5% Malt extract was optimum for maximum amylase production in S5 (0.071U/ml). The best percentage of inoculum level for maximum production of amylase was 4% in FS11 and 6% in M5 and S5. The crude amylases from the isolates were observed to hydrolyze all of the four raw starches provided and for some raw starches; their conversion efficiencies were even greater than commercial soluble starch. The crude amylase from FS11 was observed to release maximum sugar from sorghum flour (325.3µg/ml) with conversion efficiency of 124.3% when compared to commercial starch (261. 7µg/ml, 100%). M5 released high reducing sugar (303.8µg/ml) from maize flour and its conversion efficiency was113.2%, however, from starch the amount of reducing sugar produced was 268.5µg/ml. In the case of S5, the amylase released maximum sugar from wheat flour (195.1µmg/ml, 110.9%). This study showed that the isolated Bacillus spp. were found to be promising in their potential of amylase secretion which could be maximized by optimization of the cultivation conditions. Since their crude amylases have the potential of hydrolyzing raw starch sources, their large-scale cultivation seems to be promising for use in industrial and non-industrial applications.

Introduction

Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view. High temperatures or pressures needed to drive reactions lead to high-energy costs and may require large volumes of cooling water downstream. Harsh and hazardous processes involving high temperature, pressures, acidity, or alkalinity need high capital investment, and specially designed equipment and control systems. Unwanted by-products may prove difficult or costly to dispose off. High chemicals and energy consumption as well as harmful by-products have a negative impact on the environment. These drawbacks can be virtually eliminated by using enzymes (Pandey *et al*. 2013).

Enzymatic processes are fast replacing chemical processes as the merit of enzymes as industrial catalysts shoulder on their unique properties: enzymes are highly specific and efficient in catalysis; they are biodegradable and carryout catalytic reactions under mild conditions thereby saving energy and resources (Tewari, 2007). With annual growth rate of 6%, the global market for industrial enzymes reached at 3.3 billion US$ in 2010 and is expected to reach 4.4 billion US$ by 2015 (Beyer, 2011; Pandey *et al*. 2013).

The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Proteases, amylases and cellulases are the largest group of hydrolytic enzymes used in different industries such as the dairy, starch, textile, detergent and baking industries (Kirk *et al*. 2002). They account for approximately 75% of the industrial enzymes produced, of which 30% is covered by amylases (Mobini-Dehkordi and Javan, 2012).

Amylases are capable of digesting glycosidic linkages found in starch to give diverse products including dextrin and progressively smaller polymers composed of glucose units. They are among the most important enzymes and are of great significance in present day biotechnology. Amylases are obtained from diverse sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. In spite of the wide distribution of amylases, microbial sources are mainly used for industrial production. Amylases from filamentous fungal and bacterial sources are the most commonly used in industrial sectors (Pandey *et al*. 2000; Senthilkumar *et al*. 2012). This is due to their advantages such as cost effectiveness, consistency, less time and space required for production as well as ease of process modification and optimization (El-Fallal *et al*. 2012).

Though both filamentous fungi and bacteria are the most known and important sources of amylase, amylases of bacterial origin are generally preferred due to several characteristic advantages that they offer (Pandey *et al*. 2000) and the attention on amylases of bacterial origin has been increased greatly in present-day enzyme technology. Amylase production from bacteria is economical as the enzyme production rate is higher in bacteria as compared to other microorganisms (Kaur *et al*. 2012). The ability of bacterial stains to secrete large amount of extracellular proteins has made

them well suited for the industrial enzyme production (Singh *et al*. 2012). The ubiquitous nature of bacteria and their capability of inhabiting both mesophilic and extremophilic environments (hot springs, alkaline and acidophilic etc.) has widened the isolation sites and isolation of potential bacteria with unique characteristics. Bacterial amylases show unique characteristics such as thermophilic, thermotolerant, alkaline and acidophilic properties (Kandra, 2003).

Indeed, bacterial enzymes are known to possess more thermostability than fungal amylases (Eke and Oguntimehin, 1992). Thus, many bacterial amylases have been found to withstand the harsh industrial conditions. Such enzymes are particularly obtained from bacteria belonging to the genus *Bacillus*. These bacteria are industrially so important because of their rapid growth rates that lead to short fermentation cycles. Besides,their capacity to secrete proteins into extra-cellular medium, and general handling safety (Pandey *et al*. 2000; Schallmey *et al*. 2004), their heterogeneity and very versatile nature in their adaptability to the environment (Bozic *et al*. 2011; Khan and Priya, 2011), their broad biochemical diversity and susceptibility to genetic manipulations (Al-Allaf, 2011).

It is estimated that the enzymes of *Bacillus* species (spp.) make up 50% of the total global enzyme market (Schallmey *et al*. 2004). *Bacillus* spp. amylases have applications in a number of industrial processes, such as the food, fermentation, textile, and paper industries (Pandey *et al*. 2000). That is why the current study focused on production of amylase from bacterial isolates mainly from *Bacillus* spp.
Conventionally, industries prefer Submerged Fermentation (SmF) to Solid State Fermentation (SSF) for the large-scale production of microbial enzymes due to the ease of controlling the process parameters such as pH, temperature, aeration and oxygen transfer and moisture (Souza and Magalhaes, 2010; Benjamin *et al*. 2013). The production of amylases from bacteria is dependent on type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements, metal ions, pH, temperature, time of incubation and thermostability (Haq *et al*. 2010). Thus, optimization of fermentation conditions, particularly physical and chemical parameters, is important in the development of fermentation processes due to their impact on the economy and practicability of the process (Akcan, 2011).

Amylases have diverse applications in a wide variety of industries such as food (including the clarification of haze formed in beer or fruit juices), fermentation, textile, paper, and detergent and sugar industries. It can be also used in field related to biotechnology such as, removing of environmental pollutant. With the advent of new frontiers in biotechnology, the spectrum of amylase application has expanded into many other fields, such as clinical, medicinal and analytical chemistry. Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders (Pandey *et al*. 2000; Mobini-Dehkordi and Javan, 2012). In addition, amylases have an application for pretreatment of animal feed to improve digestibility (Khan and Priya, 2011).

In the East Africa region, there is a huge potential for the production of starch-based products for food and non-food applications. Cassava, sweet potato, *enset* and other root crops widely grown in the region can be used as starting material for the production of starch hydrolysates using amylase enzymes. For example, starch from cassava can be converted to glucose and fructose syrups, maltose syrups, maltodextrins, etc. with huge potential application in the food and non-food industries. One application of starch hydrolysate is to replace the pricey malt used for brewing. Currently one ton of malt costs about US$1000 and barley produced in the region do not meet the demand for malt. For example, in Ethiopia more than half of the malt is imported where more than 40,000 ton of malt with a cost of US$ 40 million is imported every year. In addition, because of its potential to grow large quantities of cotton, the region has an enormous potential for the growth and expansion of the textile industry. In this industry, amylases are among the important enzymes that would be used during the fabric process (Amare Gessesse *et al*. 2011). Despite the fact that the region has such huge potentials, to date no one uses these processes for value addition because of the cost factor of pure amylases, which could be imported for the above-mentioned purposes.

In Ethiopia, studies on hydrolytic enzymes have been carried out over the last two decades mainly in Addis Ababa University, which resulted in the publication of some papers and several unpublished graduate student thesis researches. Among the hydrolytic enzymes, amylases were one of the focus areas; and accordingly some researches on amylase production using bacteria and fungi have been carried out (Mamo *et al*. 1999; Mamo and Gessesse, 1999a and 1999b; Muluye Teka, 2006; Ayalew Damtie, 2011). Many of these enzymes are potentially attractive for large-scale industrial applications. Most of the studies conducted at Addis Ababa University were focused on production from mostly bacteria and fungi inhabiting extremophilic habitats (alkaline soda lakes, hot springs).

In contrast, very little attention has been given to amylase production from mesophilic bacteria inhabiting other sites such as forest soils, agricultural wastes, kitchen waste etc. and as a result, the potential of such bacteria to serve as source of amylase has remained largely neglected. Undoubtedly, the search for potent amylase producers from among the mesophilic bacteria would be of practical value in alleviating the shortage of amylase supply in our cottage industries and thereby boosting the Ethiopian enzyme-based industrial biotechnology. Thus, this study was designed to isolate amylase-producing mesophilic bacteria and produce potent amylases of various industrial and non-industrial applications.

Specific objectives

- To isolate amylase producing bacteria from forest soil, selected agricultural waste and kitchen waste and identify the isolates based on their morphological, physiological and biochemical characteristics;
- To optimize cultivation conditions of the isolates for maximum amylase production and
- To evaluate the potential of crude amylase extract on the hydrolysis of raw starches.

Materials and Methods

The study area

The experiment was conducted at Holetta Agricultural Research Center (HARC), Microbial Biotechnology Laboratory, which is located 34 Km West of Addis Ababa, 09004'N latitude and 380,30'E longitude and at an elevation of 2390 meters above sea level. The annual average rainfall is 1100 mm and the temperature of this location ranges from 6.13 to 22.2°C (EIAR, 2012). The study period lasted from December 26 2012 to September 20 2013.

The research design

The design of the research involved screening for amylase producing bacterial species from randomly selected forest soil, agricultural waste and kitchen waste samples and experimentally determining both the optimal growth and reaction conditions for maximum production and activity, respectively, of amylases. All experiments for amylase production were conducted in duplicates with Erlenmeyer flasks of 250 ml capacity and average values were taken. The experimental design used for optimization of cultivation conductions was conducted following the OVAT (one variable at a time) method (Abdel-Fattah *et al*. 2013).

Sample collection

Samples used as source of amylase producing bacteria were collected from different sites within the premises of HARC. Forest soil, kitchen waste (sludge), decomposed wheat straw and manure (Agricultural wastes) were the sample sources. For the forest soil, the soil samples were taken from 3 to 5 cm depth beneath the outer layer of the forest ground. From agricultural wastes, manure was taken from the dairy farm, whereas the decomposed wheat straw was taken from the area around wheat yield harvesting place ("Awduma"). Samples from kitchen waste were collected from the sludge (semi-solid waste) which is disposed from the cafeteria. The sample from each sample source was taken to the laboratory using plastic bag.

Media composition and preparation

(Santos and Martins, 2003; Swain *et al*. 2006) stated that solid media used for isolation, screening and selection of amylase producing bacteria were composed of the following nutrients:

- (g/ml): 1% soluble starch ;
- 0.2% yeast extract ;
- 0.5 % peptone ;
- 0.05% $MgSO_4$;
- 0.05% NaCl;
- 0.015% $CaCl_2$ and
- 2% (w/v) agar at pH 7

While liquid media used for production of amylase and preparation of inoculums were composed of the same nutrients except that the carbon sources and nitrogen sources

were replaced by different defined and non-defined carbon sources as well as organic and inorganic nitrogen sources as indicated in sections 3.11.4 and 3.11.5 and no agar was added. Before autoclaving, the pH of the final medium was adjusted to 7 using solutions of 1M NaOH and 1N HCl. Then the resulting media were sterilized by autoclaving at a temperature of 121ºC for 15 minutes (Qader, 2006; Jogezai, 2011).

Isolation, screening and selection of amylase producing bacteria

Isolation and screening of amylase producing bacteria

Ten grams of sample from each source was added to flask containing each 100 ml of normal saline solution (0.85% NaCl) (Punitha *et al*. 2012). The contents of the flasks were mixed for 30 minutes on a shaker set at 120 rpm. After 30 minutes of shaking, 20 ml of the suspension was heat shocked at 80°C for 10 minutes in water bath for the purpose of killing the vegetative cells and retaining spore forming microbes (Altayar and Sutherland, 2006; Maal *et al*. 2011; Wahyudi *et al*. 2011). The heat-shocked sample was left at room temperature for 10 minutes until it cooled down and was serially diluted to give 10^{-1} to 10^{-6} dilutions following standard methods. From the diluted samples, 0.1ml of each of 10^{-3} to 10^{-6} dilutions was aseptically spread-plated on to starch agar plate containing 1% starch. The resulting plates were incubated at 37ºC for 24-48 hrs (Punitha *et al*. 2012).

After 24 hrs of incubation, bacterial isolates were screened for amylolytic activity by starch hydrolysis test (Aneja, 2003; Ashwini *et al*.2011). Gram's iodine solution was over-layered on the agar plates that contained mixed isolated colonies. The excess solution was poured offafter 15 minutes (Meenakshi *et al*.2009), and observation was made to note the starch hydrolysis zone around the colonies. Presence of blue color around the growth indicated negative result and a clear zone of hydrolysis around the growth indicated positive result. Isolates that produced a clear zone of hydrolysis were considered as amylase producers. For each positive colony, a code was given and the diameter of the zone of clearance around and beneath the colonies was recorded and used for subsequent investigations. Some positive colonies having relatively higher clear hydrolysis zone from each source were selected and sub cultured for further investigation.

Selection of the best amylase-producing bacteria

Selection of best amylase producing bacteria from the three samples was done independently. i.e. isolates of forest soil were compared with each other and the best was selected. The same is true for isolates of agricultural and kitchen wastes (one isolate per sample source). Even though presence of a halo around the colony and the relative diameter of the clear hydrolysis zone is indicative of amylase production (Sohail *et al*. 2005), additional criteria was used for selection of most potent isolates. Since there is no standardized figure limit for the diameter of clear hydrolysis zone and for best induction of amylase production, quantitative test of amylase activity using liquid medium was added in addition to the comparison of the diameter of clear hydrolysis zone (Castro *et al*. 1993).

Therefore, the selection was done based on the comparative results of two tests. The first test was made by measuring the diameter of the hydrolyzed zone formed by the colony of the respective isolates while the second one was made by measuring of the amylase activity using the DNSA method. Determination of the amylase activity using DNSA was done both after 24 and 48 hrs of incubation at 37°C and pH 7 in liquid medium containing 1% starch (Ibrahim *et al*. 2013).

Characterization and identification of the selected isolates

Morphological characterization

The isolates were characterized morphologically based on their microscopic and macroscopic appearance (i.e. cellular morphology and colonial morphology, respectively).

Microscopic appearance of isolates

Gram staining

Gram staining technique was performed using fresh cultures of 24 hrs old following standard procedures (Harley and Prescott, 2002) to study the microscopic appearance of the isolates. The gram stained cells were examined under the bright field compound microscope (LABOMED) using the 4x to 100x (oil immersion) objectives to observe cell shape and gram reaction.

Endospore staining

Endospore staining was performed to find out the presence or absence of endospores. Slide preparations were made for fresh cultures grown for 72 hrs, stained and examined microscopically for endospores using the methods described in Harley and Prescott (2002).

Macroscopic appearance of isolates

Colony morphology

Colony characteristics such as size, color, shape, margin, texture and elevation were studied after growing the isolates on agar medium containing 1% starch for 24 hrs at 37°C (Ashwini *et al*. 2011; Tom-Sinoy *et al*. 2011).

Motility test

Motility test agar media were inoculated with 100µl of broth culture (Harley and Prescott,2002). Then using inoculating needle the agar containing broth culture was stabbed in a straight line and then incubated at 37°C for 24. After 24 hr incubation, observation was made for migration of the isolates away from the original line of inoculation which means that the test organism were motile (Muruga and Anyango, 2013).

Physiological characterization

Each isolate was physiologically characterized by studying its responses to the following three tests: growth in 7% NaCl, growth at different levels of temperatures and pH (Jyoti *et al*. 2009).

Growth at 7% NaCl

A fresh 24 hr old pure colony from each isolate was streaked on starch agar medium (1% starch, 0.2% peptone, 0.5% yeast extract, 0.05% $MgSO_4$, and 0.015% $CaCl_2$) containing 7% NaCl and observed for growth after 24 hr of incubation (Jyoti *et al*. 2009).

Growth at different incubation temperature

Twenty-four hours old pure colony from each isolate was streaked on a starch agar media and their growth profile was observed by incubating at different incubation temperatures (20, 25, 30, 37, 40, 45, 50 and 55°C) for 24 hr of incubation (Jyoti *et al*. 2009).

Growth at different pH conditions

To study the effect of pH on the growth of the isolates**,** 24 hrs old pure colony from each isolate was streaked on a starch agar media adjusted at different pH levels (3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13) for 24hr of incubation (Jyoti *et al*. 2009).

Biochemical characterization

The biochemical characterization of the isolates was performed using tests for catalase production, casein hydrolysis, urease production, starch hydrolysis, H_2S production and carbohydrate fermentation patterns for 12 carbon sources (Ashwini *et al*. 2011; Tom-Sinoy *et al*. 2011; Sivakumar *et al*. 2012).

Catalase test

Catalase production was detected by adding 1to 3 ml of 3% H_2O_2 to 24 hrs starch agar slant culture and using an uninoculated medium as a negative control. Formation of effervescence confirms a positive test for catalase while its absence indicates negativity (Harley and Prescott, 2002).

Casein hydrolysis test

The isolates were streaked on casein containing media (1% casien and 2.8% nutrient agar) and incubated for 24 hrs. After incubation, TCA (trichloro acetic acid) was added over the cultures to observe the clear hydrolysis zone formed by the colony if the organism is positive for casien hydrolysis (Vishwanatha *et al*. 2010).

Urease production test

Two hundred micro liters of culture broth from each isolate was inoculated into 10 ml of urea broth media with pH indicator phenol red (0.1g Yeast extract, 0.09g Mono-potassium phosphate, 0.095g Disodium phosphate, 20.0g Urea and 0.01g phenol red in 1000ml distilled water, pH 6.9) and incubated at 37ºC for 24-48 hrs. After 24 hrs

of incubation, observation was made if the organism is positive for urease production or not. When urea is hydrolyzed, ammonia accumulates in the medium and makes it alkaline. This increase in pH causes the indicator to change from orange-red to deep pink or purplish red (cerise) and is a positive test for urea hydrolysis. Failure of a deep pink color to develop is a negative test (Harley and Prescott, 2002).

Starch hydrolysis test

Twenty-four hours old fresh culture was streaked on starch agar media in a straight-line form and incubated for 24 hrs at 37°C. After 24 hrs, each of the line streaks on the starch agar plate was flooded with Gram's iodine reagent. Presence of a clear area surrounding the streaked line indicates starch has been hydrolyzed, and so positive for the test (Harley and Prescott, 2002).

H_2S production test

TSI (Triple Sugar Iron) agar slants containing 1% lactose, 1% sucrose, 0.1% glucose, 2% peptone,0.5% NaCl, 0.3% beef extract, 0.03% ferric citrate, 0.03% Sodium thiosulfate,0.0024% phenol red, and 1.2 % agar with pH 7.4 was inoculated with 100ul culture broth by streaking the slant surface using a zig-zag streak pattern and stabbing the butt using inoculating needle. The tubes were incubated for 24 hrs at 37ºC. H_2S production was detected by blackening of the agar slant (Harley and Prescott, 2002).

Carbohydrate fermentation tests

Ten milliliters of basal media (1% peptone, 0.02% yeast extract, 0.3% NaCl, 0.05% KH_2PO_4, 0.01% $MgSO_4$ and 0.01% CaCl) containing 0.1% of one of the 12 carbohydrates (Arabinose, manitol, sorbitol, xylose, inositole, glucose, galactose, sucrose, maltose, lactose, cellulose, dextrose). Besides, 0.0025% phenol red as pH indicator was inoculated with 200 µl culture broth and incubated at 37°C for 48 hrs. After 48 hrs of incubation, change in the color of the medium from red to yellow indicated a positive result for the test (a positive test for acid production from the fermentation of the carbohydrate tested) (Harley and Prescott, 2002).

Identification of the selected isolates

Identification of the selected bacterial isolates was done using data obtained from morphological, physiological and biochemical characterization (Vaseekaran *et al*. 2010; Oseni and Ekperigin, 2013).The isolates were identified to the genus level according to Bergey's Manual of Determinative Bacteriology (Logan and DeVos, 2009).

Maintenance of pure bacterial cultures

The selected pure isolates of amylase producers were maintained temporarily on starch agar slants at 4ºC (Qader *et al*. 2006) and then preserved for long time using 60% v/v glycerol in eppendorf tube at -20 and -80°C to be used as stock culture throughout the study (Bozic *et al*. 2011; Anonymous, 2004).

Amylase production using submerged fermentation

The production of amylase was performed under submerged fermentation using 250 ml capacity Erlenmeyer flasks incubated under microbiological incubators (BINDER, BD series, Model FD 115) adjusted at different temperatures.

Inoculum (seed culture) preparation

Inocula were prepared by introducing 1ml of a stock culture preserved at -20°C into 100 ml Erlenmeyer flask containing 50 ml liquid media and incubating it for 24 hrs at 37°C.

Determination of inoculum size

From the 24 hrs old seed culture, 2 ml (2% v/v) of inoculum from each isolate was serially diluted using 98 ml of distilled water to produce 2×10-2 to 2×10-10 dilutions. From each dilution, 200µl was taken and spread plated on starch agar medium and incubated at 37ºC for 24 hrs. After 24hrs of incubation, the colonies were counted and used to determine the CFU/ml of the 2% (v/v) inocula of the respective isolates using the following formula (Harley and Prescott, 2002).

$$\text{CFU} = \frac{\text{No. of colony count x DF}}{\text{Sample volume (ml)}}$$

Where, CFU is colony forming unit; DF is the dilution factor for which the viable colony counted was taken and sample volume was 2 ml culture.

Production medium preparation, inoculation and amylase production

For each isolate, the production media were prepared in duplicate Erlenmeyer flasks (250 ml capacity) containing 100 ml liquid medium following the chemical compositions and methods described in section 3.4. Following media preparation, from the 24 hrs old seed culture, 2 ml (2% v/v) of inoculum (Swain *et al*. 2006) of each isolate, which was determined in 3.8.2. Above as 2.875×10^7, 3.25×10^7, and 3×10^9 CFU/ml of FS11, M5 and S5, respectively, was introduced to each production medium. The inoculated production medium was then incubated at different temperatures, pH and incubation period and the activities of amylase determined for the respective parameters.

Crude enzyme extraction (Amylase recovery)

To obtain crude enzyme, culture broth was transferred to separate micro-centrifuge tubes and centrifuged at 10,000 rpm for 10 min at 4°C. After centrifugation, the pellet was discarded while the supernatant was used as the crude enzyme intended for assay (Punitha *et al*. 2012; Abdel-Fattah *et al*. 2013).

Measurement of amylase activity

DNSA reagent preparation

DNSA solution/reagent was prepared with Sodium Hydroxide (10 g/l), Dinitro salicylic acid (DNSA) 10 (g/l), sodium potassium tartarate 300(g/l), sodium sulfite 0.5(g/l) and phenol 2 (g/l). The first two ingredients were dissolved gently in hot plate at 80°C by adding small quantity of water drop by drop until a clear solution was obtained. Subsequently, the rest of the chemicals were added turn by turn and made to dissolve completely. After dissolving the above ingredients, the final volume was leveled to 1l using distilled water. The solution was then stored at room temperature in an amber colored bottle to avoid photo-oxidation (Abdullah, 2005).

The dinitrosalicylic acid method

The amylase activity determination (assay) was done spectrophotometrically using the dinitrosalicylic acid (DNSA) method (Sexena and Singh, 2011; Senthilkumar *et al*. 2012). The method involves mixing 0.5 ml of the substrate solution (1% soluble starch in 0.05 M Sodium phosphate buffer, pH 6.2), with 0.5 ml of appropriately diluted supernatant (crude enzyme) in a test tube and incubating the mixture for 15 minutes at 50ºC. The dilution factor for supernatants of F11 and M5 was 5x (1ml crude enzyme: 4ml distilled water) whereas for S5, it was 2x (1ml crude enzyme: 1ml distilled water). Then 2.0 ml DNS reagent was added to the test tube to stop the reaction and then kept in boiling water bath for 5 minutes.

Blanks were prepared in parallel by incubating only 0.5 ml portions of the substrate solution in separate test tubes and the same amount of crude enzyme was added after the reaction was stopped by DNSA reagent. After incubation, the reaction mixture was cooled down to room temperature in running water for 5 minutes (Thippeswamy *et al*. 2006; Ashwini *et al*. 2011; Punitha *et al*. 2012). Subsequently, its absorbance was measured at 540 nm using a spectrophotometer (Novaspec III Visible Spectrophotometer). All assays were conducted in duplicate and average values were reported.

Glucose standard curve (calibration curve)

Reducing sugar, glucose, concentration was determined from a standard curve constructed using OD readings versus known concentration of glucose. One hundred mg of glucose was dissolved in 100 ml distilled water in a measuring flask. From this solution 1 mg/ml was used to make 10 dilutions ranging from 0.1 to 1 mg/ml. Then 1 ml of each dilution was taken in separate test tubes followed by the addition of 2 ml of DNS reagent. A blank was run in parallel replacing the glucose dilution with 1 ml of distilled water. The tubes were incubated in a boiling water bath for 5 minutes prior to cooling at room temperature. Absorbance was measured at 540 nm using a spectrophotometer. A graph was plotted taking the absorbance at the ordinate and sugar concentration at the abscissa (Abdullah, 2005).

Reporting amylase activity

The amylase activity is reported in units as shown in the formula provided below. where 1 unit of amylase activity is equal to the amount of amylase required to release 1µmol glucose equivalent per minute per ml of enzyme used under the assay conditions.It was thus calculated using the following formula described by Senthilkumar *et al*.(2012) with slight modification.

$$\text{Amylase activity}\left(\frac{\text{U}}{\text{ml}}\right) = \frac{\text{Microgram of glucose produced} \times \left(\frac{1}{180}\right)\text{µmole}}{(\text{Volume of enzyme solution})(\text{Incubation time})}$$

In using the above formula, the following considerations were made:

(A) the concentration of released reducing sugar was first calculated based on glucose standard curve, described in Appendix Fig. 1,

$$Y = 0.0056X - 0.2805$$

(B) with a correlation coefficient (r^2) value of 0.997; where, X is the concentration (µg/ml) of the reducing sugar released, and Y is the absorbance (OD) of the sample.

(C) The amount of microgram of glucose produced was calculated by:

$$\text{Microgram of glucose produced(M)} = \left(\frac{\text{OD} + 0.2805}{0.0056}\right)\text{µg/ml} \times 1\text{ml}$$

$$Z = M \times \left(\frac{1}{180}\right)\mu mole$$

(D) The amount of glucose equivalents (Z) was calculated by:

$$Z = \left(\frac{OD + 0.2805}{0.0056}\right)\mu g/ml \times 1ml \times \left(\frac{1}{180}\right)\mu mole$$

Where, Z is the amount of glucose equivalents, M is the amount of micrograms of glucose produced and OD is optical density (absorbance) of the sample.

Process optimization for maximum amylase production

The factors that were optimized for maximum amylase production include initial pH of the production medium, incubation temperature, incubation period, inoculum size, supplementation of different defined and non-defined carbon sources, organic and inorganic nitrogen sources. To determine the optimum condition for maximum enzyme production of one of the above factors, all of the other factors were held constant or at the already determined condition during the optimization process. Once an optimum condition was determined, it was used in all the succeeding experiments, i.e. one-factor or variable at a time approach (OVAT method) (Abdel-Fattah *et al*. 2013).

Effect of incubation period

The optimum incubation period for maximum amylase production by isolates F11, M5 and S5 was determined by growing the isolate at a temperature of 37°C for 72 hrs in a liquid medium containing 1% starch as carbon source and whose initial pH was adjusted to 7 (Jogezai *et al*. 2011). To determine the incubation period of maximum enzyme production, 2 ml of culture was drawn aseptically from the production medium every 12 hrs beginning from the 12^{th} and continuing up to 72^{nd} hour (12, 24, 36, 48, 60 and 72). Crude enzyme was extracted from the 2 ml culture by centrifugation and its activity was determined using the dinitrosalicylic acid (DNSA) method as described in section 3.10.2.

Effect of incubation temperature

To study the effect of temperature on amylase production by F11, M5 and S5, the production medium was set at different temperatures (30, 37, 40, 45 and 50°C). The culture was incubated for 48 hrs at pH 7. Amylase production at each incubation temperature was determined using the dinitrosalicylic acid (DNSA) method (Mishra and Bereha, 2008; Ashwini *et al*. 2011; Jogezai *et al*. 2011).

Effect of initial pH of the media

The production medium was prepared by varying pH values to 5, 6, 7, 8, 9 and10 to evaluate the effect of pH on amylase production by F11, M5 and S5 at a temperature of 37°C for 36 and 48 hrs (Jogezai *et al*. 2011).

Effect of different carbon sources

The effect of carbon sources on amylase production by F11, M5 and S5 was studied by introducing different defined and non-defined carbon sources (glucose, maltose, maize flour, wheat flour, and sorghum flour and potato starch) to the cultivation medium. The effect in production was determined by growing the organism in liquid medium containing 1% w/v of a single carbon source at pH 7 and at temperatures of 37, 40 and 45°C for S5, M5 and F11, respectively. Commercial soluble starch was used as a control. Crude enzyme was extracted and assayed for its activity at the end of 36 and 48 hrs of incubation (Swain *et al*. 2006; Akcan, 2011).

Effect of different nitrogen sources

To determine effect of nitrogen sources on amylase production, 0.5% w/v of Yeast extract, Peptone, Urea, Malt extract, Ammonium sulphate, Potassium nitrate and Ammonium chloride were introduced separately to the basal growth medium. The combination of yeast extract and peptone was used as a control (Kaur and Vyas, 2012; Sivakumar *et al*. 2012). The production medium containing each of these nitrogen sources was incubated at the same temperature and initial pH indicated in section 3.11.4 above. At the end of incubation period, crude enzyme was harvested and assayed to determine its activity.

Effect of inoculum size

The effect of different inoculum size of the experimental isolates on amylase production was studied by inoculating the production medium at pH 7 with 2%, 4%, 6%, 8% and 10% v/v of inoculum from each isolate and incubating for 36-48 hrs (Jogezai *et al*. 2011).

Evaluation of the Potential of crude Amylase on the hydrolysis of raw starch

The ability of the crude amylase from each isolate to hydrolyze raw starches from selected cereals and tuber crops was studied using maize flour, potato starch, wheat flour and sorghum four (Omemu *et al*. 2005). Commercial soluble starch was used as the standard substrate for the hydrolysis test.Ten ml of five times diluted crude enzyme solution from isolates F11 and M5 and two times diluted from S5 were placed into test tubes. Ten ml of each of raw starch (1% in 100 ml 0.05M Sodium phosphate buffer, pH 6.2) was added into the enzyme preparation and incubated at 50°C for 1hr. After 1hr of incubation, 2ml solution was taken and centrifuged at 10,000 rpm for 10 minutes. Then, 1ml of the supernatant was placed into a separate test tube to which 2 ml DNS reagent was added. Subsequently, the tubes were boiled for 5 minutes at 100°C.

Blanks were also prepared in such a way that the solutions of raw starches were incubated in parallel without enzyme for 1hr. At the end of incubation period, the same amount of diluted enzyme supernatant was added and immediately 2ml was drawn from the mixture and centrifuged to be used in the preparation of the blank for each of the reaction tubes.

The susceptibility of each raw starch to amylase hydrolysis was described in terms of relative conversion efficiency (R.C.E) which was calculated from the quantity of reducing sugar (µg/ml) produced by raw starch relative to the quantity of reducing sugar (µg/ml) produced from commercial soluble starch during 1hr of incubation. The reducing sugar in the supernatant was quantified using the glucose standard curve developed following the dinitrosalicyclic acid method mentioned earlier. The conversion efficiency of the amylase of each isolate for commercial soluble starch was used as the standard (100%).

$$\text{R. C. E (\%)} = \left(\frac{\text{R. S released from raw starch (ug/ml)}}{\text{R. S released from commercial starch(ug/ml)}}\right) \times 100$$

Where, R.C.E is relative conversion efficiency in percent; R.S is amount of reducing sugar released.

Data presentation

The averages of results of the duplicate experiments were taken and presented in tables and graphs using Microsoft Office Excel 2007.

Results and Discussion

Isolation, screening and selection of amylase producing bacteria

Isolation and screening of amylase producing bacteria

One hundred and fifty-seven colonies were isolated from four different sample sources, out of which screening tests resulted in 50 colonies (31.8%) positive for amylase production (Table-1) and Appendix table-1).

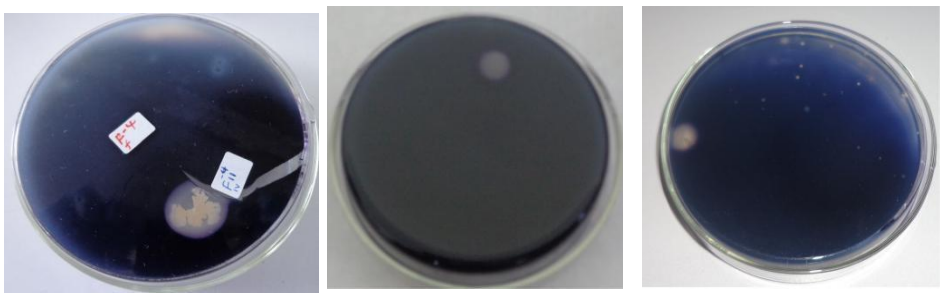
Table 1., Number of total isolated colonies and positive colonies per sample source

Forest soil	50	13
Manure	24	10
Decomposed wheat straw	58	17
Kitchen waste (sludge)	25	10
Total	157	50

As can be seen from Appendix table-1, the isolates showed great variation in the sizes of the clear zone of hydrolysis they produced on starch agar plates ranging from the least 1mm to the largest 16 mm. The results of this study are in good agreement with earlier studies that reported clear zone of starch hydrolysis ranging from 1-18 mm (Jyoti *et al*. 2009; Akpomie *et al*. 2012; Ogbonnaya and Odiase, 2012; Verma *et al*. 2011).

Selection of the best amylase-producing bacteria

From the 50 positive isolates16, isolates with relatively large clear zone of hydrolysis were selected for further investigation. The selection of best bacteria was done by comparing the isolates with each other in terms of both their diameter of clear zone of hydrolysis and their amylase activities (Table 3). The results showed that the isolates with relatively higher zone of hydrolysis also gave higher amylase production in liquid media (Table 2). This step resulted in selection of three isolates, i.e. FS11 from forest soil, M5 from agricultural waste and S5 from kitchen waste (sludge). The detection of these three selected isolates obtained from three different sources using starch agar media is shown in Figure 1.



(A) Isolate FS11 **(B) Isolate M5** **(C) Isolate S5**

Figure 1. Detection of colonies of the three selected isolates by starch hydrolysis test

Table 2. Screening of the 16-Amylase producing bacterial isolates

Sample sources	Positive isolate	Zone of Hydroly-sis (mm)	Amylase activity after 24hr (U/ml)	Amylase activity after 48 hr (U/ml)	Remark
	FS2	10	0.051	0.064	Not selected
	FS7	3.5	0.046	0.047	Not selected
Forest Soil	FS8	16	0.049	0.063	Not selected
	FS10	7	0.038	0.050	Not selected
	M1	16	0.046	0.075	Not selected
	M5	16	0.052	0.077	Selected
	M6	12	0.048	0.071	Not selected
Agricultural	M7	8	0.038	0.043	Not selected
(manure and DWS)	DWS4	7	0.042	0.057	Not selected
	DWS5	9	0.048	0.052	Not selected
	DWS6	8	0.041	0.060	Not selected
DWS7		7	0.044	0.055	Not selected
Kitchen	S1	1.5	0.039	0.041	Not selected
(Sludge)	S4	2	0.038	0.047	Not selected
S5	10	0.045	0.054	Selected	

Characterization of the selected isolates

Morphological characterization

Results of the gram staining procedures showed that all of the three isolates were rod shaped, gram-positive bacteria. Their colonies on the other hand, were circular (all) in their shape; flat (FS1 1and M5) and raised (S5) in their elevation; lobbed (FS11), crenate (M5) and Entire (S5) in their margin; pale yellow (M5); pale white (FS11) and creamy (S5) in their color and having dry (FS11 and M5) and moist (S5) surfaces (Appendix table -3). The isolates were also non-motile and endospore formers.

Physiological characterization

Physiological characterization revealed that the isolates were capable of growing at 7% NaCl and in a wide range of pH (4, 5, 6, 7, 8, 9,10,11, and 12) as well as temperature (20, 30, 37, 40, 45 and 50℃ for F11 and M5; and 20 to 45℃ for S5).

Biochemical characterization

Effervescence has been observed when they were tested for catalase. This indicates that the organisms are able to produce catalase enzyme that catalyzes H_2O_2 molecule in to oxygen and water molecules. The isolates formed a clear zone around their growth on starch and casein supplemented media, i.e. they showed positive result for starch and casein hydrolysis tests (Figure 2 and 3). This shows that the selected isolates can produce starch and protein hydrolyzing enzymes, respectively. On the other hand, they were unable to produce urease enzyme to utilize urea as nitrogen source. Different earlier reports had shown that starch degrading *Bacillus* spp. were also capable of degrading protein substrates such as casein and gelatin (Thippeswamy *et al*. 2009; Jyoti *et al*. 2009; Elkhalil and Gaffar, 2011; Kaur *et al*. 2012). However, the isolates were unable to produce urease enzyme to utilize urea as nitrogen source (Vaseekaran *et al*. 2010). Regarding the H_2S production test, the result showed that not all isolates produced the gas.

In the carbohydrate fermentation test, the isolates were found to ferment most of the tested carbohydrates (Appendix table-3). Isolate FS11 and M5 were able to ferment 10 of the 12 carbon sources provided whereas isolate S5 fermented 9 of them. However, the degree of fermentation was different from isolate to isolate and from carbohydrate to carbohydrate. In relative terms, glucose, sucrose and dextrose were the strongly fermented carbohydrates by all isolates. Cellulose and lactose on the other hand, were not fermented in this study by all isolates as observed in the works of Punitha *et al*.(2012) and Jyoti *et al*. (2009). In case of isolate S5, it was unable to ferment sorbitol. It was also observed that there was a difference in the degree of fermentation of galactose by the isolates, where S5 fermented galactose more than FS11 and M5 did.

(A) Isolate FS11 (B) Isolate M5 (C) Isolate S5

Figure 2., The clear hydrolysis zone on starch agar medium (starch hydrolysis test).

(A) Isolate FS11 (B) Isolate M5 (C) Isolate S5

Figure 3. The clear hydrolysis zone on casein agar medium (Casein hydrolysis test).

Identification of the selected isolates

The gram positivity and rod shape characteristics of theses isolates directed us to narrow the probability of the genus of these organisms to fewer than five genera (i.e. *Bacillus* spp., *Clostridium* spp., *Cornyebacterium* spp., *Lactobacillus* spp. and *Mycobacterium* spp.). Moreover, the more specific test, i.e. the endospore formation narrowed more to two probable genera (*Bacillus* spp. and *Clostridium* spp.). Here, the isolates were differentiated from *Clostridium* spp. based on characteristics such as catalase positivity and the ability to grow in an ordinary incubator. Because *Clostridium* spp. is usually catalase negative and strict anaerobes (Rainey *et al*. 2009) and indeed the sources from which the samples were collected were not conducive for their growth because they were from the open air. Therefore, based on these properties and some other additional tests done in the characterization (Appendix table- 3), the isolates were identified as members of the genus *Bacillus*.

Process optimization for maximum amylase production

Time course of amylase production

The production of amylase was studied for a period of 12 to 72 hours using the submerged fermentation process described in section 3.9 and the results are depicted in Figure 4.

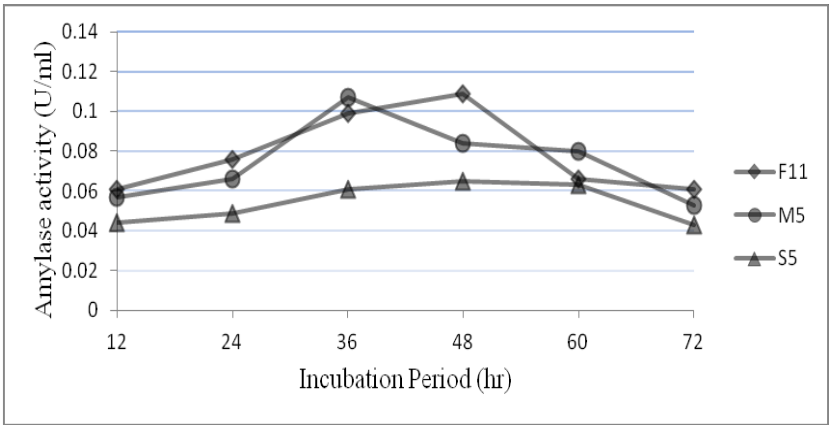


Figure 4. Effect of incubation time on amylase production

As can be seen from the figure, amylase production increased with time and reached maximum, i.e. 0.107 U/ml for isolate M5 at 36 hrs, 0.109 U/ml for isolate FS11 and 0.065U/ml for isolate S5 at 48 hrs. Further increase in incubation period, however, did not show any increase rather it resulted in a markedly decreased enzyme production. At 72hrs, amylase production was reduced to 0.061, 0.053 and 0.043 U/ml for FS11, M5 and S5, respectively.

These results are in agreement with those reported for *Bacillus* sp. (Santos and Martins, 2003), *Bacillus subtilis CM3* (Swain *et al*. 2006), *Bacillus megaterium* (Oyeleke *et al*. 2010), *Bacillus subtilis* (Jogezai *et al*. 2011) and *Bacillus amyloliquefaciens* P-001(Deb *et al*. 2013) and in which the maximum amylase production was shown to occur at 36 and 48 hrs after inoculation. The reason for the decrease in amylase production after 36 and 48 hrs might be due to the depletion of nutrients, or the death phase of the organism or due to the accumulation of by-products such as proteases, toxins, inhibitors and proteolytic activities in the medium. These by-products are known to inhibit the growth of organisms and hence enzyme formation (Mulimani *et al*. 2000; Teodoro and Martins, 2000; Aiyer, 2005).

Effect of incubation temperature on the production of amylase

Maximum amylase production for isolate FS11 and M5 (0.091 U/ml and 0.101 U/ml) was obtained at 45°C and 40°C, respectively. In addition, for isolate S5, the maximum (0.064U/ml) was obtained at 37°C. However, a considerable decrease in activity was observed with further increase in temperature beyond the maximum for the respective isolates (Figure 5). It might be because at high temperature, the growth of

the bacteria was hindered. According to the report of Aiba *et al*. (1983), high temperature may inactivate the expression of the gene responsible for the synthesis of starch degrading enzyme. At relatively low temperature (30°C), amylase production was very low for a simple reason that at low temperature bacterial growth was relatively slows (Declerck *et al*. 2003).

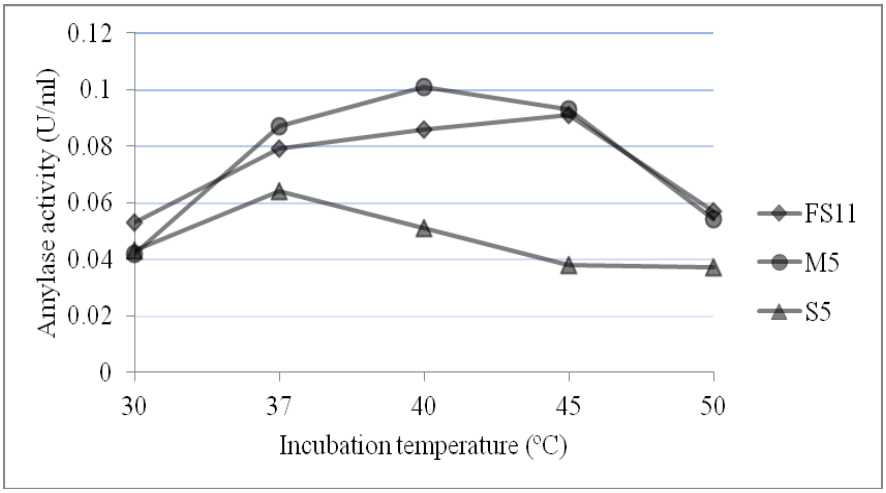


Figure 5. The effect of incubation temperature on amylase.

The above results are supported by earlier studies carried out for production of amylase from *Bacillus* sp. *marini* (Ashwini *et al*. 2011), *Bacillus* sp.5250 (Meenakshi *et al*. 2009), *Bacillus subtilis* (Jogezai *et al*. 2011), thermostable *Bacillus* sp.(Teodoro and Martins, 2000; Asad *et al*. 2011). Jogezai *et al*. (2011) and Meenakshi *et al*. (2009) reported that the optimum temperature for alpha amylase production by their respective *Bacillus* spp. was 37°C. Ashwini *et al*. (2011), on the other hand, reported that the production of amylase by *Bacillus* sp. *marini* was optimum at 40°C and as the temperature increased or decreased, there was gradual decrease in enzyme production.

Asad *et al*. (2011) and Jyoti *et al*. (2009) reported that the growth and production of amylase by thermophilic *Bacillus* was found to be optimal at 45°C. In addition, Teodoro and Martins (2000) found that the optimum temperature for amylase production by thermophilic *Bacillus* was between 45 and 55 °C and a reduction in activity was observed above 60°C. The influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic (Sivaramakrishnan *et al*. 2006). In contrast to this finding, the report from Mamo and Gessese (1999b) showed that the optimum temperature for production of highly thermostable amylase by *Bacillus* sp. WN11 was 55°C. Raharjo *et al*. (2010) also found an optimum temperature of 60°C for the production thermostable alpha amylase by *Bacillus* sp. This is due to reason that these bacteria are highly thermophilic and they were isolated from hot springs.

Effect of initial pH on amylase production

The results showed that in all isolates, the amylase activity gradually increased and reached maximum at pH 7 (0.079 U/ml, 0.097 U/ml and 0.051U/ml for isolates FS11, M5 and S5, respectively). Further increase in initial pH values resulted in the decrement of amylase production. In all isolates, pH 10 gave extremely low amylase production relative to the rest of the pH values (Figure 6). This might be because the isolates prefer neutral pH for optimum growth (Gangadharan *et al*. 2006). Normally, *Bacillus* spp. prefer neutral or slightly alkaline or a range between 6.8 and 7.2 pH for amylase production at the initial stage of fermentation (Benjamin *et al*. 2013).

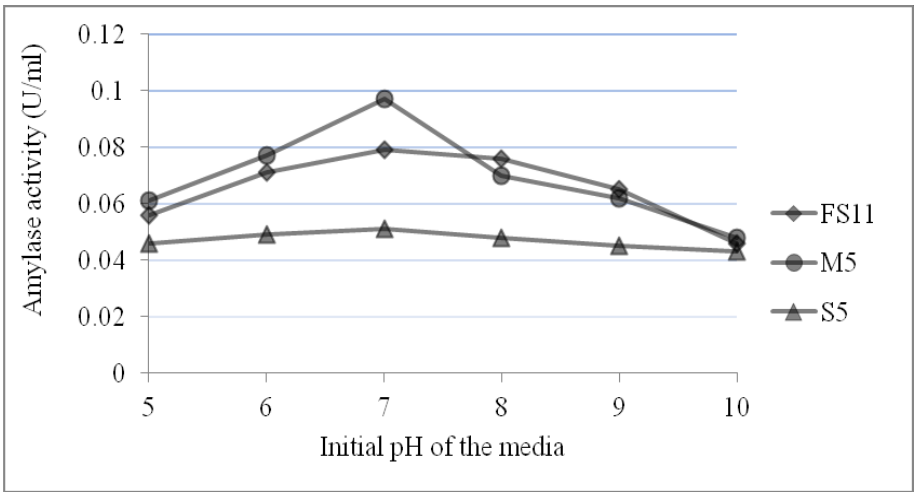


Figure 6. The effect of initial pH of the media on amylase production.

Isolate FS11 and S5 showed a wide range of pH profile for amylase production. Their production curve showed a gradual increase from pH 5 to pH 7 and the decrease in smooth from pH 7 to pH 10. In the case of M5, there was a sharp increase in enzyme activity as the pH approaches to 7 followed by a decrease as the pH further rises up suggesting that the enzymes were inactive at acidic and alkaline pH values. For bacteria isolated from meophilic environments, reports from earlier studies revealed that an optimum pH for amylase production was pH 7 (Meenakshi *et al.* 2009; Ashwini *et al*. 2011; Sivakumar *et al*. 2012).

Effect of different carbon sources on amylase production

For isolate FS11, maximum amylase production was recorded when grown on no defined potato starch (0.096U/ml) followed by wheat flour (0.074 U/ml) and commercial soluble starch (0.066U/ml). *Bacillus* sp. M5 also produced maximum amylase in media containing non-defined potato starch (0.112 U/ml) followed by commercial starch (0.094U/ml) and maltose (0.075 U/ml). In contrast, S5 achieved the highest amylase activity (0.072 U/ml) on maltose containing media followed by maize four (0.059U/ml) (Figure 7).

Several studies had revealed that maximum amylase production was found when soluble starch and maltose were used as a carbon sources. It has been found that maltose induced amylase production to the same level as that of starchor even more than starch (Santos and Martins 2003; Thippes wammy, *et al*. 2006; Jogezai *et al*. 2011). This supports the results observed in the current study in that maltose gave the highest amylase activity in isolate S5 and defined soluble starch was found to be as moderate inducer in case of isolate M5 next to potato starch. In fact, these carbon sources are the most common carbon sources that can effectively induce bacteria to produce amylase (Santos and Martins, 2003; Qader *et al*. 2006; Sexana *et al*. 2007; Meenakshi *et al*. 2009; Joshi, 2011; Bozic *et al*. 2011; Ashwini *et al*. 2011; Jogezai *et al*. 2011). Many studies had suggested that non-defined carbon sources such as corn (maize) flour, potato starch, barely flour, wheat flour etc. could be used as carbon sources because they are capable of inducing high amylase production (Mamo and Gessesse 1999b; Swain *et al*. 2006; Ayalew Akcan, 2011 Deb *et al*. 2013). In this study too, wheat, maize and sorghum flours were noted as moderate inducers of amylase production in addition to potato starch (Figure 7).

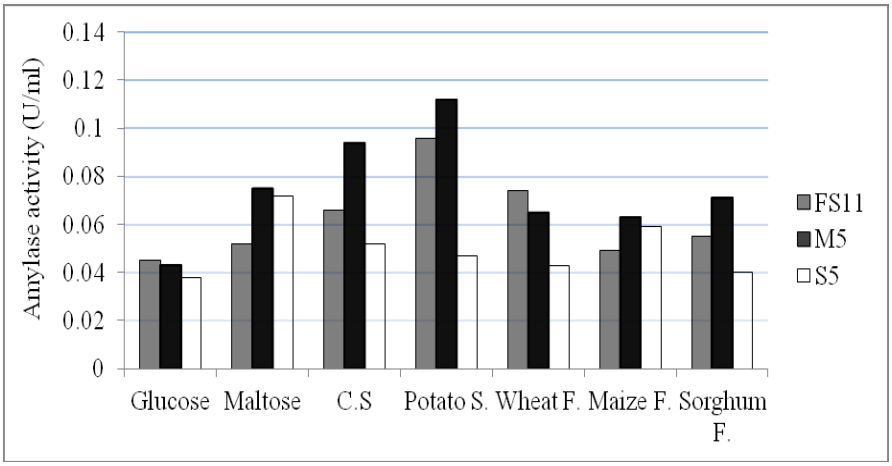


Figure 7. The effect of different carbon sources on amylase production

Supplementation of media with glucose resulted, however, in the least amylase production by all three isolates (Figure 7). It has been reported that the synthesis of carbohydrate degrading enzymes in most species of the genus *Bacillus* is subjected to catabolic repression by readily metabolisable substrates such as glucose and fructose (Meenakshi *et al*. 2009; Sivakumar *et al*. 2012). Therefore, the results of the current study are in good agreement with the findings of these studies. Meenakshi *et al*. (2009), Ayalew Damte (2011) and Sivakumar *et al*. (2012) that glucose gives the lowest amylase activity when compared to other carbon sources also reported similar results. Moreover, Santos and Martins (2003) had provided the proof for the fact that the amylolytic system was subject to catabolic repression by adding glucose to *Bacillus* sp. grown in a liquid medium containing soluble starch or maltose as a carbon source after 30 hrs.

Effect of supplementation of media with nitrogen sources on production

The nature of nitrogen source added to the production medium whether in the form of organic or inorganic form affects the amylase production. Figure 8 shows the influence of inorganic and organic sources on amylase production by isolates FS11, M5 and S5. In isolate FS11 yeast extract and its combination with peptone induced higher amylase activity, 0.085 U/ml and 0.076 U/ml, respectively. In addition, peptone and malt extract induced a considerably high level of amylase production corresponding to 0.071 and 0.064 U/ml, respectively. M5, on the other hand, produced high activity of amylase (0.101U/ml) in the presence of a combination of yeast extract and peptone followed by peptone alone (0.091U/ml). In contrast, S5 secreted maximum amylase in the presence of malt extract (0.071 U/ml) and a combination of yeast extract and peptone (0.055U/ml) (Figure 8).

Yeast extract alone and its combination with peptone have been reported to be the best nitrogen sources for amylase production (Teodoro and Martins, 2000; Swain *et al*. 2006; Meenakshi *et al*. 2009; Ashwini *et al*. 2011; Sivakumar *et al*. 2012). Many researchers had also reported that peptone was the best nitrogen source for high amylase production (Qader *et al*. 2006; Thippeswamy *et al*. 2006; Ayalew Damte 2011; Ogbonnaya and Odiase, 2012).Similar results have been also observed in the present study.

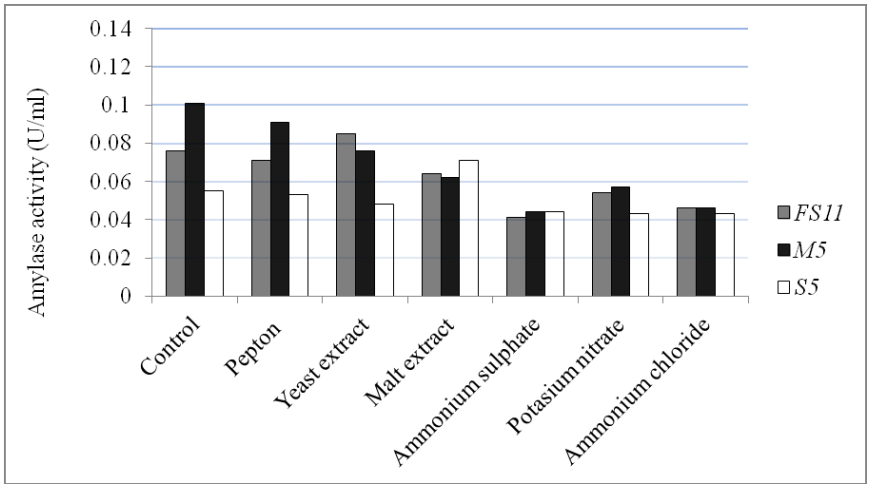


Figure 8.. Effect of nitrogen source on amylase production.

The ability of yeast extract to enhance amylase production could be due to its high content of minerals, vitamins, coenzymes and other nitrogenous components (Guerra and Pastrana, 2002). Moreover, it is believed that simple and organic nitrogen sources like peptone have a stimulatory effect on both the growth rate and amylase synthesis and thereby shortening the lag phase of the culture (Teodoro and Martins, 2000; Tiwari *et al*. 2007). Some researchers had also reported malt extract was a good inducer for high amylase production (Demirkan, 2011; Sivakumar *et al*.

2012; Swain *et al*. 2006). Thus, all these results suggest that the current study is in good agreement with other studies.

Figure 8 also shows that all isolates produced low levels of amylase in liquid media containing inorganic nitrogen sources as compared to those produced in media containing organic nitrogen source. Among the inorganic sources of nitrogen, however, potassium nitrate stimulated higher levels of enzyme production in FS11 and M5 than in S5. On the other hand, ammonium sulphate was relatively a good inducer in the isolate S5 (Figure 9). Sivakumar *et al*. (2012) and Meenakshi *et al*. (2009) had also reported a similar finding in that ammonium sulphate resulted in either maximum amylase production or in promoting amylase activity amongst the inorganic nitrogen sources tested.

Effect of inoculum sizes on amylase production

The effect of inoculum size (2 to 10%) on the production of amylase by the three selected isolates was studied and the results are shown in Figure 9. In FS11, 4% (v/v) inoculum resulted in maximum amylase production (0.146U/ml) whereas in M5 and S5, 6% (v/v) inoculum level was found to be the optimum inoculums size for maximum production of amylase (0.164 U/ml and 0.085U/ml respectively) (Figure 9).

As shown in Figure 9, in all isolates, at low inoculum level, the production is relatively low and as the inoculum level increases, the amylase production increases to a certain extent. Further increase from the optimum value resulted in a gradual decrease in amylase production.

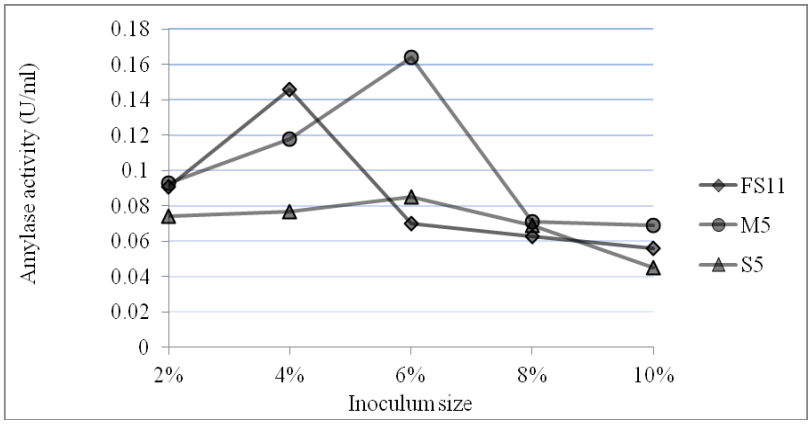


Figure 9. The effect of inoculum size on amylase production.

The result is expected because at low inoculum level growth of the organism might be reduced resulting in insufficient biomass and prolonged time for the organism to enter the stationary phase. This in turn increases the time needed for consuming the substrate and synthesizing the desired product (enzyme). On the other hand, at high concentration of inoculum, the bacteria grow rapidly and the nutrients present in the media become insufficient to support the increased number of bacteria and making it

very difficult for them to synthesize the desired product. In addition, higher inoculum size may result in a rapid over population of the bacteria and may cause problem of aeration, rapid pH change of the medium. This may affect the amylase activity of the bacteria. Thus, the production of amylase was affected at higher concentration of inoculums in this study as in previous studies (Baysal *et al*. 2003; Balkan and Ertan, 2007; Jogezai *et al*. 2011; Deb *et al*. 2013). The result of this study, specifically that of FS11, is strongly supported by the report of Jogezai *et al*. (2011) in which 4% (v/v) was recorded as being the optimum inoculum size for maximum amylase production.

Evaluation of the potential of crude amylase on the hydrolysis of raw starch

Raw starch hydrolyzing potential of crude amylases of isolate FS11, MS and S5 were tested using various raw starch granules (Table 3). It was observed that the enzyme preparation could efficiently hydrolyze different raw starch granules other than commercial soluble starch, the control.

Table 3. Raw starch hydrolyzing potential of the Amylases of Fs11, M5 and S5

	Control		261.696		100.000
*Bacillus sp.*FS11	Potato starch		281.518		107.574
	Maize flour		216.518		8
	Wheat flour		312.054		119.243
	Sorghum flour		352.268		124.292
	Control		268.482		100.000
Bacillus sp. M5	Potato starch		289.554		107.848
	Maize flour		303.839		113.169
	Wheat flour		280.268		104.390
	Sorghum flour		239.196		89.092
	Control		175.982		100.000
Bacillus sp. S5	Potato starch		173.661		9 8
	Maize flour		179.554		102.029
	Wheat flour		195.089		110.857
	Sorghum flour		159.732		90.766

Amylase from FS11 released high amount of sugar units (325.3µg/ml) from sorghum flour with in1 hr. The next higher values were from wheat flour (312.1µg/ml) and potato starch (281.5 µg/ml). Amylase from M5, on the other hand, released higher reducing sugar (303.8 µg/ml) from maize flour followed by potato starch (289.6µg/ml) and wheat flour (280.3 µg/ml). In S5, the amylase released a maximum sugar from wheat flour (195.1µg/ml). Maize flour (179. 6µg/ml) and soluble starch (176 µg/ml) were the next higher values (Table 3).

The conversion efficiency (Table 3) revealed that relative to the soluble starch used as the standard (100%), sorghum flour starch had conversion efficiency of 124.292% while wheat flour, potato starch and maize flour starches had119.243%,107.574%, 82.747% respectively when the enzyme preparation from FS11 was used. When the amylase from M5 was used, maize flour, potato, wheat and sorghum flour starches had conversion efficiency of 113.169%,107.848%, 104.390 and 89.092% respectively, relative to commercial soluble starch (100%). Amylase from S5 on the other hand, hydrolyzed these starches with conversion efficiency of 110.857%, 102.029%, 98.681 and 90.766% for wheat flour, maize flour, potato and sorghum flour starch, respectively. Similar results were reported by Liu and Xu (2007) and Gashaw Mamo and Amare Gessesse (1999) that the amylases they extracted were potent in hydrolyzing the raw starch of maize, potato starch, and wheatgranules.

Summary and Conclusion

Summary

The objectives of this study were to isolate potential amylase producing bacteria (*Bacillus spp*.) from three different sources (forest soil, kitchen and agricultural wastes) and to optimize their cultivation condition for maximum amylase production. The potential of extracted amylases on hydrolyzing raw starches has also evaluated. From 157 pure bacterial colonies, 50 (31.8%) were found as amylase positive. Out of the 50 amylase positives, following two step selection, i.e based on their hydrolysis zone diameter and amylase activity in liquid media, FS11 from forest soil, M5 from agricultural waste, and S5 from kitchen waste were found to be the best potent amylase producing isolates. Based on the result of different morphological, physiological and biochemical tests done, these isolates were found to be member of the genus *Bacillus* and assigned as *Bacillus* sp.FS11, *Bacillus* sp.M5 and *Bacillus* sp.S5.

The effects of different physical and chemical parameters are incubation period (12, 24, 36, 48, 60 and 72), temperature (30, 37, 40, 45, 50), initial pH of media (5, 6, 7, 8, 9, 10) and defined and non-defined carbon sources (glucose, maltose, starch, potato starch, wheat, maize and sorghum flours with commercial starch as control). Besides, organic and inorganic nitrogen sources (peptone, yeast extract, malt extract, ammonium sulphate, ammonium chloride and potassium nitrate) and inoculums size (2%, 4%, 6%, 8%, and 10% v/v) on amylase production by these isolates were studied. The potential of the crude amylases harvested from each isolate were also evaluated to hydrolyze raw starches (potato starch, wheat, maize and sorghum flours) after 1 hr of incubation.

Time courses of amylase production in all isolates indicate that the production increase as time increases up to the optimum time of incubation and decline after wards. The maximum amylase was harvested after 36 hrs in case of *Bacillus* sp.M5 and after 48hrs inoculation for *Bacillus* sp. FS11 and *Bacillus* sp. S5. The effect of different incubation temperatures show as the incubation temperature increase production increases up to the optimum temperature, but beyond the optimum, production decreased. *Bacillus* sp.S5 produces maximum amylase at 37°C,

whereas 40°C was optimum for *Bacillus* sp.M5. *Bacillus* sp.FS11 was able to produce high amylase at 45°C. All isolates produced maximum amylase at pH 7 when compared to some slight acidic and alkaline pHs. The production curve of Bacillus sp.FS11 and S5 indicated that they have a wide range of pH for production, as the curve increased and decreased in very gradual manner with a small unit differences.
Bacillus sp.FS11 gave maximum amylase in non-defined potato starch supplemented medium followed by wheat flour. *Bacillus* sp. M5 also produced maximum amylase in the presence of non-defined potato starch followed by commercial starch. In *Bacillus* sp.S5, maltose gave the highest amylase activity and maize four was the next. The effect of nitrogen sources indicated that in all isolates organic nitrogen sources resulted in maximum amylase production as compared to inorganic nitrogen sources. *Bacillus* sp.FS11 produced maximum amylase in a medium containing yeast extract followed by the control flasks where a combination of yeast extract and peptone was used. *Bacillus* sp. M5 on the other hand, produced higher amylase in the presence of yeast extract with peptone (control) and the next value was by peptone alone. *Bacillus* sp.S5 secreted maximum amylase in the presence of malt extract and in medium containing yeast extract and peptone.

The Production curve of effect of different size of inoculums on amylase production revealed that amylase production increased when the percent of inoculum increased up to the optimum and then decrease beyond the optimum size. In *Bacillus* sp. FS11, maximum amylase was harvested when the inoculum size used is 4% v/v to the production medium. *Bacillus* sp.M5 and *Bacillus* sp.S5 on the other hand give maximum amylase in 6%v/v inoculum. The potential of raw starch hydrolysis indicated, amylase from *Bacillus* sp. FS11 released higher amount of sugars from sorghum flour followed by wheat flour and potato starch. Amylase from *Bacillus* sp.M5 on the other hand, released higher reducing sugar from maize flour followed by potato starch and wheat flour after. In case of *Bacillus* sp.S5, its amylase produced maximum reducing sugar from wheat flour with the next value from maize flour. The conversion efficiency of most raw starches showed the crude amylases from each *Bacillus* spp. were capable of degrading other raw starches in higher efficiency than commercial starch.

Conclusion

The results of the optimization process and evaluation of their amylases showed that the isolated *Bacillus* spp. would have great importance in large-scale amylase production for different applications. Regarding their optimum incubation periods, these isolates could be a potential amylase producing bacteria because they could allow us harvesting of the enzyme in a shorter time when compared to known amylase producers such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, which are the main sources of commercial amylases at present and are reported to produce maximum amylase after 72hrs.

The optimum temperature of *Bacillus* sp. FS11 is somehow promising, because its optimum temperature is very close to those of some thermostable *Bacillus* spp.

reported earlier. Therefore, this isolate could be a potential candidate for the production of amylases that could be used in starch sugar industries.

The wide range of pH profile of amylase production in FS11and S5 will be promising because some amylase-based industrial and non-industrial applications are processed under acidic or alkaline conditions. Their ability to utilize and preference for non-defined carbon sources compared to defined carbon sources is the other very interesting feature of these isolates. From an economic point of view, it is interesting that the isolates, especially FS11 and M5 produce higher level of amylase on cheaper and more easily available resources than on expensive and refined substrates.
The ability of the isolates to produce amylases that could hydrolyze raw starches such as wheat, potato, maize and sorghum which are the most abundant and cheap and easily available starch sources .They are economically attractive as they can increase the range of starch sources for direct hydrolysis of starch in the following areas.

- In the sugar industry ;
- In food industry (baking and brewing) and
- In fuel alcohol industry.

In addition, the potential of these enzymes could also give great importancein animal feed processing because these raw starches especially, sorghum and maize are high grain diets used for fattening of farm animals like sheep.

Recommendations

Based on the findings of this study, the following recommendations were made.

- Optimization methods such as factorial design could give best interactions of the factors for maximum production. Therefore, if enough materials and equipment were there, the factorial design would be recommended.
- For bacterial cultures cultivating in liquid media, continuous shaking of the growth media is necessary. Therefore, fermentation of cultures in an incubator shaker would improve the performance of the isolates in producing amylase.
- Amylases of different biochemical properties are needed when they are used at large-scale applications. Therefore, characterization of their activity and stability with respect to pH, temperature, metal ions etc. is necessary.
- Identification of the type of the amylase that the isolate produced would give full information of the amylase for which application could be used.

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Production and Partial Characterization of Laccase from Oyster Mushroom (*Pleurotus ostreatus)*

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Abstract

The aim of the current study was to evaluate the effect of different growth substrates on laccase production and to partially characterize it in terms of the effect that pH, temperature and substrate concentration had on its activity. The study was conducted at the National Agricultural Biotechnology Research Center, Holetta. The fruiting bodies of ***Pleurotus ostreatus*** *were collected from live old Eucalyptus tree barks. The potential of* ***Pleurotus ostreatus*** *in laccase production was evaluated on PDA plate containing guaiacol. After the 10th day of incubation, the fungus was able to produce dark brown color around the colonies. In solid-state fermentation (SSF), bean straw produced more laccase with an activity of 0.1248 U/ml. The lowest activity was recorded from Cupressus lusitanica sawdust. Eucalyptus sawdust that was the second-best medium in SSF was considered for further parameter optimization under SSF. Optimal laccase production (0.1198 U/ml) was obtained on the 10 day of incubation period and the optimum temperature and pH were 28°C and 5.5, respectively. Soluble starch and peptone were found to be the most preferred carbon and nitrogen sources for laccase production. Copper sulfate and asparagine induces more laccase production Copper sulfate being the most potent inducer. Generally, there was a 1.72-fold increase of laccase production in optimized media of Eucalyptus sawdust when compared to unoptimized one. The crude laccase extract showed maximum activity at pH 6.0 and a temperature of 50°C. When incubated overnight, laccase was found to be thermostable at lower temperature (20- 30°C). At pH 6.0, laccase nearly maintained 70% of its initial activity after 12 hrs incubation. There was a linear relation between laccase activity and its substrate concentration. The apparent calculated Vmax and Km were 0.922U/ml and 4.14mM, respectively showing its high affinity towards itssubstrate. Optimized medium of Eucalyptus sawdust gave more laccase production than unoptimized one and hence recommended for large-scale production.*

Introduction

The white rot fungi, *Pleurotus* species are commonly known as oyster mushrooms. Most of the known species of the genus *Pleurotus* are edible. One of the most important aspects of *Pleurotus* species is related to the use of their lignocellulolytic system for a variety of applications. These includes, bioconversion of lignocellulosic wastes into valuable products, for animal feed and other food products and the use of their ligninolytic enzymes for the biodegradation of organopollutants, xenobiotic and industrial contaminants (Stamets, 1993). Oyster mushrooms are efficient destructors of lignocellulosic substrates, which leave a white rot residue during degradation, hence, called as white rot fungus (Ortega *et al.* 1993). *Pleurotus ostreatus,* which is well-known among cultivated oyster mushroom species, are characterized by its white to lilac-gray spore print and in their natural growing environment, easily recognized by way it grows on the wood in aggregate shelf-like clusters, relatively large size and its whitish gills that run down a stubby nearly-absent stipe.

Oyster mushrooms are naturally growing in temperate or tropical forests on dead and decaying wooden logs or sometimes on outer barks of living trees. The fruit bodies of these mushrooms are distinctly shelly or oyster shaped with different colour of white, cream, gray, yellow, pink, or light brown depending upon the species. *Pleurotus* species can synthesize and release appreciable amounts of the cellulolytic, ligninolytic and xylanolytic enzymes. Cellulolytic enzymes have gained much importance in lignocellulosic degradation while ligninolytic enzymes degrade lignin to humus, water and carbon dioxide (Singh and Gautam, 2004).

White-rot fungi can degrade lignin either selectively or non-selectively. Selective white- rot fungi degrade lignin rather than the cellulose components, whereas in non-selective fungi all components of lignocelluloses are degraded. Selective lignin degraders are especially interesting from the standpoint of biotechnological applications, since they remove lignin and leave the valuable cellulose intact. Such lignin-selective fungi include, *Phanerochaete chrysosporium, Ceriporiopsis subvermispora, Pleurotus ostreatus, P. eryngii, Dichomitus squalens* and *Phlebia radiata* (Friday, 2010).

Basidiomycetes or wood- rotting fungi can be divided into white-rot, brown rot and litter decomposing fungi. The white rot fungi have been described as the most efficient ligninolytic organisms. They are common inhabitants of forest litter and fallen trees (Kersten and Cullen, 2007). The unique capacity of white-rot fungi to degrade lignin is dependent on the production of their oxidative extracellular ligninolytic enzyme system. This enzyme system primarily comprises lignin peroxidase (LiP), manganese peroxidase (MnP), laccase, and hydrogen peroxide-generating oxidases. In addition to the ligninolytic enzymes, white-rot fungi also produce cellulases, xylanases, and other hemicellulases (Hammel and Cullen, 2008).

Laccase is copper-containing protein, which belongs to the large and diverse superfamily of multicopper oxidases (Arora and Sharma, 2010). It is widely distributed in higher plants and fungi (Kiiskinen *et al.* 2004) and has been found in insects and bacteria (Viswanath *et al.* 2014). Fungal laccase degrades lignocellulosic materials/phenolic compounds with the concomitant reduction of molecular oxygen to water (Makela, 2009). Laccase of fungi is of particular interest with regard to potential industrial applications because of its capability to oxidize a wide range of industrially relevant substrates. Oxidation reaction is comprehensively used in industrial processes, for instance, in the textile, food, wood processing and pharmaceutical and chemical industries. Enzymatic oxidation is a potential substitute to chemical methods since enzymes are very specific and efficient catalysts and are ecologically sustainable.

Laccase is currently studied intensively for many applications and it is already used in large scale in the textile industry. Together with low molecular weight redox-mediator compounds, laccase can generate a desired worn appearance on denim by bleaching indigo dye. Another potential environmental application for laccase is the bioremediation of contaminated soils as laccase is able to oxidize toxic organic pollutants, such as polycyclic aromatic hydrocarbons and chlorophenols. The most useful method for this application would probably be inoculating the soil with fungi that are efficient laccase producers because the use of isolated enzymes is not economically feasible for soil remediation in large scale. The current practical applications of the use of laccase have led to a search for source of the enzyme from white-rot fungi and the use of mediators, which promote or facilitate enzyme action (Viswanath *et al.* 2014).

Owing to its vivid biotechnological applications, studies on laccase producing organisms have been intensified in recent years and several groups (Sivakumar *et al.* 2010) are carrying out the optimization of laccase production from different microorganisms. The ever-increasing demand for this enzyme requires the production process to be economical. Identifying inexpensive raw materials for enzyme production could be viewed as a solution to make the entire process cost effective. Laccase production by fungi is influenced by culture conditions such as nature and concentration of carbon and nitrogen sources, media composition, pH, temperature, and presence of inducers. The nutritive substances employed in the culture medium constitute significantly to the total production costs. Hence, it has been a matter of concern to find environmentally sound and economically feasible media constituents for laccase production. To this end, this study was undertaken to isolate white rot fungus (*Pleurotus ostreatus)* from its natural habitat, i.e. bark of old *Eucalyptus* trees, and detect laccase activity on potato dextrose agar (PDA) plate containing guaiacol with the aim of using the isolate for laboratory scale production of laccase. Therefore, the main objective of the current study was to investigate the potential of *Pleurotus ostreatus* for laccase production on six different lignocellulosic substrates and determine the optimal levels of physicochemical conditions required for the production.

The specific objectives were to:

- Evaluate the effects of various growth substrates on laccase production by *Pleurotus ostreatus;*
- Determine the optimal levels of physicochemical conditions required by *Pleurotus ostreatus* for laccase production and
- Partially characterize laccase produced by *Pleurotus ostreatus* in terms of pH and temperature on its activity and stability and substrate concentration on its activity.

Materials and Methods

The study area

The study was conducted in National Agricultural Biotechnological Research Center, in Holetta, which is geographically located at 34 km West of Addis Ababa. It has a latitude and longitude of 9° 3' N and 38°30'E, respectively, and an altitude of 2390 meters above sea level. The area has a bimodal rainfall pattern with a mean annual precipitation of 1100 mm. The main rain season extends from June to September while the short rain falls between February and April. Mean annual maximum and minimum temperatures are 21°C (ranging from 20°C to 27°C) and 6°C (ranging from 2°C to 9°C), respectively. Mixed crop/livestock farming is the major agricultural production system practiced in the area with the major crops comprising teff, wheat, barley, chickpea and faba bean. The predominant soil type in the area are nitosols and vertisols varying between reddish brown to dark reddish brown clays (www.eiar.gov.et).

Oyster mushroom collection, identification and maintenance

Fruiting bodies of *Pleurotus ostreatus* were collected from bark of live old *Eucalyptus* tree around Holetta Agriculture Research Center (HARC) by using sterile forceps and handled in sterile polythene bags. The fungi were collected based on their morphology following the methods of *Pleurotus* species identification guideline given by Consensus Document on the Biology of Pleurotus species (OECD, 2005). The major morphological traits considered during fruiting body collection were; occurrence, stem/stipe, odor, shape and color of fruiting body. The fungi grown in shelf-like clusters, nearly absent stem, smooth and thick flesh with whitish kidney shaped cap having anise odor were considered. The fungi having the above mentioned features were collected from 10 (ten) different trees in early September, 2014 and were immediately kept in a freezer at -21°C until cultured on potato dextrose agar (PDA) media.

One week later PDA media were prepared and the mushrooms were inoculated on it under aseptic condition following standard microbiological procedures. First the fruiting bodies (gills of the fungi) were cut into pieces of nearly 3 mm size and subjected to surface sterilization by dipping successively in sterile distilled water, 70% ethanol and 5% NaOCl, each for 4 minutes and finally repeatedly washed with sterile distilled water. Then after the pieces were kept on sterile filter paper for few minutes for drying and then placed on prepared PDA (pH 5) plates. All plates were incubated at 28°C and daily observation was made. After a week, sub-culturing was

done by taking fungal mycelia from incubated plates and transferred to new PDA plates to get purer cultures. The major microscopic features considered during sub-culturing were spore print and colony morphology. The fungi having smooth white to lilac-grey spore with cylindrical to long elliptical shaped morphology were considered as *Pleurotus ostreatus* (www.mushroomexpert.com/) and (OECD, 2005). After pure *P. ostreatus* cultures were obtained, they were maintained on agar slants by sub-culturing ever month and preserved at 4 degreecentigrade in a refrigerator according to Naraian *et al.* (2010).

Source and preparation of growth substrates

The growth substrate like *Cupressus lusitanica* sawdust (CSD), *Eucalyptus* bark (EB) and *Eucalyptus* sawdust (ESD) were collected from local small wood processing business center. Bean straw (BS), Teff straw (TS) and Wheat straw (WS) were collected from HARC farm field. All lignocellulosic substrates were air-dried and then oven dried in- order to free all substrates from moisture for ease of grinding. After that, all substrates were ground by using a blender separately. Then all substrates were sieved through a 1mm size mesh, and the powder passed through 1mm size mesh was used for submerged fermentation and above 1mm size (remain on mesh) was used for solid state fermentation. Hence, the powder was stored at room temperature in capped glass bottles separately until used.

Cultivation of *Pleurotus ostreatus* and laccase extraction

Cultivation of *Pleurotus ostreatus* in SSF and laccase extraction

The above described powdered substrates (CSD, ESD, BS, EB, TS, and WS) were transferred to flasks of 250 ml each in 10g separately. Then 30 ml of distilled water was added to all bottles following Demir *et al*. (2011). The experiments were performed in duplicate. Then the glass bottles were wrapped and autoclaved at 121˚C for 20 minutes. After cooling, all the glass bottles were inoculated with 5 discs of the fungal culture (Patel *et al.* 2009) of 5-day old under aseptic condition, and then incubated at 28°C for complete growth of mycelium. After the fourteenth day of incubation, 30 ml of 100 mM acetate buffer (pH 5.0) was added to the glass bottles containing different solid substrates for fungal growth, under aseptic condition (Kumari and Negi, 2014). Then, the glass bottles were kept on shaker at 120 rpm at room temperature at least for 30 minutes to allow the buffer to extract the enzyme. Later, the contents were filtered with whatman No 1 filter paper and the culture filtrate was centrifuged at 10,000 rpm for 15 minutes at 4°C to remove spores and insoluble. The clear supernatants (crude enzymes) were then collected in separate containers by decantation (Elsayed *et al*. 2012). This was used for the determination of laccase activity.

Cultivation of *Pleurotus ostreatus* in SmF and laccase extraction

For production of laccase in submerged fermentation, liquid media were prepared from CSD, ESD, BS, EB, TS, and WS by adding 10 g from each in 100 ml distilled water in separate bottles. The culture media were then autoclaved and inoculated with a disk of *P. ostreatus* 5-day old culture under aseptic condition and kept on rotatory shaker at

120 rpm for 14 days at room temperature (24 ±2 °C). The extracellular enzyme present in the culture filtrate was obtained by filtration through Whatman No. 1 filter paper. The culture filtrate was then centrifuged at 10000 rpm for 15 minutes and the clear supernatant was collected in separate tube by decantation and used for Laccase activity assay (Elisashvili *et al.* 2001).

Enzyme Assay

Qualitative assay

Guaiacol was used for qualitative enzyme assay. Agar discs of 5-day-old cultures were placed on PDA petri-dishes containing 4 mM guaiacol and incubated at 28°C. The intense brown color development around the colony due to oxidation of guaiacol was considered as an indication for laccase activity. Therefore, if the plate changes color to brown, it was concluded that there was laccase production by *P. ostreatus* (Desai *et al*. 2011).

Quantitative assay

Laccase activity was assayed according to the procedure suggested by Gedikli *et al.* (2010) and Demir *et al.* (2011) with minor modification (incubation temperature 50°C was used additionally). For measuring laccase activity, 0.1 ml of culture filtrate (enzyme source) was added to 4.9 ml of 0.1 M sodium acetate buffer (pH 4.5) and 1 mM guaiacol as substrate. The reaction mixture prepared was incubated at 37°C for 15 minutes. The same amount of reaction mixture was incubated at 50°C for 15 minutes. Incubation with distilled water was used as control. From these two parallel experiments, the best temperature was identified and used in the successive optimization experiments of the various physicochemical parameters. Enzyme activity was measured by reading absorbance in the UV-Visible spectrophotometer adjusted to 465 nm wavelengths. The absorbance of a sample is directly proportional to concentration (Beer's Law) and sample thickness (Lambert's Law). When these two relationships are combined, we get the Beer-Lambert equation (Manole *et al.* 2008).

$$A = \varepsilon c l$$

Where; ε = extinction coefficient, a characteristic constant for a given absorbing substance

c = concentration of substrate in M (mole/L)

l = thickness of the sample in cm (usually 1.00 cm for standard sample cuvette)

Where, c is the concentration of the absorbing material (tetraguaiacone; product formed from guaiacol oxidation by laccase in this case), A is the absorbance measured at 465 nm, and *l* is the length of the light path (1.0 cm for current spectrophotometer). The extinction coefficient, ε, for guaiacol at 465nm is 12,100 L/M.cm. using this value and the Beer-Lambert equation; it was possible to convert an absorbance reading into an actual amount of product formed.

The UV-Visible spectrophotometer was adjusted to zero by using the above-mentioned buffer and guaiacol as substrate and distilled water instead of crude enzyme extracts. The reading was adjusted to zero and used as a benchmark for actual reading, because the difference in the two reaction mixtures is only due to crude enzyme. If the absorbance of control (buffer, guaiacol, and distilled water) is adjusted to zero, then the absorbance of the experiment (buffer, guaiacol, and crude enzyme) is only because of crude enzyme activity. The molar extinction coefficient of guaiacol is 12,100 L/ (mol.cm), and one activity unit of laccase was defined as the amount of enzyme required to catalyze 1 µmol of guaiacol per minute (Ping *et al*. 2008). Enzyme activity was expressed as Enzyme units (U), where 1U is defined as the amount of enzyme required to oxidize 1micromole of guaiacol (substrate) per min and laccase activity in U/ml was calculated with the following formula (Kalra *et al.* 2013).

$$U/ml = (A \times V) / (\varepsilon \times t \times l \times v)$$

Where; U = Enzyme unit t = time

A = Absorbance l = sample thickness V = Total reaction volume

v = Enzyme volume ε = Molar extinction coefficient

Optimization of *Pleurotus ostreatus* growth condition for laccase production

ESD was selected among the growth substrates and optimized for the following parameters under solid state fermentation. For each experiment, five mycelial discs (nearly 6 mm diameter) were used as inoculum in each flask. The experiments were done classically; only one factor at a time and on duplicate for all parameters. One factor was optimized at a time and was used for the next parameter optimization.

Effect of growth time on the production of laccase

In order to determine the optimum time of incubation for the maximum laccase production, growth medium was prepared in flask (250 ml) from 10 g of ESD with 30 ml of distilled water. Totally 16 flasks of production medium were prepared and autoclaved. To these, five discs (6 mm in diameter) of 5-day old culture was inoculated and incubated at 28°C. The cultures of two flasks were harvested at every 2-day interval starting from the second day of incubation. The cell-free supernatant obtained after centrifugation was used to determine laccase activity.

Effect of incubation temperature on the production of laccase

To determine the effect of incubation temperature on laccase production, growth substrate was inoculated with 5 days old mycelial plugs and the flasks were incubated at 20, 25, 28, 30, and 37ºC, (2 flasks for each incubation temperature). At the end of incubation period, the cultures were harvested and enzyme activity was determined.

Effect of pH on the production of laccase

The effect of pH on laccase production was tested by incubating the flasks of growth medium adjusted to different pH from 4.5-6.5 with 0.5 interval. The initial pH was adjusted to appropriate level by using 1% diluted NaOH and HCl. Then, the production medium was inoculated with five mycelial discs and incubated at optimal temperature. At the end, harvesting and enzyme assay was carried out.

Effect of carbon source on the production of laccase

The effect of different carbon sources: monosaccharides (glucose, galactose, fructose, and mannitol), disaccharides (maltose, lactose and sucrose) and polysaccharides (cellulose and soluble starch) on the production of laccase from *P. ostreatus* was studied. The carbon sources were added as amendment into the production media at the concentration of 2% (optimal level of carbon source) following Abdulah (2008). Then, five mycelial discs (6 mm diameter) of 5-days old culture were transferred to flasks containing production medium and then incubated at the optimum time and temperature. The control flasks were inoculated with fungal plugs without amendment of any carbon source and incubated along with other flasks.

Effect of nitrogen source on the production of laccase

In order to determine the suitable nitrogen source for maximum production of laccase by *P. ostreatus*, the following organic and inorganic nitrogen sources, namely: sodium nitrate, peptone, beef extract, ammonium sulphate, and ammonium chloride were used for amendment at concentrations of 0.2% (optimal level of nitrogen source for laccase production) in the production media (Abdulah, 2008). Flasks without any nitrogen source amendment were used as a control.

Effect of divalent metallic ions on the production of laccase

To determine the effects of different metal ions on the production of laccase by *P. ostreatus*, different metal ions or salt solution were used with final concentrations of 5 mM (Elsayed *et al.* 2012): Mn^{2+} ($MnCl_2$), Zn^{2+} ($ZnCl_2$), Fe^{+2} ($FeSO_4$), Mg^{2+} ($MgSO_4$), Ca^{2+} ($CaCl_2$), Cu^{2+} ($CuSO_4$). The control flasks were inoculated and incubated without addition of metallic ions (salt solution).

Effect of different inducers on laccase production

Different inducers were investigated for their capacity to increase laccase production such as $CuSO_4$ (1 mM), L-methionine, L-tryptophan, L-valine, L-leucine and biotin with (0.2% w/v) concentration following Krishna *et al. (*2005). The control flasks were inoculated without any inducers.

Partial characterization of laccase

Effect of pH on the activity and stability of laccase

The pH optima for laccase activity were determined over a range of pH 3.5–9.0. The optimum pH for laccase activity was investigated by using three different buffer systems comprising of 100 mM sodium acetate buffer (pH 3.5–5.5); 100 mM sodium phosphate buffer (pH 6.0–8.0) and 100 mM Tris–HCl buffer (pH 8.5–9.0) following Patel *et al.* (2013). The reaction mixture was prepared from 0.1ml of culture filtrate (enzyme source), 4.9 ml of 100mM buffer and 1 mM guaiacol as substrate. Then, the reaction mixtures were incubated at 50°C for 15 minutes and enzyme activity was measured following section 3.5.2. For determining laccase stability over different pH ranges, similar reaction mixtures were incubated overnight and their activities were measured as an indicator of stability of laccase.

Effect of temperature on the activity and stability of laccase

The effect of temperature on laccase activity was determined following the procedures similar to section 3.5.2, by incubation of reaction mixture for 15 min at temperatures ranging from 20–80°C with an interval of 10°C by using optimal pH. The thermal stability was determined under same assay conditions but incubated overnight in a temperature range of 20–60°C.

Effect of substrate concentration on activity of laccase

Rate of laccase catalysis was determined by incubating substrate guaiacol at various concentrations (2, 4, 6, 8, 10, and 12 mM) with 1 ml of laccase crude extracts. The incubation time was 15 min for determination of the effect of substrate concentration on laccase activity. The enzyme incubated without guaiacol served as control. The kinetic constants Km and Vmax were estimated by curve fitting of the reciprocal plot of reaction rate in enzyme activity (U/ml) versus substrate concentration using the Line Weaver-Burk equation plot transformation of the Michaelis-Menten rate equation (Asgher *et al.* 2012).

Data analysis

The study was carried out experimentally with CRD design, meaning experimental units were randomly taken for treatments and vice versa. The results were expressed as the mean of data obtained from duplicate experiments. The data were first entered in Microsoft Excel and analyzed using appropriate statistical function. One-way analysis of variance (ANOVA) was used to compare the mean values among different parameters. P- Values less than 0.05 were considered statistically significant.

Results and Discussion

Identification of *pleurotus ostreatus*

Identification of *Pleurotus ostreatus* was done at their natural habitat (living *Eucalyptus* tree) based on color difference of their fruiting bodies. This well-known mushroom is easily recognized by the way it grows on the wood in shelf-like clusters

(Figure 1A), its relatively large size, its whitish gills that run down a stubby, nearly-absent (short) stipe, and its whitish to lilac gray spore print. Only white colored fruiting bodies forming overlapping shelves or clusters on logs were collected, as those were the features of *Pleurotus ostreatus* (www.mushroomexpert.com/ and www.messiah.edu/).

In the laboratory, the fungus was identified based on its colony morphology as well as microscopic visualizations (spore print). The organisms producing smooth and white to lilac-gray colored spores print with cylindrical to long-elliptical shape were considered. The microscopic observations showed smooth, cylindrical to narrowly kidney shaped features (Figure 1B). These colony characteristics and microscopic visualizations were similar to the genus *Pleurotus ostreatus* (www.messiah.edu/ and OECD, 2005); hence, the isolated fungus was concluded to be *Pleurotus ostreatus*. The fungus was further sub- cultured for laccase production detection and then for growth parameter optimization.

Figure 1. Growth of *Pleurotus ostreatus* in natural habitat on *Eucalyptus* tree bark (A) and on PDA plate (B).

Detection of laccase production by *Pleurotus ostreatus*

Five days old culture of *P. ostreatus* was placed on PDA petri-dishes containing 4mM guaiacol and incubated at 28°C for 5 days. The culture was unable to grow quickly as expected compared to guaiacol free plate; although there was suppression, effect from guaiacol there was mycelial growth initiation. After the tenth day, the culture was able to develop intense brown color around the colony (Figure 2) due to oxidation of guaiacol by laccase, which can be correlated to its activity (Kumari and Negi, 2014). Therefore, it can be concluded that the fungus under investigation (*P. ostreatus*) was a laccase producer.

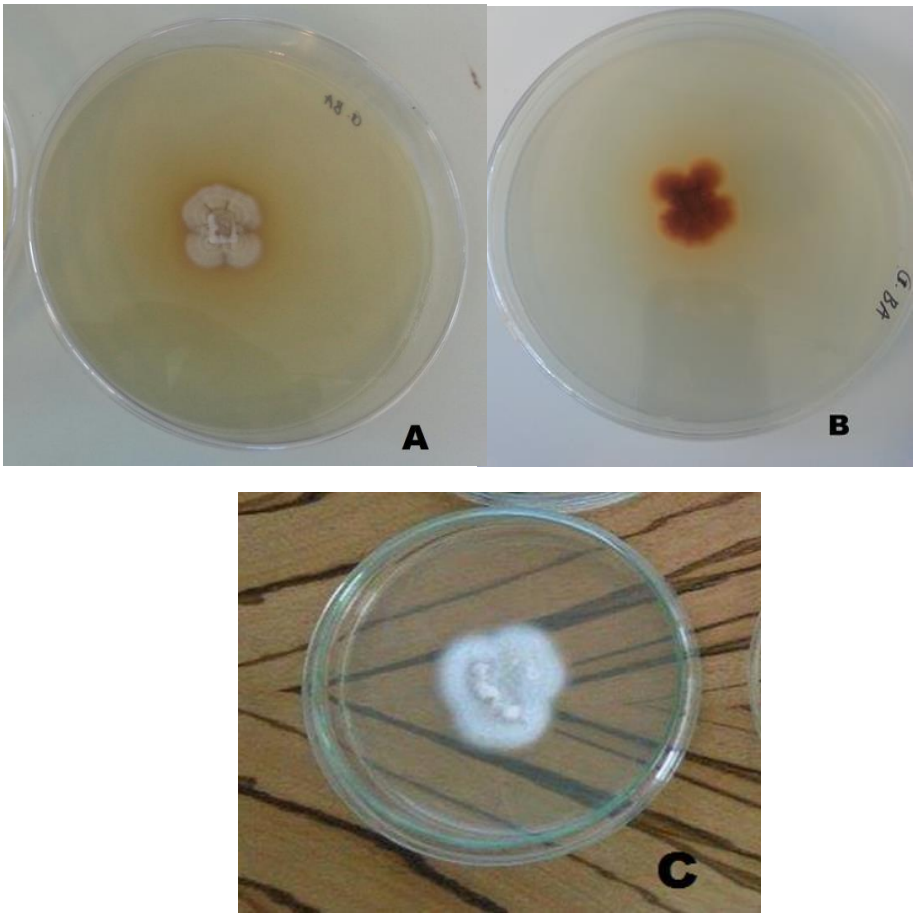

Figure 2. Detection of laccase production by *Pleurotus ostreatus* on PDA plate (A- PDA plate with guaiacol front view, B- PDA plate with guaiacol backside view and C- PDA plate no guaiacol).

Production of laccase by *Pleurotus ostreatus* cultivated on different lignocellulosic. Substrates

Production of laccase enzyme by *P. ostreatus* grown on different lignocellulosic substrates; bean straw (BS), *Cupressus lusitanica* sawdust (CSD), *Eucalyptus* bark (EB), *Eucalyptus* sawdust (ESD), teff straw (TS) and wheat straw (WS) under solid state and submerged fermentation condition was evaluated and the results are summarized and presented in Figure 5. Each substrate was served as a sole nutrient source in both media form.

The highest enzyme production was obtained from bean straw in SSF and wheat straw in case of SmF, which were 0.1248, and 0.0985 U/ml, respectively (Figure 3). This indicates that laccase could be used in animal feed treatments in order to improve their nutritional value and palatability. The least was produced in *C. lustanica* sawdust in both media types. These were 0.011 U/ml in SSF and 0.0109 U/ml in SmF.

Eucalyptus sawdust was the second highest in SSF which was 0.0889 U/ml; and the third highest in SmF which was 0.0774 U/ml. *Eucalyptus* tree bark is the natural substrate for the study organism, but the study showed that it had moderate activity of laccase in both fermentation methods probably because of the poor nutrient content available in bark.

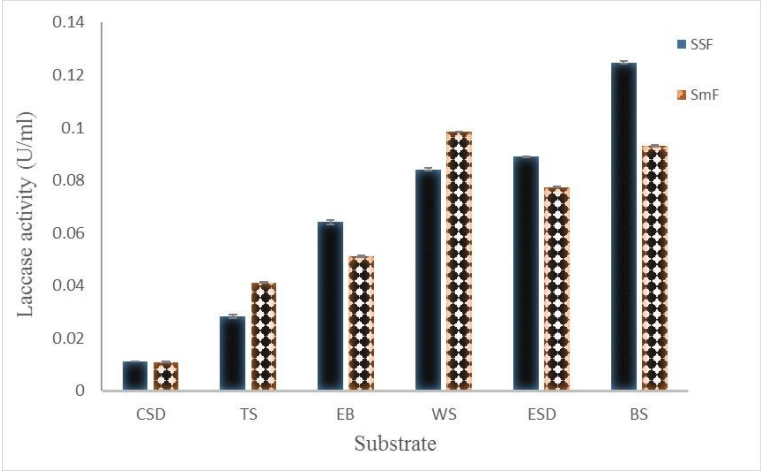

Figure 3. Laccase production by *P. ostreatus* grown on different lignocellulosic substrates under SSF and Sm.

The production of laccase and other enzymes by white rot fungi were higher in SSF compared to SmF (Toca-Herrera *et al*. 2007). This is because the organisms are exposed to continuous hydrodynamic forces in SmF but in SSF they are growing on surface of solid material. The success of SSF was directly related to the physical characteristics of the support material (particle size, porosity, consistency), which favor both gas and nutrient diffusion and the attachment of the microorganisms. Smaller substrate particles provide a larger surface area for microbial colonization but if they are too small may result in substrate agglomeration as well as poor growth. In contrast, larger particles provide better aeration but a limited surface for microbial colonization. Therefore, a compromised particle size must be selected for the process. In SmF, the particle size of the substrate was too small in order to make them dissolved in water, and hence the fungi suffer aeration problem. Although, they face aeration problem under SmF, they had a potential to produce laccase as seen in TS and WS, which was higher than their respective SSF. Other reason for SSF to be better than SmF is that the organisms are growing on similar or nearly similar environmental conditions to those of the natural habitats like wood and decayed organic matter (Toca-Herrera *et al*. 2007; Desai *et al*. 2011). Kalra *et al*. (2013) reported that laccase produced with the help of both submerged and solid-state fermentations, but higher production was achieved under solid-state conditions where rice bran was taken as support substrate and higher levels of laccase was obtained from SSF, which was 11.7 U/ml.

In the current study, there were significant variations in laccase production in both media form i.e., SSF and SmF. Other than CSD, all substrates showed statistically different results (Table 1). Laccase activity obtained from BS, ESD, EB, TS and WS in SSF are significantly different from the one obtained in SmF of similar substrate. Compared to all six substrates, BS showed more laccase activity in SSF. But due to its scarcity in study area and difficulty in preservation, it was not selected for further physicochemical growth parameters optimization of *P. ostreatus* and hence for laccase production. Rather ESD which was the second best in SSF was considered as a good substrate and taken for further physicochemical growth parameter optimizations of *P. ostreatus* and hence for laccase production under solid state fermentation.

Table 1. Mean laccase activity of six different substrates in Ssf and Smf (values are mean ± standard Error, N=2). average laccase activity (U/Ml)

Substrate	SSF	SmF	P-Value
BS	0.1248±0.0005	0.0931±0.0005	0.00047223
CSD	0.0110±0.0004	0.0109±8.2E-5	0.47380652
EB	0.0641±0.0008	0.0510±0.0004	0.01520793
ESD	0.0889±0.0002	0.0774±0.0001	0.00144129
TS	0.0281±0.0006	0.0410±0.0002	0.01773512
WS	0.0841±0.0005	0.0985±4.1E-5	0.0229853

Means having p-value less than 0.05 are statistically different between SSF and SmF within substrate.

Determination of optimum conditions for laccase production

Determination of optimum incubation period

Many of the wood degrading white rot fungi were found to be excellent producers of laccase. Nevertheless, variations prevailed in relation to optimum expression period of this enzyme among the organisms (Sivakumar *et al.* 2010). In the present study, the time course of laccase production by white rot fungus *P. ostreatus* was evaluated by harvesting enzyme every other day for 16 days. The results in Figure 4 show that the optimum incubation time was the tenth day, at which laccase activity reached 0.1198 U/ml. There were increments in laccase production from the second day up to the sixth day of incubation. After the sixth day, laccase production was increased slightly and reached its maximum on the tenth day. After the tenth day, activity decreased slowly up to the fourteenth day, and then sharply dropped up to the sixteenth day. So, the maximal incubation period of *P. ostreatus* for laccase production was the tenth day. Activity observed on the 6th and 14th and 8th and 12th are not statistically different, but in general incubation time had a significant effect on laccase production by *P.ostreatus*

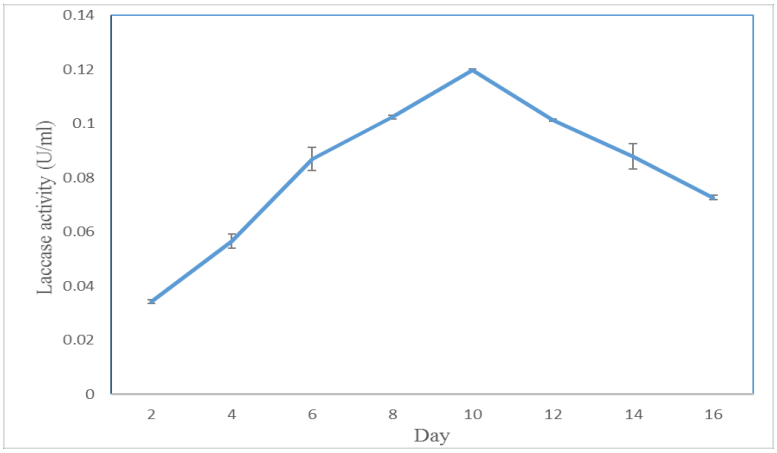

Figure 4. Effect of incubation period on laccase production by *P. ostreatus* grown on ESD under SSF.

Kumar *et al*. (2011) who reported that maximal laccase activity in P. ostreatus was found nearly similar results on the 9th day (570 U/L). Nadeem *et al*. (2014) reported that, there was a sharp increase in laccase production by *P. ostreatus* from 2-6th day and after 10thday its activity decrease slightly. Laccase produced in late phase of *P. ostreatus*, i.e. on 6th and 8th day, reached its maximum value with activity of 5.53±0.11 and 5.68±0.08 U/ml, respectively and after that decrease subsequently. The laccase production on day 8 was 61.7 and 41.4 fold more than the 2nd and 16th day of incubation, respectively. Ling (1994) also reported maximum laccase activity on the 10th day from *P. sajor-caju*. In addition to this, Sivakumar *et al*. (2010) also found optimum laccase production by *Ganoderma species* after 10 day of incubation. Sterjiades *et al*. (1992) also reported Laccase activity in sycamore maple cell suspension medium (*Acer pseudoplatanus)* reached a maximum approximately 12 days after inoculation. The production of laccase activity occurred on day 2 and reached its maximum (484.72 U/L) on day 10 and then declined gradually as indicated by Gao *et al*. (2011).

On the other hand, Abdulah (2008), found maximum laccase production on the 5th day of incubation which was 0.127 U/ml? The report also indicated that after 5 days there was a sharp decline in enzyme activity observed and reached (0.043 U/ml) after 15 days. Maximum level of laccase activity (0.395 U/ml) was observed after 15 days of incubation and there was no activity observed even after 25 days of incubation as reported by Hashim (2012). Time course study of laccase showed that maximum production was 75 IU/ml in 8 days of SSF of banana stalk by *Schizophyllum commune* IBL-06 as reported by Irshad and Asgher (2011).

Determination of optimum temperature

Temperature is considered as a crucial and effective factor in the growth of microorganisms and their metabolism i.e. temperature significantly influences the production of mycelial biomass, protein and laccase (Nadeem *et al*. 2014).

The results of the present study indicate that an incubation temperature 25, 28 and 30ºC was good for laccase production with 28°C being an optimal with activity of 0.1197 U/ml (Figure 5). But at 37 and 20°C the fungus growth decreased and consequently laccase's units dropped, because high temperature causes the cell membrane composition alteration and stimulation of protein catabolism, and lower temperature results in suppression of nutrient transformation which was in line with the report given by Nadeem *et al*. (2014). At 37°C, laccase's activity dropped to 0.0654 U/ml, which is nearly 54.64% of its maximum activity at 28°C. Generally, temperature more than 30°C and less than 25°C does not favor the growth of *P. ostreatus* and subsequently lesser laccase produced. There was no significant different in laccase activity obtained at 25 and 30°C. However, there was significant difference in laccase activity at different temperatures in general (Appendix Table 7). This indicates that cultivating *P. ostreatus* under optimized temperature is important for more laccase production.

The above result is in line with Abdulah (2008), who reported that the highest laccase production was obtained at 28˚C; with an enzyme activity of 0.132 U/ml. Patel *et al*. (2009) who showed that the best temperature for laccase production by P. ostreatus was 28˚C. While Snajder and Baldrain (2007) indicated that the highest laccase production was obtained at 25-30 ° C with the same organism also reported the same result. The optimum temperature for maximum laccase production by *P. sajor-caju* was found to be 30°C on day 9 with an activity of 0.2844 U/ml.

Very little ligninolytic activities were observed at temperatures above 30°C probably due to the fact that increasing the temperature could have inhibited the fungal growth and hence, decreased enzyme activities (Patrick *et al*. 2011). Sivakami *et al*. *(*2012) also reported that the optimal temperature for laccase production by *P. ostreatus* was 30°C. On the other hand, Patrick *et al*. (2009) indicated that the optimum temperature for maximum production of ligninolytic enzymes was determined to be 25ºC for laccase on day 6 with an activity of 0.36 U/ml.

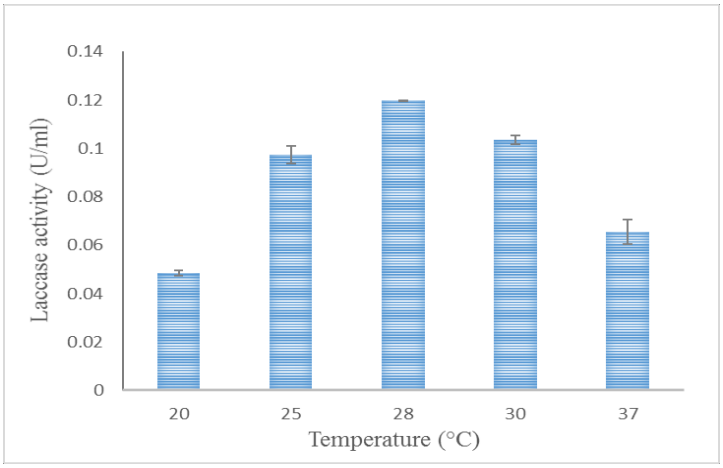

Figure 5. Effect of temperature on laccase production by *P. ostreatus* grown on ESD under SSF

Determination of optimum pH

pH is an imperative and significant factor, influencing the extracellular laccase production in *P. ostreatus* and other microorganisms since it affects the characteristics of the medium including nutrient solubility and transportation and thus it affects the nutrient availability to the growing microorganism. Secondly, it also affects the enzyme ionizable group and thus affects the stability of an enzyme (Abdulah, 2008). Sivakumar *et al*. (2010) also indicate that pH is an important factor of culture medium for fungus to grow, for ligninolytic enzyme production and xenobiotic degradation. In the present study, after determination of optimum incubation time and temperature, which was tenth day and 28°C, respectively, the fungus was cultivated at a series of initial pH values ranging from 4.5 to 6.5 with 0.5 intervals. During the course of growth of fungal cultures, pH of culture medium was not regulated as it is barely possible.

The highest laccase activity was obtained when the initial pH of the medium was adjusted to 5.0-6.0; with 5.5 being an optimal with activity of 0.1266 U/ml (Figure 6). It was also observed that laccase activity was raised with increased pH from 4.5 up to 5.5, later on it showed a declining trend in activity as the pH approached neutral. There was no significant different in laccase activity at pH 5.0 and 6.0.

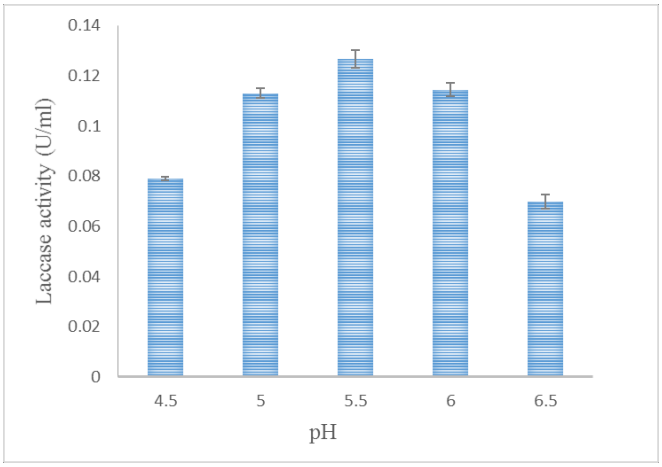

Figure 6. Effect of pH on laccase production by *P. ostreatus* grown on ESD under SSF.

The results obtained in the present study are in agreement with Nadeem *et al.* (2014) who reported that most of the fungal cultures preferred a slightly acidic pH of medium for growth and enzyme production. Prasad *et al.* (2005); Adejoye and Fasidi (2009); and Sivakami *et al*. (2012) also showed that in *Schizophyllum commune* and *P. ostreatus* the optimal pH for fungi mycelia biomass yield and laccase activities was 5.5. Selim *et al*. (2013) reported that enzyme production was found to be increasing gradually with the increase in initial pH, reaching the maximum at 7 (0.472 U/ml) and then decreasing at higher pH values such as pH 9 (0.005 U/ml). The culture pH strongly affects many enzymatic processes and transport of various components across the cell membrane.

Determination of the most preferred carbon source by *Pleurotus ostreatus* for laccase production

In order to determine the most preferred carbon source by *P. ostreatus* for laccase production, different supplementary carbon sources at 2% concentration were used in production media. The highest laccase activity was obtained when soluble starch was used as additional carbon source and laccase activity reached up to 0.1416 U/ml, showing a 1.31-fold increase compared to control, while galactose resulted in low laccase level (0.0757 U/ml). Glucose, sucrose and maltose shows equal laccase activity with the control, while all the remaining carbon source shows less laccase activity compared to control unit (Figure 7).

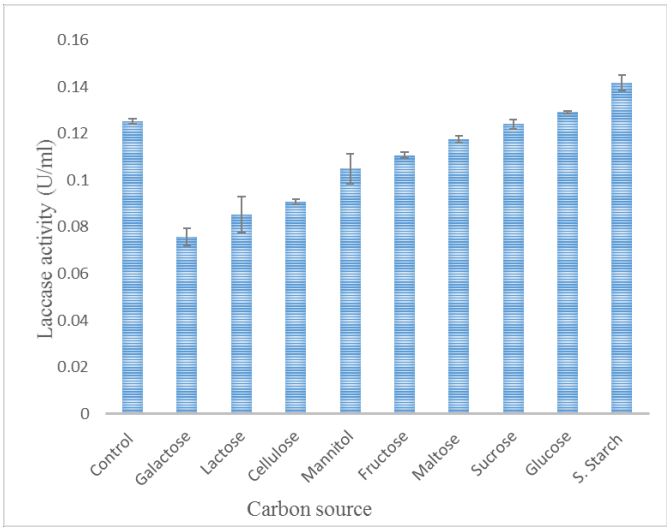

Figure 7. Effect of supplementation of ESD with different carbon source on laccase production by *P. ostreatus*.

There was no significant difference in laccase activity obtained from control unit and glucose, maltose and sucrose. Fructose, maltose and mannitol, as well as cellulose and lactose also show insignificant variation. However, there was statistically significant variation in laccase activity obtained from different supplementary carbon sources, which indicate that the variation is most probably brought by type of supplementary carbon sources.

In agreement with the current study, Elsayed *et al.* (2012) reported that soluble starch was found to be the best carbon source for laccase production. However, galactose, fructose, and maltose significantly repressed laccase formation by *P. ostreatus*, which was in line with the current finding. Additionally, Sivakumar *et al. (*2010) confirmed that among six different carbon sources such as mannitol, maltose, sucrose, glucose, lactose and starch tested at 2% for laccase production in *Ganoderma* species. Starch supported a maximum laccase activity of 0.18 U/ml. Abdulah (2008) also reported that the highest laccase activity was obtained when glucose was used as carbon source, but cellulose resulted in low laccase levels (0.01 U/ml).

On the other hand, Ticlo *et al*. (2006) described that among several of the carbon sources tested; maximum laccase activity was detected when fructose was used as the carbon source.

Determination of the most preferred nitrogen source by *Pleurotus ostreatus* for laccase production

Ligninolytic enzyme production by the wood-rotting basidiomycetes has been influenced by the nature of nitrogen sources and they are the most important factors for regulation of ligninolytic enzyme production (Bakkiyaraj *et al.* 2013). The results depicted in Figure 8 indicate that nitrogen source like peptone and beef extract gave better laccase activities than ammonium chloride, ammonium sulfate and sodium nitrate. Maximum laccase activity was recorded with peptone, which was 0.1578 U/ml. When compared to control, laccase obtained with inorganic nitrogen source showed less activity, while results from organic nitrogen showed more or equal laccase activity. There was no significant variation in laccase activity obtained from beef extracts and control unit. However, generally there was significant variation in laccase activity, which brought by addition of supplementary nitrogen source to ESD production media.

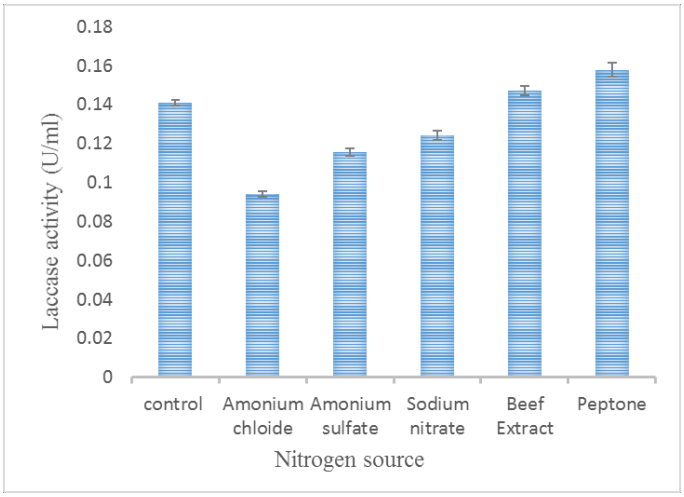

Figure 8.Effect of supplementation of ESD with different nitrogen source on laccase production by *P. ostreatus*.

Inorganic nitrogen sources like ammonium chloride, ammonium sulfate and sodium nitrate have failed to produce high laccase when using them as supplementary nitrogen source compared to organic ones i.e. peptone and beef extract. The current result is in line with the reports of Arora and Gill (2004); Strong (2011); Abdel-Azeem and Salem (2012); Kenkebashvili *et al*. (2012); Bakkiyaraj *et al.* (2013); Junyao He *et al.* (2014) which shows that organic nitrogen sources such as peptone were more suitable than inorganic sources for different fungi.

Determination of the most preferred divalent metallic ions by *Pleurotus ostreatus* for laccase production

In order to select the best salt solution for laccase production by *P. ostreatus* 5 mM of each salt (metal ions) was used namely: manganese chloride, zinc chloride, iron sulfate, magnesium sulfate, calcium chloride, and copper sulfate. The results showed that all compounds gave good fungal growth other than copper sulfate and iron sulfate although there was a little variation in mycelial biomass (Figure 9). Maximum laccase activity was observed in medium with calcium chloride, which was 0.1781, U/ml and the minimum was0.0043 U/ml with iron sulfate medium. Copper sulfate and iron sulfate inhibited the growth of the fungus in the production media. There were only very little mycelia on the media, which were brought along with the PDA plugs used as inocula. In general, other than calcium chloride medium, all salt solutions (metal ions) reduced laccase production compared to control unit without any salt solutions (Figure 9). There was statistical insignificant difference in results obtained from control unit and manganese chloride, iron sulfate and copper sulfate, magnesium sulfate and manganese chloride. In general, supplementation of ESD production medium of *P. ostreatus* with different metal ions highly affects its laccase production.

Metal ions can significantly affect the growth and extracellular enzymes production capabilities of white rot fungi (Baldarian 2004). Selim *et al.* (2013) reported similar results by showing that laccase production reached the maximum value with supplementation of Ca^{2+} (0.730 U/ml) and decreased with Fe^{+2} (0.49U/ml). Mongkolthanaruk *et al*. (2012) also indicated that Ca^{2+} activated more laccase production. The requirement for specific metal ions depends on the organisms i.e. different organism prefers different metal ions and thus the product varies. Ions such as iron may interrupt the electron transport system of laccase and substrate conversion and thus results in little or no activity (Kim and Nicell, 2006).

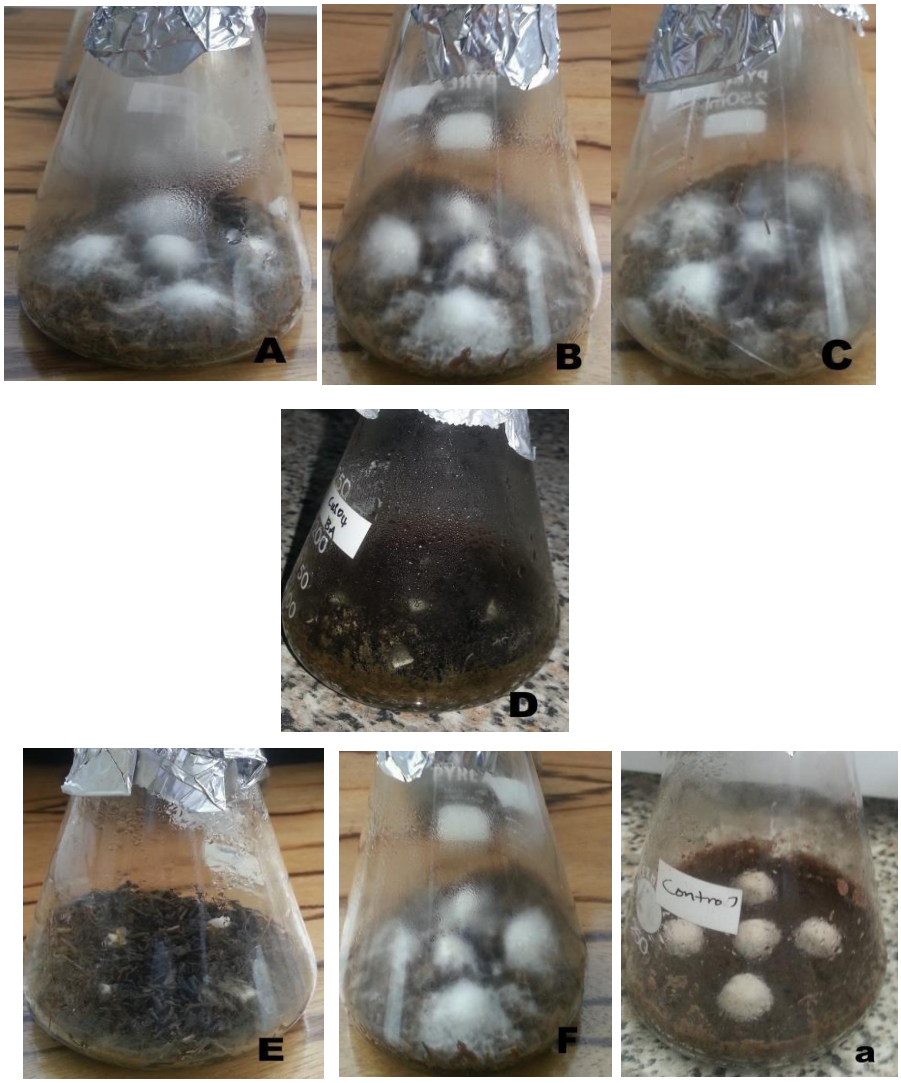

Figure 9. Fungal culture on medium with different supplementary metal ions (salt solutions).

(A- Calcium chloride, B- Magnesium sulfate, C- Manganese chloride, D- Copper sulfate, E- Iron sulfate, F- Zinc chloride and a- Control).

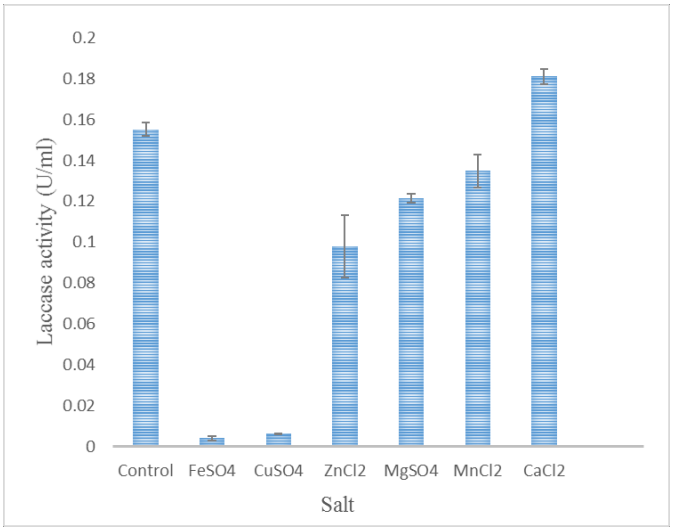

Figure 10. Effect of supplementation of ESD with different metal ions on laccase production by *P. ostreatus*.

Determination of the most potent inducers for laccase production by *Pleurotus ostreatus*

Different inducers were investigated for their potential of laccase induction, namely biotin, alanine, asparagine, cystien, leucine, lysine, phenylalanine, glycine and copper sulfate. The production media were amended with the aforementioned inducers at a concentration of 0.2% (w/v) amino acids and 1 mM of $CuSO_4$. Inducer compounds have been widely employed to enhance laccase production by different organisms and the nature of the compound that induces laccase activity differs greatly with the species (Junyao He *et al*. 2014). When $CuSO_4$ was added to culture medium before inoculation, it resulted in in fungal growth inhibition. There was no/little fungal growth initiation when $CuSO_4$ was added to growth medium before inoculation. Therefore, in order to overcome this problem, the fungus was first inoculated on free production media and incubated at 28°C. Then $CuSO_4$ was added on the third day following Cavallazzi *et al.* (2005). After that, since the fungus already established its growth system there was no or little suppression effect from $CuSO_4$.

After full growth period, the recorded activity showed that asparagine induces more laccase production with an activity of 0.1928 U/ml. Alanine showed similar laccase production with control while all the other remaining compounds resulted in decreased laccase production with lysine being the least inducer resulting in an activity of 0.0290 U/ml (Figure 11). In addition to this, $CuSO_4$ which was added on the third day of inoculation showed more laccase production with an activity of 0.2105 U/ml, which was (1.2-fold increase) compared to the control. In addition to asparagine, $CuSO_4$ was also concluded to be the potent inducers for laccase

production by *P. ostreatus.* Laccase activity obtained from different inducers shows that there was statistically significant variation among different inducers.

The results are in agreement with those of Selim *et al*. (2013), where they reported that enhanced laccase production was observed in the presence of CuSO4, L-tryptophan, L-leucine and L-methionine. Copper is a laccase cofactor which presents four cupric ions each associated with one single polypeptide chain, but it also has been proved that this element may play an important role in laccase gene regulation at transcription level. Junyao He *et al.* (2014) confirmed that copper sulfate is capable of inducing laccase synthesis when added at 1 mM concentration after 24 h of cultivation. Addition of various amino acids in the medium stimulates the ligninolytic enzyme production. The higher laccase activity (57.25 U/ml) was recorded with alanine at the concentration of 0.01% in the medium and moderate to good level of enzyme activities were obtained with asparagine (Johnsy *et al*. 2014).

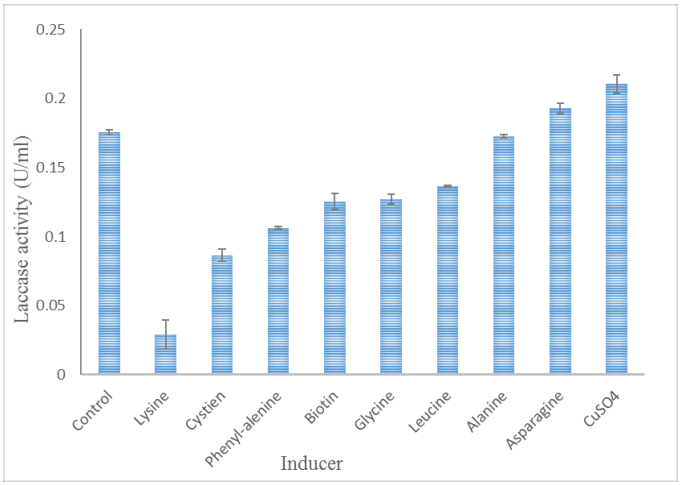

Figure 11. Effect of supplementation of ESD with different inducers on laccase production by *P. ostreatus*.

Production of laccase under optimized and non-optimized media

At the end of growth parameter optimization, the fungus was grown under optimized and non-optimized media of SSF to compare their significant difference. In optimized media, ESD was amended with 2% soluble starch as supplementary carbon source, 0.2% peptone as supplementary nitrogen source and 5 mM $CaCl_2$ as most preferred salt solution. Non- optimized media was simply prepared from ESD as a sole nutrient source. The pH of both media were adjusted to 5.5 and incubated at the same temperature 28°C for 10 days. For optimized media 1mM of $CuSO_4$ was added as a potent inducer on the third day of incubation. The fungus grew on optimized medium shows more mycelial biomass than fungus grew on non-optimized medium (Figure 12).

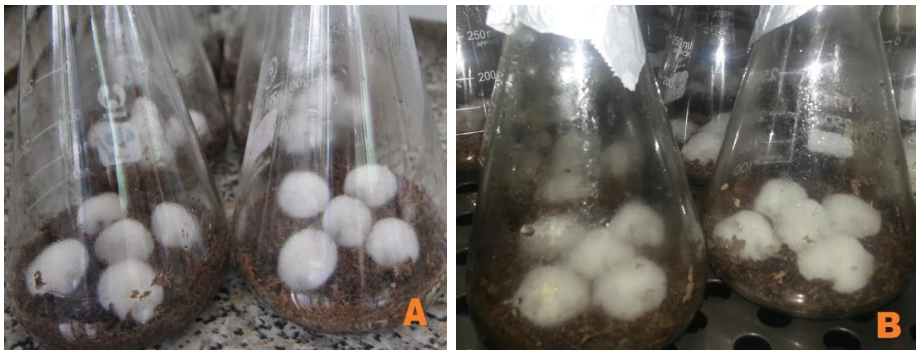

Figure 12. Production of laccase by *P. ostreatus* on optimized and non-optimized media of ESD under SSF. (A- Non-optimized media and B- Optimized media).

After full incubation time, enzyme was harvested and their activity was recorded. The activity obtained in optimized medium was 0.2187 U/ml, whereas non-optimized medium is 0.1269 U/ml. There was a total of 1.72-fold increment of laccase activity in optimized media compared to non-optimized media. This indicate that, cultivation of *P. ostreatus* under optimized media resulted in more laccase activity and hence more laccase production (Figure 13).

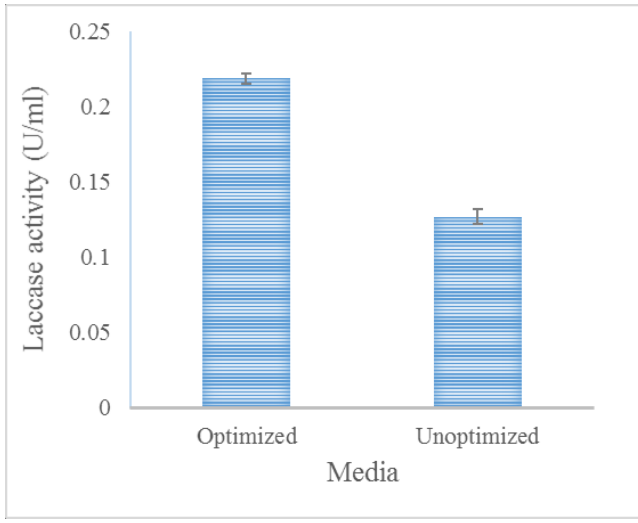

Figure 13. Effect of optimized and non-optimized media on laccase production by P. ostreatus under SSF of ESD.

Partial characterization of laccase

Effect of pH on laccase activity

In order to determine the optimum working pH for laccase enzyme, the crude extract was first harvested and incubated at 50°C within different pH range from 3.5-9.0 for 15 minutes. The results of this test showed that the optimum pH for laccase activity was 6.0 and there was a sharp decline in activity with further increase in pH

(Figure 14). It was also observed that there was less activity of laccase in acidic pH values compared to slight acidic pH ranges. There was statistically significant variation in laccase activity recorded in different pH, which indicate that the level of working pH had direct influence on laccase catalysis.

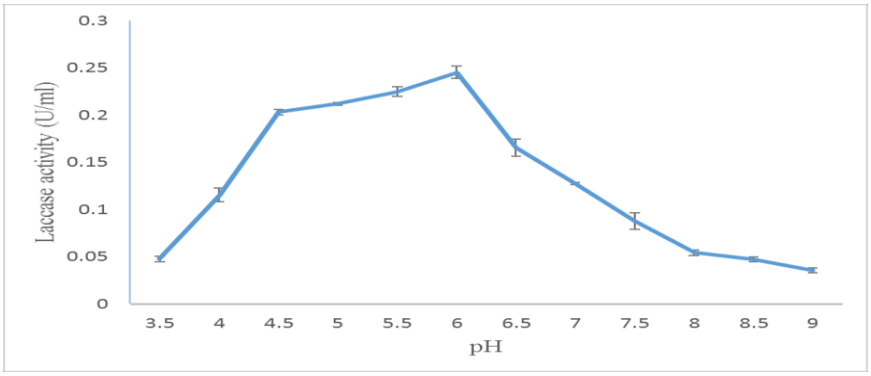

Figure 14. Effect of pH on laccase activity

The result is in agreement with Abdulah (2008) who indicated that enzyme activity has a bell shaped profile with an optimum pH that varies considerably. The report also showed that the optimum pH for laccase activity was 6.0. The dependence of laccase activity on pH usually renders a bell-shaped profile. This bi-phasic profile is the result of two opposing effects. The first is due to the redox potential difference between a reducing substrate and the Type 1 copper center of laccase, where the substrate docks. Here, the electron transfer rate is favored for phenolic substrates at a high pH. The second is generated by the binding of a hydroxide anion to the Type 2/ Type 3 copper center of laccase, which inhibits the binding of O_2, the terminal electron acceptor, and therefore inhibits the activity at a higher pH because of the increased Amount of OH^- ions (Bar, 2001). In addition, Ravikumar *et al*. (2012) also reported that the optimum pH of the purified laccase was found to be 6.0, and Ashger *et al*. (2012) indicated that laccase activity increased initially with pH and peaked up at 5.0. The enzyme had a good activity over a wide pH range (5.0 to 8.0), which may be a useful characteristic for various industrial and biotechnological applications.

Effect of pH on laccase stability

To determine the stability of laccase over a range of pH, crude laccase extracts was incubated overnight (nearly 12 hrs) at 50°C and pH range from 3.5-9.0. Laccase activity was recorded and compared with the initial activity. Table 2 shows initial and final activity of laccase and percentage of remaining activity after 12 hrs incubation. There was significant difference in laccase activity recorded initially and after overnight incubation. This indicates that overnight incubation had an effect on laccase activity of *P. ostreatus.*

Result of this test confirmed that crude laccase nearly retains 71 % of its initial activity at pH 6.0 when incubated overnight. At pH 6.5-7.5, it maintains 66-56% of its initial activity, while it was 33-43% at lower pH values (3.5-4.5). The reason for

declining in enzyme stability at acidic pH is due to the effect of acidic environment in enzymatic structure, which causes ionizing groups in active site. The declining in enzyme activity at pH above the optimum pH may be due to irreversible denaturation of enzyme molecule that leads to change in enzyme structure associated with the formation of enzyme dimerization that leads to enzyme autolysis (Abdulah, 2008).

Table 2. Comparison of laccase activity (U/Ml) recorded in different Ph after overnight incubation (values are mean)

3.5	0.0477±0.0004	0.0159±0.0004	33.42	0.0003
4	0.1155±0.0009	0.0430±4.1E-5	37.27	0.0079
4.5	0.2033±0.0028	0.0880±0.0017	43.29	0.0025
5	0.2120±0.0015	0.1059±0.0051	49.93	0.0197
5.5	0.2248±0.0049	0.1439±0.0009	64.02	0.0338
6	0.2453±0.0065	0.1737±0.0018	70.82	0.0433
6.5	0.1658±0.0026	0.1102±0.0012	66.47	0.0118
7	0.1277±0.0010	0.0763±0.0021	59.72	0.0077
7.5	0.0879±0.0004	0.0497±0.0019	56.55	0.0269
8	0.0545±0.0012	0.0273±0.0006	50.07	0.0105
8.5	0.0472±0.0006	0.0216±0.0002	45.75	0.0077
9	0.0357±0.0024	0.0147±0.0017	41.15	0.0266

±*Standard error, N=2).*

Activity within pH Sivakam enzyme retains more than 50%. Activity in the pH range 4.0-6.0. Naturally, laccase is very stable over a broad range of pH. The pH stability profile indicated that the enzyme was unstable at pH values above 6.0 but reasonably stable over pH values from 1.5 to 5 maintaining 80% of its original activity after incubation for 1h as reported by Junyao He *et al.* (2014). They also cited that most laccase from fungi such as *Coltricia perennis, Paraconiothyrium variabile* and *Scytalidium thermophilum* have an optimal pH range of 3−6 and have good stability in near neutral conditions. Laccase from *P. ostreatus* is stable at different pH values and more stable at alkaline range and it retains about 85-88% of its initial activity after 5 h of incubation at pH range from 5-7 (Othman *et al.* (2014).

Effect of temperature on laccase activity

Determination of optimal working temperature was tested by incubating crude enzyme extract at different temperature ranging from 20-80°C with 10°C interval (Figure 15). Phosphate buffer (pH 6.0) which was an optimized pH for laccase activity was use Activity within pH.

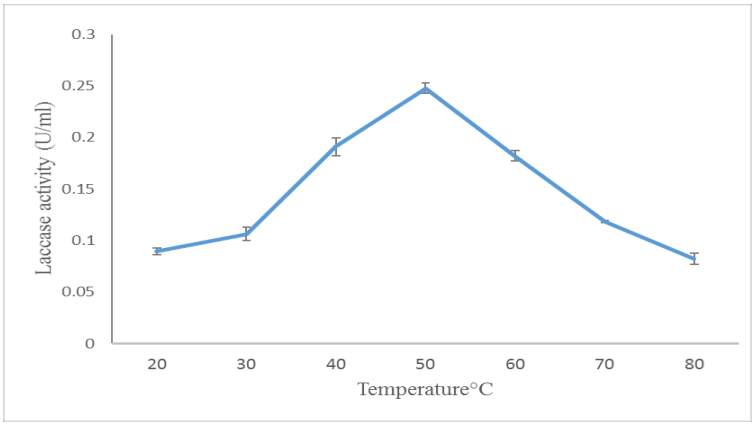

Figure 15. Effect of temperature on laccase activity.

The temperature versus laccase activity curve showed an increase in activity towards 50°C with an activity of 0.2478 U/ml. After 50°C the activity decline and the minimum activity was recorded at 80°C which was 0.0824 U/ml that was only 33.3% of its optimum activity at 50°C. The reason for declining of an activity towards higher temperature is that the speed of enzyme interaction increase with increasing temperature within a certain range. This is due to increased energy kinetics and the collisions between enzyme molecules and substrate except that the high temperatures within certain limits lead to denaturation of the enzyme and loss of three dimensional structure and then decline in enzyme activity (Abdulah 2008). Generally, there was significant variation in laccase activity obtained at different incubation temperatures.

Othman *et al.* (2014) reported that *P. ostreatus* purified laccase displayed a maximum activity at 50ºC. The optimum activity for laccase from *Trametes hirsuta* is ranged from 40 to 60°C as reported by Castillo *et al.* (2012). The optimal temperature for laccase was determined to be 60°C and laccase activity declined when the temperature was increased from 60 to 80°C, with 40% of the optimal enzyme activity observed at 80°C (Ding *et al.* 2012). On the other hand, Ashger *et al.* (2012); Junyao He *et al.* (2014) indicated that the optimum temperature for the crude laccase was 40 and 45 °C, respectively.

Effect of temperature on laccase stability

To determine the thermostability of crude laccase extract, incubation was done overnight (nearly 12 hrs) at temperature range from 20-60°C. The result indicated that laccase only lost 4 and 12% of its initial activity at 20 and 30°C, respectively when incubated overnight (Table 3). At 40°C, moderate stability was observed maintaining 60% of its initial activity. Above 40°C, the stability declined sharply and only 32% of its initial activity remains at 60°C. This indicates that crude laccase extracts are more stable at lower temperature and needs to be stored at low temperatures. The reduction of activity above 40°C is due to sensitivity to high temperature, reflecting

the effect of temperature on the three dimensional structure of protein by damaging R-groups of amino acids, which lead to denaturation of protein and losing its activity. There was significant variation in laccase activity at different temperature after overnight incubation. Table 3 shows activity recorded initially and after overnight incubation and the remaining activity in percentage.

Table 3.Comparison of laccase activity (U/Ml) recorded at different temperature after overnight incubation (values are mean ±standard Error, N=2).

Temp (°C)	Initial	Final	Activity	P- value
20	0.0893±8.2E-5	0.0858±0.0021	96.11	0.1707
30	0.1064±0.0014	0.0936±0.0005	88.06	0.0233
40	0.1908±0.0071	0.1146±0.0029	60.04	0.0169
50	0.2478±0.0034	0.0912±0.0032	36.81	0.0005
60	0.1821±0.0017	0.0585±0.0032	32.14	0.0017

Means having p-value less than 0.05 are statistically different between initial and final within temperatures.

For a variety of industrial applications, relatively high thermostability is an attractive and desirable characteristic of an enzyme. However, most of white rot fungi laccases are stable and almost fully active in a temperature range of 30-50°C (Ashger *et al.* 2012). Othman *et al.* (2014) revealed that the *P. ostreatus* purified laccase could sustain heating up to 30°C without apparent loss of activity for 120 min, and the residual activity was regularly decreased as a function of both time of exposure and temperature. Incubating the enzyme at 40, 50 and 60ºC for 120 min resulted in a loss of about 4, 20 and 40% of its activity, respectively. Junyao He *et al.* (2014) also discovered that at 45°C, laccase retained approximately 50% of its initial activity. However, it was almost completely inactive at temperatures above 60°C. Ding *et al*. (2012) also found that there was no loss in laccase activity detected over 80 min at 30, 40 or 50°C. At 60°C, 46% of the residual laccase activity remained after 80 min, indicating that *G. lucidum* laccase is a thermo-stable enzyme. Less than 10% of laccase activity was retained after 20 minutes when incubated at 70°C, and complete inactivation occurred after 10 min at 80°C. The greatest stability of the laccase enzyme was observed at 30°C after 48 hrs of incubation (Stoilova *et al.* 2010).

Effect of substrate concentration on laccase activity and study of kinetic Parameters

Different concentrations of guaiacol substrate (from 2-12 mM) were prepared to check the effect of substrate concentration on laccase activity and to find the kinetic parameters of laccase. The result indicated that there was a linear relation between laccase activity and its substrate concentration. There was increase in activity with increasing substrate concentration. After certain concentration, the rate of increase in the velocity decreased due to occupation of active sites of enzyme by the substrate

and finally there was no increase in the rate of reaction (Saqib *et al.* 2015). Further, addition of the substrate had no effect on laccase activity.

The reciprocal of laccase activity 1/V in U/ml was plotted against reciprocal of substrate concentration (1/[S]) in mM. Kinetic parameters like Km and Vmax values for *P. ostreatus* laccase were calculated using Lineweaver Burk plot. The regression equation for the Lineweaver Burk slope was y = 4.4898x+1.0842 (R^2 = 0.9862). From this, the apparent calculated Vmax and Km were 0.922 U/ml and 4.14 mM, respectively (Figure 16). The relationship between rate of reaction and substrate concentration depends on the affinity of the enzyme for its substrate expressed as Km (Michaelis constant) of the enzyme.

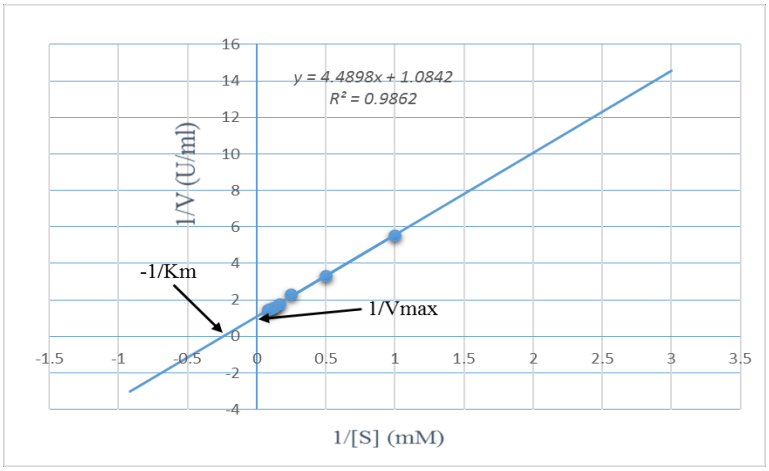

Figure 16. Linweaver Burk plot of laccase activity vs substrate concentration.

Laccase has different Km and Vmax values for different kind of substrates used. Saqib *et al.* (2015) reported that the calculated values of Km and Vmax for laccase were found to be 0.666 mM and 20.8 µM/min, respectively. Shraddha *et al.* (2011) also reported that Km using guaiacol reagent as substrate for laccase found to be 0.405 mM. The Km and Vmax values of *T. versicolor* laccase by using ABTS as a substrate was 73 uM and 780 U/ml, respectively as indicated by Asgher *et al.* (2012). There was significant variation in laccase activity at different guaiacol concentration.

Summary and Conclusion

Summary

Pleurotus ostreatus was identified based on its colony morphology as well as microscopic visualizations. The microscopic observations showed smooth and cylindrical to narrowly kidney shaped features, which was the features of *P. ostreatus* fungi. The culture was able to develop intense brown color around the colony after

10th days of incubation due to oxidation of guaiacol by laccase, which can be correlated to its activity. Production of laccase by study organism was better in SSF system than SmF system and among tested lignocellulosic substrates; *Eucalyptus* saw dust was selected for further growth parameter optimization. Crude laccase extract was harvested by sodium acetate buffer (pH 4.5) and centrifuged at 10000 rpm at 4°C for 15 minutes and later the clear supernatant was collected through decantation that was used for laccase activity detemination.

The production of laccase was higher at 10th day of incubation period, and the maximal pH and incubation temperature were 5.5 and 28°C, respectively. Soluble starch and peptone was found to be the most preferred carbon and nitrogen source for laccase production by *P. ostreatus*. When added on the 3rd day of incubation, copper sulfate was found to be the best inducers. In addition, asparagine was also found to be good inducers for laccase production by *P. ostreatus*. Generally, there was 1.72-fold increase of laccase production in optimized media compared to non-optimized one.

The optimum working pH and temperature of laccase were found to be 6.0 and 50°C, respectively. After 12 hrs, incubation at pH 6.0 laccase was found to maintain 71% of its initial activity. Laccase only lost 4 and 12% of its initial activity at 20 and 30°C, respectively, when incubated for 12 hrs at its optimum pH value. Generally, laccases are stable and almost fully and/or partly active in a temperature range of 20-50°C. We hope that these characteristics would make this enzyme potentially attractive in variety of industrial applications like animal feed treatments. There was a linear relation between laccase and its substrate concentration, there is increase in activity with increasing substrate concentration. The relationship between rate of reaction and substrate concentration depends on the affinity of the enzyme for its substrate expressed as Km (Michaelis constant) of the enzyme. The lower the Km, the higher affinity of the enzyme towards its substrate.

Recommendations

At the end, the following recommendations were made based on the findings of current investigation; from current study, solid-state fermentation would be recommended for laccase production. Optimized medium of *Eucalyptus* sawdust gave more laccase production than unoptimized one and hence recommended for large scale production. Further study should be made to optimize the potential of *P. ostreatus for* production of other ligninolytic enzymes such as peroxidases.Additional research should be made on large scale production, further enzyme purification and detailed characterization of laccase from *P. ostreatus*. Further study is recommended at a molecular level to enhance the production of laccase from *P. ostreatus* for animal feed processing and other possible industrial application.

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Production, Purification and Characterization of Xylanase from Oyster Mushroom (*Pleurotus Sp.*)

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Abstract

*The aim of this study was to screen **xylanase** producing oyster mushroom species collected from Eucalyptus tree bark around Holetta, to evaluate the In vitro production of **xylanase** by **Pleurotus spp**. using different lignocellulosic substrates, and to partially purify and characterize the **xylanase** produced by **Pleurotus spp.** with respect to changes in pH, temperature, and concentration of substrates. One hundred mushroom specimens were randomly collected from Eucalyptus tree bark in the premise of Holetta Agricultural Research Center campus. 100% of the collected mushroom specimens were identified morphologically and biochemically as **Pleurotus ostreatus** and also screened for **xylanase** production depend on the clear zone formation on malt extract agar plate containing xylan as sole carbon source. This mushroom specimen were cultivated using both solid state fermentation and submerged fermentation systems supplemented with different substrates (wheat straw, teff straw, bean straw and Eucalyptus tree bark) to identify the most suitable medium for the production of **xylanase.** The highest enzyme production was obtained on bean straw (2.38U/ml and 1.77 U/ml in solid-state fermentation and submerged fermentation, respectively) and the lowest was obtained in media containing Eucalyptus tree bark (0.36 U/ml and 0.58 U/ml in solid-state fermentation and submerged fermentation, respectively). Optimal production of **xylanase** was obtained when Pleurotus ostreatus was grown in solid-state fermentation using wheat straw supplemented with 5% birch wood xylan, peptone and KCl salt at pH 4.0 and a temperature of 30∘C under stationary conditions for four days. The **xylanase** from **P. ostreatus** was partially purified to homogeneity using different concentrations (30 to 80% (wt/vol) of ammonium sulphate. **Xylanase** having highest specific activity (11.47U/ml) and total protein content of 0.631mg/ml was recovered from the culture supernatant when precipitated with 40% (wt/vol) ammonium sulphate. The optimum activity was observed at 50∘C and pH 6.0. The enzyme was very stable at a wide range of temperature and pH. Its apparent Km and Vmax were 186.67 µ g/ml and 11.58µ mole/min, respectively, showing its high affinity towards its substrate.*

Introduction

Mushrooms are wide spread saprophytic macroorganisms belonging to fungi growing on dead organic matter of plant origin. They can utilize agricultural wastes as their substrates for their growth and metabolism. During the growth of mushroom, various biochemical changes are known to occur because of which enzymes are secreted extracellularly to degrade the insoluble materials in to simple and soluble molecules. These enzymes affect the food and nutrient value, flavour and shelf life of these fungi (Chandra, 2011).

There are at least 12, 000 species of fungi that can be considered as mushrooms with at least 2000 species are edible. Over 200 species have been collected from the wild vegetation and used for various traditional medicinal purposes. Thirty-five species have been cultivated commercially and 20 are cultivated on an industrial scale (Chandra, 2011). The most cultivated mushrooms worldwide are *Agaricus bisporus* (button mushroom), followed by *Lentinus edodes* (shiitake), *Pleurotus* spp. (oyster mushrooms), *Auricula auricula* (wood ear mushroom), *Flamulina velutipes* (winter mushrooms) and *Volvariella volvacea* (straw mushroom) (Chandra, 2011). The edible mushrooms are excellent foods that can be incorporated into well balanced diets due to their low content of fat and energy, and high content of dietary fiber and functional compounds. Their benefit to health includes immunomodulatory, antitumoral, and hypocholesterolemic effects (Rajarathnam and Bano, 1987). Innumerable molecules synthesized by macrofungi are known to be bioactive compounds such as polysaccharides, glycoproteins, terpenoids, lectins, among others (Chandra, 2011).

The *Pleurotus species* are commonly known as oyster mushrooms. Most of the known members of the genus are edible. One of the most important aspects of *Pleurotus species* is related to the use of lignocellulosic system for a variety of applications, such as the bioconversion of lignocellulosic wastes into valuable products, for animal feed and other food products and the use of their enzymes for the biodegradation of organopollutant, xenobiotics and industrial contaminants. *Pleurotus* is an efficient destructor of lignocellulosic substrates, which leaves a white rot residue during degradation, hence called as white rot fungus (Ortega *et al*. 1993).

Oyster mushrooms grow naturally in the temperate or tropical forests on dead and decaying wooden logs or on outer barks of living trees. The fruiting bodies of this mushroom are distinctly shelly or oyster shaped with different color of white, cream, gray, yellow, pink or light brown depending upon the species (Charles, 2008). Pleurotus is a versatile genus of white rot basidiomycete fungi belongs to the order Agaricale of family pleuroteceae that can synthesize and release appreciable amounts of the cellulolytic, lignolytic and xylanolytic enzymes that are of much importance in lignocellulosic degradation (Singh and Gautam, 2004).

Cohen *et al*. (2002) have studied the use of lignocellulolytic enzymes from microbes such as Pleurotus spp. for the biodegradation of organo pollutants, xenobiotics and industrial contaminants previously. Plant cell walls are generally composed of three major polymeric constituents; cellulose, an insoluble polymer composed of ß-D-glucopyranosyl residues linked by ß-1, 4-glycosidic bonds; hemicelluloses, a series of heteropolysaccharides that include xylans, glucans, mannans and arabinans; and lignin, a complex polyphenol, intimately interconnected with the hemicelluloses (Eichlerova *et al.* 2006). Thus, the lignocellulolytic enzymes produced by oyster mushrooms growing on tree barks include among others, laccase, manganese peroxidase (Stajic *et al*. 2006), xylanase (Elisashvili *et al*. 2001), cellulase, β-glucosidase and β-xylosidase (Maganhotto *et al.* 2005). These enzymes have shown enormous biotechnological potentials as they can be used at a wider level for the degradation of this highly complex plant cell wall material (Ren and Buschle, 2007) and detoxification of agro industrial residues with high phenolic contents (Mata *et al*. 2005).

Lignocellulosic wastes contain significant concentrations of soluble carbohydrates that aid in inducing the synthesis of enzymes and ensuring the efficient production of lignocellulolytic enzymes (Reddy *et al*. 2003). In addition, due to the heterogeneity of xylan, the hydrolysis of hemicelluloses requires the action of a complex enzyme system that is required for a number of ain chain and side chain cleaving enzyme activities.

The main chain cleaving enzymes are endo-ß-1, 4-xylanases, ß-1, 4-xylosidases and the more recently discovered enzymes named as exoxylanases (Biely, 1993). Hemicellulases are more complex enzymes than other lignocellulolytics and are capable of degrading hemicelluloses. The xylanases are the best characterized and most widely studied of the hemicellulolytic enzymes. Xylanases hydrolyze 1, 4-β-D-xylosidic linkages in xylan to produce xylooligosaccharide. In natural environment, mainly microorganisms, marine algae, protozoans, crustaceans, insects and snails produce xylanases. Among microbial sources, filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase activities are much higher than these found in yeasts and bacteria (Sunna and Antranikian, 1997). This property makes fungal xylanases attractive to be used in various industrial applications. For instance, in pulp and paper industry, the xylanases are employed in the prebleaching process to reduce the use of the toxic chlorine chemicals. In animal feed industry, xylanases are used to increase the body weight gains of the animals. In bread and bakery industry, xylanases are used to increase the dough viscosity, bread volume and shelf life (Palaniswamy *et al*. 2008).

Oyster mushrooms are able to produce hydrolytic (cellulases and hemicellulases) and oxidative (ligninolytic) extracellular enzymes required to degrade the major components of lignocellulosic biomass into the low molecular weight compounds that can be very easily assimilated by their cells (Leatham, 1985; Cohen *et al*. 2002; Reddy *et al*. 2003). Several reports indicate that it is practically and economically

feasible to apply bioconversion processes to waste lignocellulosic materials, especially in developing countries (Chang and Steinkras, 1982; Martinez *et al*. 1991).

Industrial enzymes are produced principally by submerged fermentation (SmF) but they are also produced by solid-state fermentation (SSF) at a lower level. There are several reports analytically comparing both types of enzyme production systems [7-12]. Although SSF is advantageous in terms of higher volumetric productivity and enzyme concentration, difficulties in scaling up restrict its use as the main technology for the industrial production of enzymes. Hence, this technique supports controlled release of nutrients. SSF is best suited fo fermentation techniques involving fungi and microorganisms that require less moisture content. However, it cannot be used in fermentation processes involving organisms that require high water activity, such as bacteria (Babu and Satyanarayana, 1996). However, very little work is reported on xylanolytic enzyme production by *Pleurotus* spp; Besides, especially little attention has been given to the evaluation of the hydrolytic system of these fungi, purification, detailed characterization of their enzyme, its broad activity, within temperature, pH range and other factors need further investigation (Baldrian and Gabriel, 2003; Ahmad, 2009).

Thus, the purpose of this study was to investigate the level of xylanase production by *Pleurotus* spp. collected from eucalyptus tree bark around Holetta, at National Agricultural Biotechnology Research Laboratory in submerged and solid state fermentation using various carbon sources of different substrates. Screening for xylanase producing *Pleurotus* spp is available at Holetta. Thus the specific objectives were:

- To evaluate the *In vitro* production of xylanase by *Pleurotus* spp. using different Substrates;
- To partially purify and characterize the xylanase produced by *Pleurotus* spp. (in terms of effects of pH, temperature and concentration of substrates.

Materials and methods

The study site

The study was conducted at Holetta Agricultural Research Center, National Biotechnological Research Laboratory that is located at 34 km West of Addis Ababa in the Oromia Special Zone Surrounding Finfinne, Oromia Region. Geographically, HARC is located at a latitude, longitude, and an altitude of 9 °3’ N, 38° 30 ’ E and 2391 meters above sea level, respectively. Typically, it has a bimodal rainfall pattern with a mean annual precipitation of 1100 mm.

Collection and maintenance of oyster mushroom

The specimen collections were conducted early September, immediately after main rainy season. One hundred mushroom samples were randomly collected from 20 different *Eucalyptus* tree around Holetta area, five specimens were collected from each tree; using sterile plastic bags and maintained at Holetta in the Biotechnological Research laboratory center on potato dextrose agar slants (PDA) and preserved at 4 ∘C in a refrigerator. These cultures were sub cultured at least monthly according to Jong (1978). PDA plate cultures were incubated similarly for six to seven days prior to use as inocula for SSFs or SmFs.

Identification of *Pleurotus* spp.

Identification of *Pleurotus* spp. was done using morphological and biochemical tests, based on identification methods (Charles, 2008; Kuo, 2005; Chandra, 2011).

Screening for xylanase producing *Pleurotus* spp.

Malt extract agar medium (MEA) containing xylan as the sole carbon source was inoculated with *Pleurotus* spp. The plates were incubated for 4 days at 30°C and positive xylanolytic activity was detected by the formation of clear zones of hydrolysis on plate after flooding the plates with 0.1% aqueous Congo red followed by repeated washing with 1 M NaCl. The composition of the MEA plate agar medium was (g/l): birch wood xylan, 1.0; peptone, 5.0; Malt extract, 5.0; K_2HPO_4, 0.2 and agar 20.0 .The final pH of the medium was adjusted to 5.5 prior to sterilization (Teather and Wood, 1987).

Source and preparation of growth substrates

The growth substrates like teff straw, wheat straw, bean straw, Birch wood xylan and salt minerals were obtained from HARC and from market. The selection of substrate for enzyme production in a SSF processes depends up on several factors mainly related with cost and availability of the substrates, and thus may involve screening of several agro industrial residues (Pandey *et al*. 1994). The lignocelluloses substrates were oven dried and ground using a manual grinder. All the resulting powder could pass through a 1 mm size mesh, and then the powder passed through 1 mm size mesh used for submerged fermentation and substrate above 1 mm used for solid state fermentation.

Preparation of media for enzyme production

Media for submerged fermentation

Media used for submerged fermentation were arranged in four separate Erlenmeyer flasks of 250 ml capacity containing each 10 g of a single type of lignocellulosic substrate (eucalyptus tree bark, teff straw, wheat straw or bean straw smaller than 1mm). Experiments were performed in triplicate at room temperature (25±2∘C) with shaking at 120rpm. The respective substrates were submerged in 200ml basal medium consisting of (g/l): (NH4)2SO4, 1.4 KH_2PO_4, 2; $CaCl_2$, 0.3; $MgSO_4$,

0.3; $FeSO_4.7H_2O$, 0.5; $MnSO_4.7H_2O$, 0.16; $ZnSO_4.7H_2O$, 0.14; $CoCl_2$, 0.2; and Tween 80, 1 ml; with a final pH of 5.5. The initial pH of the medium was adjusted to 4.0 prior to sterilization by adding 1N HCl. The media was autoclaved at 121°C (15 lbs) for 20 min and cooled (Papaspyridi *et al*. 2012).

Media for solid-state fermentation

Media used for solid state fermentation were arranged in four separate flasks of 250 ml capacity containing each 10 g of a single type of lignocellulosic substrate (eucalyptus tree bark or teff straw or wheat straw or bean straw). Experiments were performed in triplicateusing Erlenmeyer flasks at 30∘C. The respective substrates were moistened with 24 ml of salt solution (KH_2PO_4 0.5 g, $MgSO_4.7H_2O$ 0.3 g, NH_4NO_3 0.1 g, $CaCl_2.2H_2O$ 0.03 g and 1 ml of 1% FeCl3), with a final pH of 5.5. The media was then autoclaved at 121∘C (15 lbs) for 20 minutes and cooled (Jemaneh, 2007).

Preparation of inoculum

PDA inocula were prepared on PDA plates where the fungal stock stored at 4°C was transferred onto PDA plates with a sterilized needle and then allowed to grow at 30°C in the dark for 4 days. The growing edges of the spore were cut with a sterilized 4 mm core borer and used as inocula. The inoculum size was 3.6×10^6 spores per ml as determined by microscopic enumeration with a cell counting hematocytometer chamber (Neubauer chamber, Marienfeld and Germany). After it was inoculated to agar plates, the myceli of the inoculum piece were placed face down so that they had good contact with the agar surface (Jemaneh, 2007).

Growth of *Pleurotus* Sp. and extraction of xylanase

Submerged fermentation

Three mm^2 square of spore containing PDA was used to inoculate the flasks containing submerged media. After 4 days of mushroom cultivation, biomass was filtered through cottogauze and the solids separated by centrifugation 10000rpm for10 min at 4∘C. The supernatant was filtered through Whatman no. 1 filter paper and the clear filtrate was used as crude enzyme extract.

Solid-state fermentation

The solid-state media were inoculated with three mm square of spore containing PDA. After mixing, the flasks were incubated at 28 ± 2°C temperatures for 4 days under static conditions After 4 days of incubation the solid-state fermentation 100ml citrate buffer (0.05M at pH 5.3) rotated on rotary shaker at 120 rpm for at least 2 h at room temperature for maximum enzyme extraction. Liquid homogenate was then filtered through cotton gauze and centrifuged at10000rpm for 10 minute at 4°C to remove solid particulate matter. Supernatant was filtered through Whatman no.1filter paper and the clear filtrate was used as crude xylanase preparation.

Enzyme assay

Quantitative assay

The.xylanolytic activity was quantitatively assayed based on enzymatic hydrolysis of birch wood xylan and the reaction of the liberated reducing sugar with 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Xylose was used as the reference reducing sugar. One unit of xylanase activity was defined as the amount of enzyme that liberated reducing sugar at the rate of 1 µ mole/min. The reaction mixture contained 900 µl xylan solution (1% birch wood xylan in 0.05 M citrate buffer pH 5.3) and 100 µl crude enzyme.

After 10 minutes of incubation at 50°C, the reaction was stopped by adding 2ml DNS reagent followed by boiling for 5minutes and cooling (Bailey *et al*. 1992). Absorbance was measured on spectrophotometer (Novaspec III) at 540 nm against a reagent blank. One unit (U) is defined as the amount of enzyme that releases1µ mol of reducing sugar equivalent to xylose per minute. The amount of xylanase produced in SSF and SmF was expressed as U/ml.

$$\frac{\text{Units/}}{\text{ml}} = \frac{\text{df = Dilution factor(µmoles of xylose liberated)}}{\text{(df) (10) (0. 10 = Time of assay (in minutes)}}$$

Protein assay of the filtrate

Protein content of the culture supernatants was assayed by the folin ciocalteau method of Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as standard.

Optimization of growth conditions and characterization of xylanase

Time course of xylanase production

P*leurotus* isolates were grown on the basal liquid medium containing xylan and incubated at30∘C for 2-14 days and samples were harvested at 48 hour time intervals. The amount of xylanase was measured as described in section 3.9.1.

Effect of pH on the production of xylanase

After preparation of the basal liquid medium containing xylan, suitable aliquots were adjusted at pH 2, 4, 6, 8, 10 and 12 using 0.1M NaOH and 0.1N HCl buffer system. After 4 days of incubation at 30∘C, the amount of xylanase was assayed as mentioned in section 3.9.1.

Effect of temperature on the production of xylanase

After inoculation, In order to determine the optimum temperature for production of xylanase by isolate, the media were incubated at various temperatures ranging from, 20◦c to 35◦c with 5◦c intervals. After 4 days of incubation, the amount of the xylanase was assayed as mentioned in section 3.9.1.

Effect of different carbon source on the xylanase production

The basal medium was supplemented with various carbon sources at concentration of 5%. These carbon sources were maltose, starch, glucose, sucrose, cellulose and xylan, wheatstraw without any supplementation (as control). After 4 days of incubation at 30◦C, the amount of the xylanase was assayed as mentioned in section 3.9.1.

Effect of different nitrogen sources on the xylanase production

Different nitrogen sources, with concentration of 5% were used to see their inductive effect on the enzyme production. These includes ($(NH4)_2NO_3$, peptone, casein, tryptone, malt extractand xylan (as control). After 4 days of incubation at 30◦, the amount of the xylanase was assayed as mentioned in section 3.9.1.

Effect of metal ions on the xylanase production

To determine the effect of metal ions,on xylanase production, the basal medium was supplemented with different metal ions with final concentration of 5%.

Partial purification of xylanase

The crude enzyme was purified from the culture supernatant fluid using ammonium sulphate in a buffer of pH 5.3 (Fialho and Carmona, 2004). For this purpose, various concentrations of ammonium sulphate, i.e. 30, 40, 50, 60, 70 and 80% were used for the precipitation of enzymes. Nine volumes of the respective levels were mixed in one volume of crude enzyme filtrate and kept at least for 10minutes at -20◦C (over-night).

After thawing, the mixture was centrifuged at 10000 rpm for 10minutes at 4 ◦C. The supernatant was then carefully discharged while the precipitate was dried by inversing the tube on tissue paper. The resulting precipitate was re-suspended in citrate buffer (pH 5.3) and analyzed for xylanase activity. The optimum xylanase activity at a specific concentration of ammonium sulphate reflects the best concentration to attain maximum enzyme recovery. The purification fold was calculated by dividing the specific activity recovered by the starting specific activity.

Characterization of xylanase Produced by *Pleurotus* sp.

Effect of temperature on xylanase activity

Thermal stability was investigated by incubating the 100 µl xylanase of *Pleurotus* sp. in 900µ l of 0.1% xylan, at temperatures ranging from 20∘C to 90∘C with 10∘C intervals and pH 6.0 for 10 minutes. The residual activity of the enzyme was determined following the standard procedure.

Effect of pH on xylanase activity

The pH of reaction mixtures was adjusted with 100 mM of the following buffer solutions: citrate buffer (pH 2.0-5.0), phosphate buffer (pH 6.0-9.0) and 0.1M of NaOH buffer (pH 10.0-12.0). 900 µl of 0.1% xylan dissolved in respective buffers was incubated with xylanase (100µ l), incubated at 30∘C for 10 min and subsequently assayed.

Effect of substrate concentration on xylanase activity

Rate of xylan hydrolysis was determined by incubating 900 µl substrate (xylan) at various concentrations (2, 4, 6, 8, 10 and 12 µ g/ml) with 100 µ l of partially purified xylanase. The enzyme incubated without xylan served as control for 10 minutes. The kinetic constants k_m and V_{max} were estimated following Lineweaver and Burk method (Bruno *et al*. 1994**).**

Results and Discusion

Identification of *Pleurotus* species

The study indicated that, out of 100 specimen collected, all (100%) of them were identified as *Pleurotus* spp. (Table 1), *Pleurotus* spp. exhibit differences in morphology, color, host/substrate and fruiting season. The oyster mushrooms prefer wood and dead debris. The study organism of selected area grow on eucalyptus tree bark may be due to the appropriate tree available was only eucalyptus tree.

Table 1. Pleurotus Spp. exhibit differences in morphology, color, host/substrate and fruiting season

tree	(cm)	shape								
5	5.6	W- B	Enrolled	Convex	Absent	White	Decurrent	Peculiar	Cylindrical	*P.* spp
5	7.5	W- B	Enrolled	Kidney	Short	Brown	Decurrent	Peculiar	Kidney shape	*P.* spp
5	3.10	W- B	Enrolled	Kidney	Absent	Brown	Decurrent	Peculiar	Kidney shape	*P.* spp
5	5.2	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	4.4	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	8.3	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	7.5	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Cylindrical	*P.* spp
5	6.24	W- B	Enrolled	Kidney	Shot	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	6.35	W- B	Enrolled	Kidney	Absent	Brown	Decurrent	Peculiar	Kidney shape	*P.* spp
5	6.5	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	3.5	W- B	Enrolled	Kidney	Absent	Brown	Decurrent	Peculiar	Kidney shape	*P.* spp
5	4.5	W- B	Enrolled	Kidney	Short	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	5.52	W- B	Enrolled	Kidney	Short	White	Decurrent	Peculiar	Cylindrical	*P.* spp
5	3.2	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	7.35	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	7.0	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	8.2	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Kidney shape	*P.* spp
5	6.0	W- B	Enrolle	Kidney	Absent	White	Decurrent	Peculiar	Cylindrical	*P.* spp
5	3.5	W- B	Enrolled	Kidney	Absent	White	Decurrent	Peculiar	Cylindrical	*P.* spp
5	4.15	W- B	Enrolled	Kidney	short	Brown	Decurrent	Peculiar	Kidney shape	*P.* spp

W-B: White to Black, P.spp: Pleurotus species

Screening for xylanase producing oyster mushroom

The growth on the PDA is the best when compared to other medium at 30°C. The study organism was able to form halo zone of hydrolysis, which confirm its ability to produce xylanase enzyme. Moreover, additional qualitative tests revealed that the culture was also found to display amylolytic and proteolytic activities.

Xylanase production on different lignocellulosic substratmes

When different substrates were used in both solid state fermentation and submerged fermentation medium, the highest enzyme production was obtained on bean straw (Figure 2) (2.38 U/ml, 1.77 U/ml respectively) and the least on eucalyptus tree bark (0.36 U/ml, 0.58 U/ml in SSF and SmF, respectively). This indicated that bean straw is the most suitable substrate for xylanase production by *Pleurotus* spp. When compared with other study substrates. This is probably due to higher protein content of bean straw. Wheat straw was the second highest in SSF, while the third in SmF.

Based on the substrate selection criteria bean straw was not selected for further study in this experiment. Study substrates were analyzed by economical point of view; its availability around study area and management to use it as substrate. Bean straw is more scare, more expensive and need high management (Easily perishable). According to Bakri *et al*. (2003) wheat straw is an inexpensive, agricultural by product, which contains a lot of xylan. Therefore, further studies were conducted using wheat straw as carbon source. Eucalyptus tree bark is natural substrate for the study organism, but the study showed that it results lowest production of xylanase. This is due to nutrient content found in it, which the organism highly need protein nutrient to grow (why it is highest in bean straw).

The production of xylanase by *Pleurotus* sp. was higher in SSF, using different substrates as carbon source, when compared to the results obtained in SmF with the same substrates (Figure 1). A higher efficiency on enzymatic production SSF is described by several authors for various enzymes and microorganisms (Kamra and Satyanarayana, 2004; Da Silva *et al*. 2005). While in submerged fermentation (SmF), the fungus is exposed to hydrodynamic forces; in SSF growth is restricted to the surface of the solid matrix. Another factor is that the use of solid systems (SSF) provides the fungus with an environment closer to its natural habitat (wood and decayed organic matter), which stimulates the fungi to produce more hemicellulolytic enzymes (Da Silva *et al*. 2005). Since this enzyme has an ability to hydrolyze hemicellulosic substrates and function at wide range of temperature and pH, it can be a great attractive to be utilized in industry after pretreatment of hemicellulose from agricultural wastes to fermentable sugars.

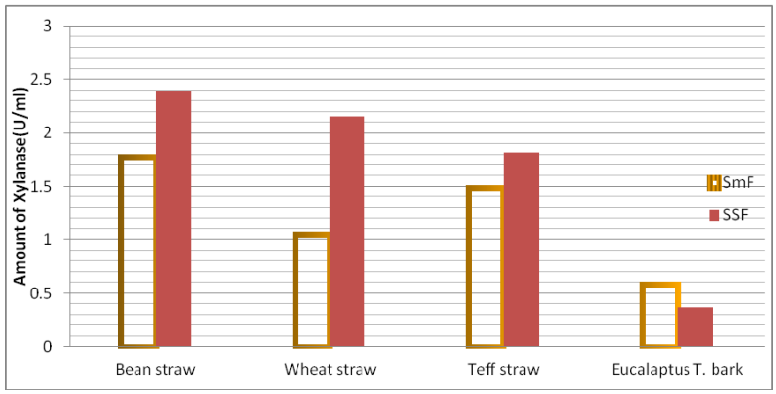

Figure 1. Xylanase production by different lignocellulosic substrates under SSF and SmF.

Optimization of growth medium for xylanase production under SSF

Time course of xylanase production

The time was observed on the 4th days (Figure 2), which was similar to xylanase course of xylanase production by *Pleurotus ostreatus* was investigated and maximum production production from *Aspergillus niger (*Widjaja *et al.* 2009); and different from that of *Penicillium canescens* in SSF which resulted in maximum production after 6 days incubation (Jayalakshmi *et al.* 2007). Further incubation after this did not show any increment in the level of enzyme production, probably due to increase in toxic waste substances and depletion of nutrients in the media, which leads to decreased growth and enzyme production.

From the application point of view, faster production of an enzyme could be advantageous which may allow appreciable reduction in the production cost of the enzyme and products can be found in short period of time. Moreover, an organism that produces maximum xylanase activity in short incubation time offers significant advantage in reducing the risk of contamination.

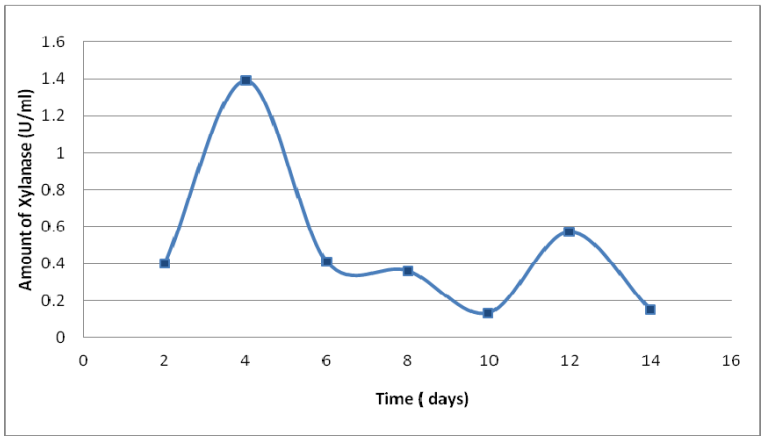

Figure 2. Effect of incubation time on the production of xylanase under SSF

Effect of pH on the production of xylanase

The results revealed that optimum of pH 4.0 to be the best for xylanase production (Figure 3), which was similar with result obtained by Fujimoto *et al*. (1995) by *Aspergillus aculeatus* xylanase. The pH value of the culture medium affected the permeability of cells as well as stability of enzyme (Mase *et al.* 1996). The effect of hydrogen ion on enzyme may be due tothe stability of the enzyme at particular pH and denaturation of the enzyme proteins occur at pH differ, the optimum pH of such enzyme. Acidic pH probably helps to keep away bacterial competition, as most bacteria require high water activity and a pH of around neutrality and above (Kalra and Sandhu, 1986).

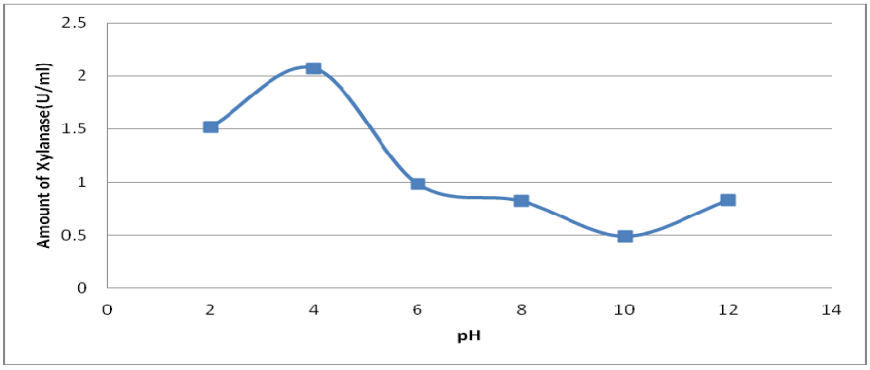

Figure 3. Effect of pH on the xylanase production under SSF.

Effect of temperature on the production of xylanase

The results revealed that the optimum temperature of production of xylanase was found to be 30°C C (Figure 4). The incubation temperature affects the level of enzyme production under SSF (Holker and Jurgen,2005). Maximum production at lower temperatures maybe advantageous; because it can

reduces the rate of evaporation during incubation. The fact that the organism produces maximum enzyme at mesophilic temperature hence facilitates the production of the enzyme without the need of incubation instrument and reduce the cost of enzyme production.

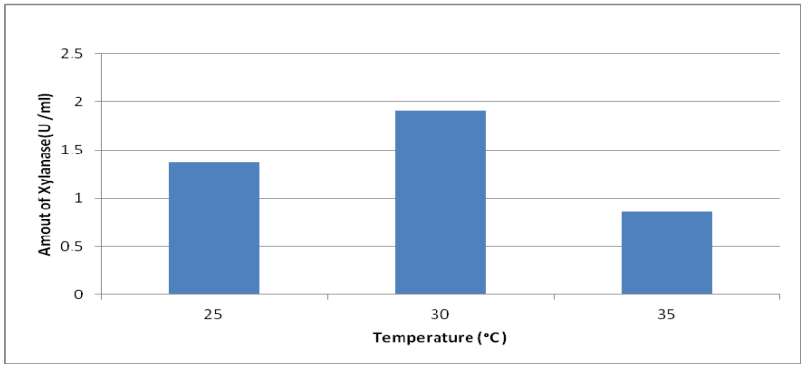

Figure 4. Effect of temperature on the xylanase production under SSF.

Effect of varying carbon sources on xylanase production

Efficient production of xylanase is dependent upon the choice of an appropriate inducer substrate and the medium composition. In this study, the production medium was supplemented with different carbon sources, maintaining other parameters constant, to find out their effect on the amount of xylanase. The results showed varying levels of xylanase depending on the kind of substrate (carbon source) used. Xylanase production was significantly increased when the production medium containing wheat straw was supplemented with xylan.

The report shows that production of xylanase was good on wheat straw without supplementation. It is might due to presence of high amount xylan in wheat straw. According to Kulkarni *et al*. (1999), xylanase production is inducible by xylan rich substrates, which is similar to the present finding. Glucose, maltose and starch were found to reduce xylanase production while on the other hand sucrose completely inhibited its production (Figure 5), Khwanchi (2009) reported that the reason was believed to be in the presence of easily metabolisable substances, production of xylanolytic enzymes were decreased (repressed).

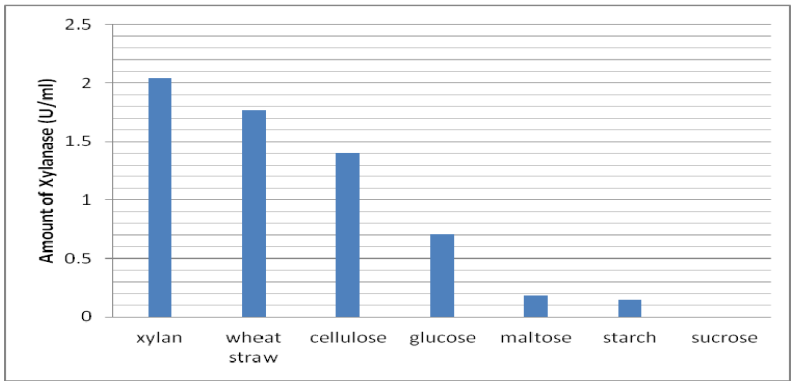

Figure 5. Effect of different carbon sources supplementation on the xylanase production under SSF

Effect of different nitrogen sources on the xylanase production

Enzyme production by *Pleurotus* sp. was seriously affected by the type of nitrogen source used in the growth medium (Figure 6). This study showed that peptone was the best of the six nitrogen sources tested in resulting relatively higher level of xylanase production, it increase xylanase production by 14% which has an important role in enzyme synthesis, probably because this complex nitrogen source contains elements that are necessary for the metabolism of fungus when compared with production on control (xylan). In contrast, low xylanase production was observed in the medium supplemented with malt extract, which is similar with the result reported by Kuhad *et al*. (2006), most of microorganism need nitrogen for growth and production of enzyme.

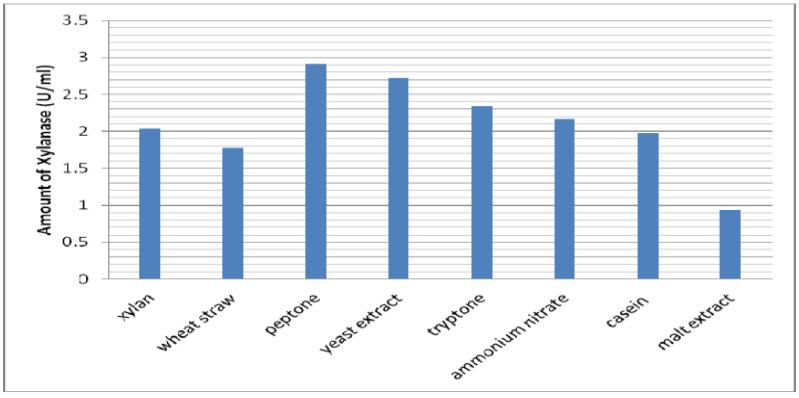

Figure 6.Effect of different nitrogen sources supplementation on the xylanase production under SSF.

Effect of metal ions on xylanase production

Figure 7 shows that, the influence of metal ions on the production of xylanase by *Pleurotus* sp. It is generally known that xylanases may be inhibited or activated by metal ions or other reagents. Many impurities like metal ions, which can inhibit the production and activity of xylanase, exist in industrial wastes. *Pleurotus* spp. xylanase production was stimulated by addition of K^{+}, which increase 13.6% when compared with control; whereas addition of Zn^{2} and Cu^{+2} inhibited the xylanase production.

Similarly, xylanase production by *Penicillium glabrum, Penicillium sclerotiorum* and *Aspergillus ficuum* was inhibited by these ions (Knob *et al.*, 2013). Ponnusami *et al*. (2013) also reported that xylanase production by *Bacillus subtilis* was inhibited and stimulated by Cu^{+2} and Zn^{+2}, respectively. This may be due to its interaction with sulphydril groups, suggesting that there is an important cysteine residue in or close to the active site of the enzyme.

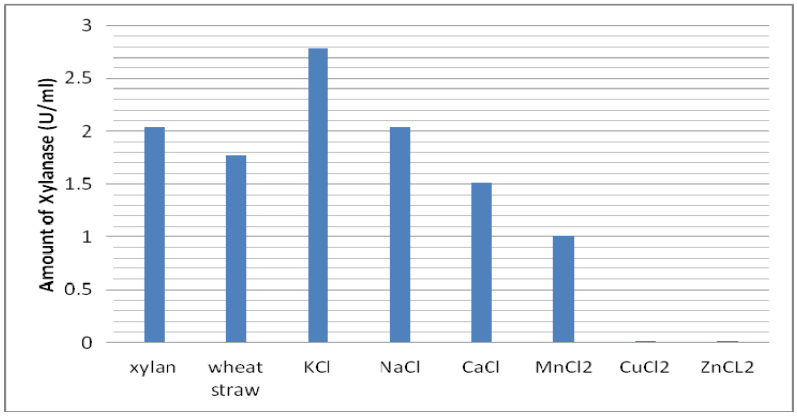

Figure 7. Effect of different metal Ion supplementation on the xylanase production under SSF.

Partial Purification of xylanase

Table 2. Partial purification of *Pleurotuss* sp. Xylanase using ammonium sulphate precipitation

Purification with D/t Conc. of $(NH4)_2SO_4$	Total activity (U/ml)	Total protein (mg/ml)	Specific (U/ml)	Activity	Purification fold
Crude extract	5.57	0.465	11.97		1
$(NH4)_2SO_4$ (30%)	3.988	0.418	9.52		0.79
$(NH4)_2SO_4$ (40%)	11.47	0.631	18.18		1.5
$(NH4)_2SO_4$ (50%)	4.59	0.361	12.7		1.06
$(NH4)_2SO_4$ (60%)	3.8	0.406	9.35		0.78
$(NH4)_2SO_4$ (70%)	1.86	0.266	6.99		0.58
$(NH4)_2SO_4$ (80%)	0.346	0.115	2.99		0.24

In the present study, xylanase was partially purified with 30-80% ammonium sulphate precipitation. The results showed that 40% ammonium sulphate saturation resulted in the highest xylanase activity with 1.5 purification fold. Similar studies also reported a xylanase with 1.25 fold purity from a fungus *Paecilomyces themophila* upon partial purification using 20-50% ammonium sulphate saturation (Lite *et al*. 2006). Enzymes could be used without purification for commercial applications (Marta *et al*. 2000) because of the fact that crude extracts have synergistic activities and the presence of some other factors in the crude extract that stabilizes the xylanase enzyme. Higher activity of crude enzyme is very important especially when the enzyme is to be applied in its crude form not in pure condition (Kulkarni *et al*. 1999). However, purification enhances the efficacy of the xylanase and hence purity is needed.

Characterization of partially purified xylanase

Effect of temperature on xylanase activity

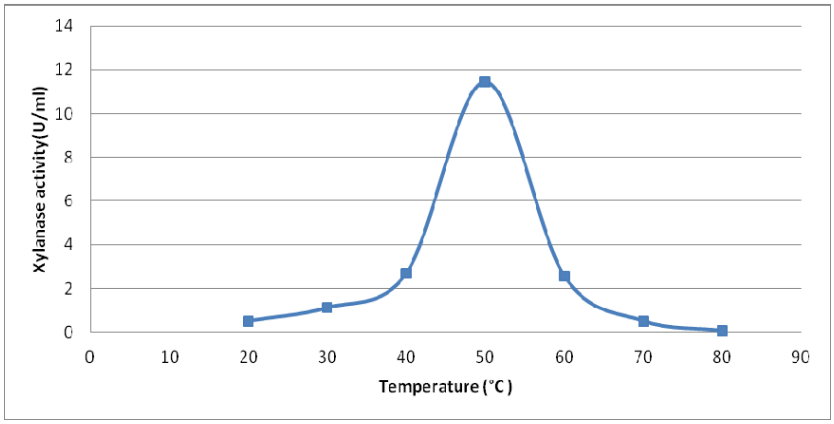

Figure 8. shows that the influence of temperature on the activity of *Pleurotus* sp. xylanase.

Effect of Temperature (∘C) on the activity of xylanase, under SSF Usually, xylanases from filamentous fungi show optimum temperature between 40∘C and 55∘C (Knob and Carmona, 2010; Dobrev and Zhekova, 2012), that was in agreement with present study. Nevertheless, other fungal xylanases show optimum temperature at 60°C or above (Fawzi, 2010). Incubation temperature for enzyme substrate reaction plays a critical role in enzyme activity; the stability of the enzyme in a wide range of temperature shows its potential application in different industries (Seyis and Aksoz, 2003).

Effect of pH on xylanase activity

The *Pleurotus* sp. xylanase showed optimal activity at pH 6.0 (Figure 9) in agreement with xylanase obtanined from *Bacillus* sp. K1 (Ratanakhanokchai *et at*. 1999) and *Bacillus* sp. C- 125 (Honda *et al*. 1985). Similarly, most of xylanases from different fungi show optimal activity in pH between 5.0 and 7.0 (Madlala *et al*. 2001). pH stability is an interesting enzyme property due to the great industrial importance. Xylanses, which are active in the acidic pH range (pH 4.8-6) are considered to be suitable for application as animal feed supplement and relatively high temperature will be of great advantage for the hydrolysis of wastes, especially in pulp and paper industry waste as it contains more dissolved xylan. (Tony *et al*. 2005).

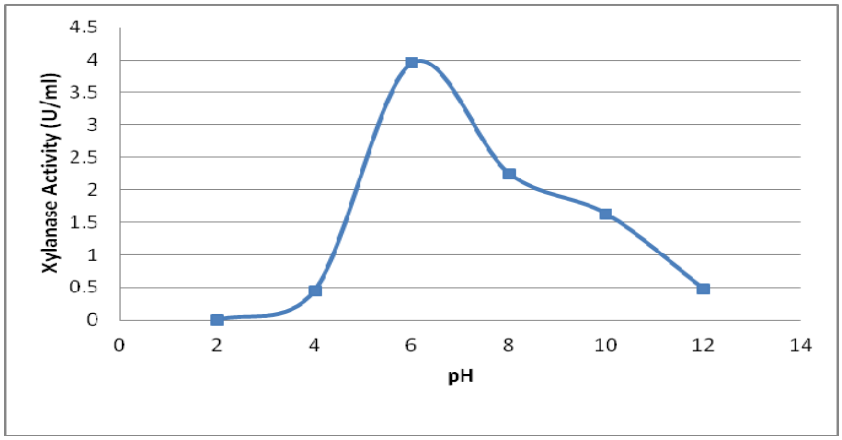

Figure 9. Effect of pH on the xylanase activity at different pH ranging from 2 to 12.under SSF.

Effect of substrate concentration on xylanase activity

The present xylanase apparent kinetic parameters were calculated using Lineweaver Burk plot. The regression equation for the Lineweaver Burk slopes of *Pleurotus* sp. xylanase was y =16.098x+0.0863 (R^2 = 0.895) an apparent Vmax of (11.58µ mole/min) and apparent Km value of 186.56µ g/ml,

According to Sa-Pereira *et al*. (2002), generally, Km value for xylanases obtained from some microbial sources was relatively low (25-1700µ g/ml) which is agreed with present study. The lower the K_m, the higher affinity of the enzyme towards its substrate (Hamilton *et al*. 1998) and the larger the velocity, the higher will be the amount of substrate binding which is a desirable quality for an enzyme. Monisha *et al*. (2009) reported that V_{max} of 0.000068µ mole/minute for partially purified xylanase from *Bacillus pumilus*, which was comparatively lesser than the present study.

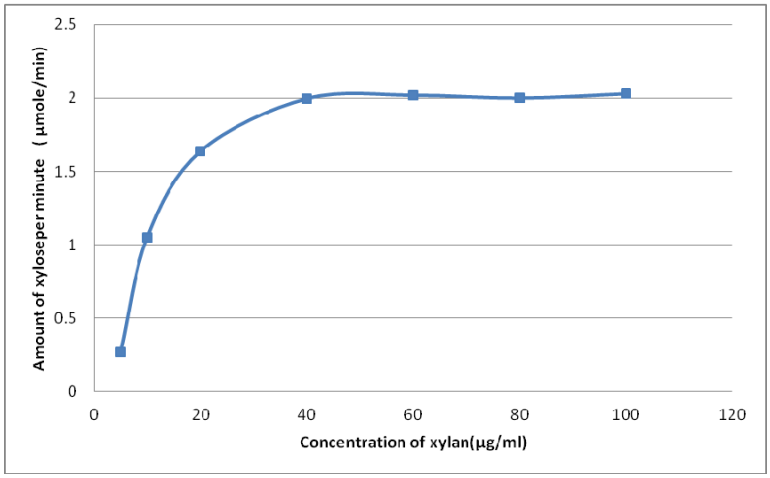

Figure 10. Rate of substrate degradation at pH 6.0 and 50°C

Summary and Conclusion

This study was conducted to screen xylanase producing oyster mushroom species collected from Eucalyptus tree bark around Holetta to evaluate the in vitro production of xylanase by Pleurotus spp using different lignocellulosic substrates and to partially purify and characterize the xylanase produced by Pleurotus spp. with respect to changes in pH, temperature and concentration of substrates.

One hundred mushroom specimens were randomly collected from eucalyptus tree bark in the premise of Holetta Agricultural Research Center campus. 100% of the collected mushroom specimens were identified morphologically and biochemically as *Pleurotus* sp. and also screened for xylanase production depend on the clear zone formation on malt extract agar plate containing xylan as sole carbon source.

Screened mushroom specimen were cultivated using both solid state fermentation and submerged fermentation systems supplemented with different substrates (wheat straw, teff straw, bean straw and Eucalyptus tree bark) to identify the most suitable medium for the production of xylanase. The highest enzyme production was obtained on bean straw (2.38U/ml and 1.77 U/ml in solid-state fermentation and submerged fermentation, respectively) and the lowest was obtained in media containing Eucalyptus tree bark (0.36 U/ml and 0.58 U/ml in solid-state fermentation and submerged fermentation, respectively).

Optimal production of xylanase was obtained when Pleurotus ostreatus was grown in solid-state fermentation using wheat straw supplemented with 5% birch wood xylan, peptone and KCl salt at pH 4.0 and a temperature of 30∘C under stationary conditions for four days. The xylanase from *Pleurotus* sp. was partially purified to

homogeneity using different concentrations (30 to 80% (wt/vol) of ammonium sulphate. Xylanase having highest specific activity (11.47U/ml) and total protein content of 0.631mg/ml was recovered from the culture supernatant when precipitated with 40% (wt/vol) ammonium sulphate. The optimum activity was observed at 50°C and pH 6.0. The enzyme was very stable at a wide range of temperature and pH. Its apparent Km and Vmax were 186.67 µ g/ml and 11.58µ mole/min, respectively, showing its high affinity towards its substrate.

Conclusion

This study was concluded that a reasonably higher amount of xylanase can be produced by *Pleurotus* sp. cultivated in SSF system than in SmF system. The cultivation system can easily be modified to enhance productivity and facilitate the scale up process for mass production of xylanase. Using this system, maximum production of xylanase can be achieved in 4 days at pH 4 and a temperature of 30∘C when a basal medium containing wheat straw is supplemented with xylan, peptone and KCl.

The enzyme seems to be very stable at a wide range of temperature and pH in addition to having high affinity toward its substrate. We hope that these characteristics would make this enzyme potentially very attractive for animal feed processing and other industrial applications.

Recommendation

This study is recommended that further research be conducted on molecular level characterization of *Pleurotus sp*. Optimization of *Pleurotus sp*. Xylanase production would be done, to be used in industrial level. Based on the *pleurotus* sp. xylanase characteristics, the enzyme was potentially very attractive for animal feed processing and other industrial applications. This study is also strongly recommended that researches have to be done on large-scale production, further purification and characterization of xylanase from *Pleurotus* sp.

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Characterization of Antimicrobial Resistance in *Staphylococcus aureus* Isolated from Bovine Mastitis in Central Ethiopia

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Abstract

Staphylococcus aureus *is commonly associated with mastitis in dairy herds with potential public health implications.* ***Bovine mastitis*** *is a common reason for therapeutic and/or prophylactic use of antibiotics on dairy farms. Overall, 303 samples were collected from September 2015 to July 2016 to characterize the phenotypic and genotypic pattern of drug resistance in* ***Staphylococcus aureus*** *isolated from cases of clinical and sub-clinical* ***bovine mastitis*** *in central Ethiopia. Milk samples were tested by using California Mastitis Test and positive samples were subjected for bacterial culture, disc diffusion test and PCR to detect the presence antimicrobial resistance. Based on CMT result and clinical examination, the prevalence of mastitis was 70.6%.* ***Staphylococcus aureus*** *was isolated from 36.9% of the milk samples cultured. The phenotypic determination of antimicrobial resistance showed that the isolates were most resistant to Ampicillin (80%) followed by trimethoprim-sulfamethoxazole (23.3%), tetracycline (15%), streptomycin (10%) and Gentamycin (3.3%) and equally to both erythromycin and chloramphenicol (1.6 %). Characterization of the antimicrobial resistance gene was done by using PCR. Most of the isolates (56%) contained blaZ gene followed by erm B (33%), ermC 13.3% and each erm A and msrA appeared only in 2% of the isolates. There was no any isolate harboring the methicillin resistance mecA gene. Thirty six percent of the isolates contained more than one antibiotic resistance genes. The highest Multidrug resistance (MDR) gene combination was observed by blaZ*ermB (31.25%) genes and the least frequently occurred MDR gene combinations were blaZ *ermA and msrA*ermB (3.12%) each. The resistance of S. aureus against antimicrobial agents is well established and the resistance markers are identified.*

Introduction

Mastitis is one of the most important diseases in dairy cows throughout the world and is responsible for significant economic losses to the dairy industry. *S. aureus* causes one of the most common types of chronic mastitis in dairy animals worldwide. The inappropriate uses of antibiotics for medication and growth enhancers in farm animals contribute for emergence of antibiotic resistant organisms. It is more than two decades since the emergence of antimicrobial resistant in staphylococci (Franklin, 2003). Staphylococci are the main etiological agents of mastitis in dairy cattle, cows are the second largest reservoir of *Staphylococcus aureus* next to human nares and up to 75

million of cows can be infected by this bacterium form the world cattle population (Olga *et al*. 2011; Somayyeh and Habib, 2014; Raney, 2009). The overall loss due to mastitis range from 31 kg -749 kg in first lactation to losses between 117 kg - 860 kg in subsequent lactation (Hultgren and Svensson, 2009; Ostergaard and Grohn, 1999).

Although there is host range barrier among *S. aureus* lineage some illustrates the potential hazard of animal origin *S. aureus* on human health, which implies possible transmission of genotypes from one species to the other (Tara, 2015; Lowder *et al*. 2009). Despite the substantial economic impact and potential public health concern, the prevalence as well as the phenotypic and genotypic antibiotic resistance nature of *S. aureus* isolates are less studied in developing countries like Ethiopia. In these countries, scarce in veterinary services, shortage of variety of drugs and poor drug regulatory frameworks could lead to under dosage medication, which may end up with development of antibiotic resistant organisms. On the other hand, low hygienic standards of housing and milking can disseminate mastitis causing pathogens including *S. aureus* among individual animals or farms (Amanu *et al*. 2016).

In Ethiopia, prevalence rate ranging from 15.3 % to 53.4% has been recorded from different parts of the country (Amanu *et al*. 2016; Sori *et al*. 2011). However, there are few trends to detect antibiotic resistance genes and to correlate their association with the phenotypic resistance. Hence, this research intended to characterize phenotypic as well as genotypic antibiotic resistance of *S. aureus* isolated from bovine mastitis.

Materials and Methods

Sample collection and preparation

Three hundred and three lactating cows were screened for subclinical mastitis from September 2015 to July 2016 using California mastitis test (CMT). All the lactating cows were examined carefully and CMT screening procedures were done. Approximately two ml of milk was taken from each teat in to the four CMT paddle indentations. Then, equal amount of CMT reagent (COX, USA) was added and swirled gently for 15 seconds. The screening was done according to the procedure stated in Quinn et al. (1994). The CMT positive samples were kept in cold box and transported immediately to the National Agricultural Biotechnology Research Center Laboratory, Holetta.

Bacterial isolation and identification

Bacteria were cultured and identified from CMT positive milk samples. The collected samples from each quarter were streaked on MacConkey agar and blood agar base plates enriched with 7% ovine blood. The inoculums were then incubated aerobically at 37°C for 24 to 48h. After primary culture, identification of *S. aureus* was done by using microscopic and biochemical methods (Quinn *et al.* 2011; OIE, 2012).

Antimicrobial sensitivity test

Antimicrobial resistance patterns were determined by Kirby-Bauer disc diffusion method on Mueller-Hinton agar (Sigma-Aldrich, USA) (Kirby *et al.* 1966). The bacteria were inoculated on the plate at a rate of $5X10^5$ Bacteria/ml after serial dilution determined by OD measurements according to CLSI recommendation. Antibiotic discs were placed and gently pressed by forceps on the bacterial culture spreaded on Mueller-Hinton agar (Sigma Aldrich, USA). The inhibition zone was measured after incubation of the plates at 37^0C for 18 hours under aerobic environment. The response of the isolates to each antimicrobial agent was evaluated by measuring the zone of inhibition categorized as sensitive, intermediate and resistant according to the standards recommended by CLSI (2007). The antimicrobials used in the experiment were ampicillin (10 µg), chloramphenicol (30 µg), gentamycin (10µg), erythromycin (15µg), tetracycline (30µg), streptomycin (10 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg).

Isolation of plasmid DNA

Plasmid DNA of the staphylococcus isolates was performed by using Plasmid Midi Kit (QIAGEN). Single colony was taken from each isolate and inoculated into a separate 5ml LB Broth and incubated over night at 37^0C in an orbital shaker. Cells were harvested by centrifugation at speed of 6000g for 15 min. The isolation procedures were performed according to the manufacturers' protocol. The concentration and the purity of the extract were measured by using a Nano-drop ND-1000 spectrophotometer (Thermoscientific, Wilmington, DE). The integrity of the plasmid DNA was assessed after electrophoresis in 1% agarose gel after mixing with gel loading dye (Thermoscientific, USA).

Polymerase chain reaction

The genes involved in antimicrobial resistance (*mecA, blaZ, erm* A, *erm* B, *erm* C and *msr*A) were detected by polymerase chain reaction (PCR) using the primers and cycle conditions described by Murakami *et al.* (1991) and Sawant *et al.* (2009) (Table 1). A single colony was picked and inoculated in to 10ml of nutrient broth (Sigma Aldrich, USA) and incubated overnight at 37^0C in shaker incubator at speed of 100rpm/min. Bacterial plasmid DNA was extracted using kit (Biobasic, USA) from well-grown broth cultures. The PCR reaction was prepared by mixing, reaction buffer (500Mm KCl,17.5mM MgCl2, 100mM Tris-HCl,0.1% TritonX-100,) (Himedia, India), 10mM dNTPs, 10pmol of each primer,1UTaqDNA polymerase (Himedia, India) and Nuclease free water which was added up to 25 µl. PCR products were electrophoresed in 1.5% agarose gel after mixing with gel loading dye (Thermo-scientific, USA) (0.5µg/mL) and observed under UV illumination.

Table 1. The genes involved and their oligonucleotide primers for the polymerase chain reactions

Gene	Oligonucleotide sequences	Reference
mecA	F- CTTTGGAACGATGCCTAATCTCAT R- AAGAGATTTGCCTATGCTTC	Murakami *et al.* (1991)
blaZ	F- GCTTGACCACTTTTATCAGC R- ATCGGATCAGGAAAAGGACA	Sawant *et al.* (2009)
ermA	R- CACGATATTCACGTTTTACCC F- AAGGGCATTTAACGACGAAA	
ermB	R- CTGTGGTATGGCGGGTAAGT F- TGAAATCGGCTCAGGAAAAG	
ermC	F- TGAAATCGGCTCAGGAAAAG R- CAAACCCGTATTCCACGATT	
msrA	F- TGGTACTGGCAAAACCACAT R-AAACGTCACGCATGTCTTCA	

Results and Discussions

Prevalence of *Stapylococcus aureus* and its antimicrobial resistance pattern by using disk diffusion method

Out of the 303 lactating cows, 214 (70.6%) of them were found positive by CMT for either of the four quarters. Among these 214 samples, 187 (87.4%) were bacterial culture positive in which 79(36.9%) of the culture was identified as *S. aureus*. Antimicrobial resistance test was conducted for 60 of the 79 isolates. Antimicrobial resistance pattern of the *S. aureus* isolates is shown in table 2. The isolates were resistant to ampicillin (80%) followed by trimethoprim-sulfamethoxazole (23.3%), tetracycline (15%), streptomycin (10%), Gentamycin (3.3%) and equally to both erythromycin and chloramphenicol with the least resistance (1.6 %) (Table 2).

Table 2. Antimicrobial resistance rate (%) Of *S. aureus* isolated from bovine mastitis

Antimicrobial Disc	Sensitivity			Total
	Sensitive	Intermediate	Resistant	
Erythromycin	30(54.5%)	29(43.9%)	1 (1.6%)	60 (100%)
Chloramphenicol	55(91.6%)	4(6.6%)	1(1.6%)	60 (100%)
Gentamycin	56(93.3%)	2(3.3%)	2(3.3%)	60 (100%)
Ampicillin	10(16.6%)	2(3.3%)	48(80%)	60 (100%)
Tetracycline	40(66.6%)	11(18.3%)	9(15%)	60 (100%)
Streptomycin	45(75%)	9(15%)	6(10%)	60 (100%)
Trimethoprim sulfamethoxazole	37(61.6%)	9(15%)	14(23.3%)	60 (100%)

Determination of antimicrobial resistance genes

Among all 45 isolates tested for the presence of antibiotic resistance gene, only 14 (35%) were found free from any of the anti-microbial resistance gene (Table 3).

Isolates containing antibiotic resistance genes were observed after electrophoresis of the PCR product (Figure 1).

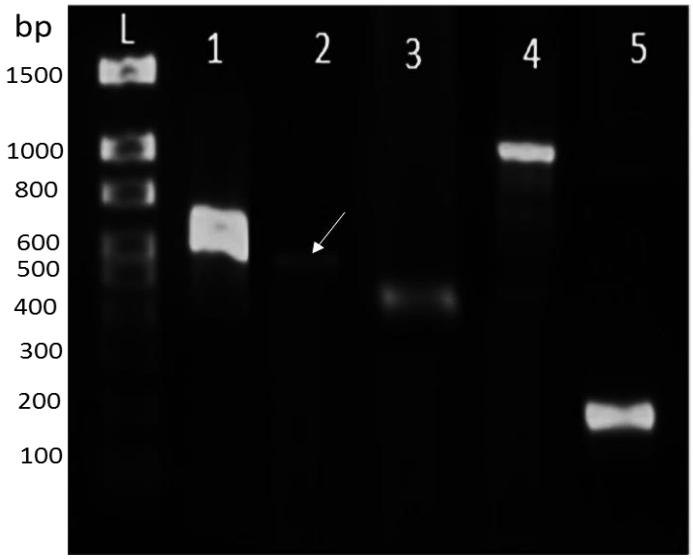

Figure 1. Agarose gel electrophoresis of representative isolates containing antibiotic resistance marker (1%).

L. Ladder (100bp -1.5 kb DNA ladder, Bio Basic), 1. blaz (517bp), 2. ermA (486bp), 3. ermB (423 bp), 4. msrA (1000bp), 5. ermC (272bp).

Most of the isolates (56%) contain *bla*Z gene followed by *erm*B (33%), *erm*C 13.3% and each *erm* A and *msr*A was detected in only 2% of the isolates. There was no any isolate harboring the methicillin resistance mecA gene.

Table 3. Distribution of antimicrobial resistance genes among isolates

Gene	All	Sole	Total
blaZ	25 (55%)	10(22.2%)	45(100%)
ermA	1 (2.2%))	0(0.0%)	45(100%
ermB	15 (33.3%)	4(4.8%)	45(100%)
ermC	6 (13.3%)	2(2.4%)	45(100%)
msrA	1 (2.2%)	0(0.0%)	45(100%)
mecA	0 (0.0%)	0(0.0%)	45(100%)

All refers the amount of the frequency for the specific gene from the whole isolates, Sole refers the frequency of the specific gene appeared solely from the whole isolates.

Assessment of multi-drug resistance genes

Thirty-six percent (36%) of the isolates contained more than one antibiotic resistance genes. The highest MDR gene combination was observed by *bla*Z**erm*B (31.25%) genes and the least frequently occurred combinations were *bla*Z **erm*A and *msr*A**erm*B (3.12%) each. Combination of *bla*Z **erm*C accounts in 12.5% and only *bla*Z gene was observed in 31.25% of the isolates. Whereas both *msr*A and *erm*A genes did not appear alone in any of the isolates. Similarly, single drug resistance markers, *erm*B and *erm*C genes were found in 12.5 and 6.25% of the isolates respectively (Table 4).

Table 4. Occurrence of multiple antimicrobial resistance genes

MDR	Genes frequency
blaZ ermB*	10 (22.2%)
*blaZ *ermC*	4 (8.8%)
blaZ, ermA*	1 (2.2%)
*ermB*msrA*	1 (2.2%)
subtotal	16 (35.5%)

Among the 45 isolates in which the PCR test was performed, 16(35.5 %) of them showed resistance on the disc diffusion test contained the respective resistance gene. High association between ampicillin resistance and the presence of *bla*Z gene has been observed. Sixty-eight percent of the isolates harboring the *bla*Z gene were found resistant to ampicillin.

Conclusion

Staphylococcus aureus causes one of the most common types of chronic mastitis in dairy animals worldwide. In this study, the prevalence of Bovine mastitis was found in 70.6%. This prevalence of mastitis is comparable with previous reports from Ethiopia who reported a prevalence rate of 71.0 and 75.2% in dairy farms located at Holetta and Jimma towns respectively (Sori *et al*.2011; Mekibib *et al*. 2009). However, higher result was found in Holetta, Bahirdar and Gondar Towns of Ethiopia (Amanu *et al.* 2016; Bitew *et al.* 2010; Moges *et al*. 2011). The difference on the prevalence of mastitis among the studies could be due to difference in management system, milking practices, productivity, breed of the animals and location (Amanu *et al.* 2016; Sori *et al.* 2011).

The prevalence of *S. aureus* was found 36.9%, which is higher than the finding of Amanu *et al.* (2016) and lower than Sori *et al*. (2011). In Kenya, Shitandi and Sternesjo (2004) reported equivalent prevalence rate (30.6%) of S. aureus. However, prevalence rate of 25.5%, 23.3% and 10% were reported from China, Iran and Finland respectively (Wang *et al*. 2007; Bahraminia *et al.* 2017; Pitkalla *et al.* 2004). The reason for the variability may be due to lactation stage of the cow, age of the cow and

milking method (Kivaria *et al*. 2007; Ergun *et al.* 2009). In this study, 85% of the *S. aureus* isolates showed resistance to at least one antimicrobial drug. This is similar with the previous finding from Jimma and central Ethiopia with overall antibiotic resistance rates of 92 and 97.5% respectively (Sori *et al*. 2011; Virdis *et al*. 2010). However, in Brazil Rabello *et al*. (2016) reported relatively higher susceptibility to antimicrobials (49.1%). This variation might occur due to difference in milking practice, purpose of use of the antibiotics and inappropriate therapeutic treatment by non-professionals.

Considerable resistance to beta-lactam antibiotic by *S. aureus* appears serious threat to the world (Franklin, 2003). In the current study, higher resistance to ampicillin (80%) was observed. This is comparable with the previous reports from Gondar (81.5%) and Italy (88%) (Moges *et al*. 2011; Franca *et al*. 2012], but higher than other reports from Hawassa (67.9%) (Teshome *et al*.2016). However, Daka *et al*. (2016) reported lower resistance rate (7.7%) from Hawassa, Ethiopia.

Tetracycline and its derivatives are the most extensively used antibiotics in Ethiopia for treatment of animal diseases. Therefore, certain level of resistance to this drug was expected. Our finding on resistance against tetracycline was 15%, which is nearly half of the figure observed at Gondar and Holetta with the resistance rate of 29.6 and 33.3% respectively (Tara, 2015; Wang *et al*. 2007). The most effective drugs in this experiment were gentamycin followed by erythromycin and chloramphenicol. These drugs are not first choice for treatment of mastitis in most part of the country hence the chance to develop resistance by *S. aurues* will be minimum. In contrast, there are evidences of erythromycin resistance development in some parts of the country (Moges *et al.* 2011; Franca *et al.* 2012).

Multiple drug resistance is the ability of an organism to resist and grow against two or more antimicrobials. In this study, the isolates had shown inverse relation between the Multi-drug resistance nature and the number of antimicrobials applied. This observation match with the finding of Sori *et al.* (2011) who demonstrate MDR pattern of 25 ,10.45 and 7% for two, three and four types of drugs respectively. However, it differs from Teshome et al. (2016) who record MDR of 34.8% of the isolates for three and 8.7% for two antimicrobials. The difference observed in the pattern of MDR could be explained by group of drugs with similar chemical structure and mechanism of action may exhibit cross-resistance by the bacteria despite the number of drug involved (Pechere, 2001).

The two main mechanisms of macrolide resistance are drug-efflux membrane pumps and modification of the drug target site in the ribosome. The third mechanism of resistance is drug inactivation (Bailey *et al.* 2008; Kayode *et al.* 2006, Jensen *et al.* 1999; Lina *et al.* 1999). It has been known that, isolates harbor *erm* genes (erythromycin resistance rRNA methylase) code for the protein called methyl tranferase, which induce N6 -dimethylation of an adenine residue of 23S rRNA. This process produces conformational changes in the phospahte site of the rRNA and

prevents the macrolide binding at the peptidyl transferase center, hence the protein production will proceed and the bacteria will survive (Kot *et al.* 2012; Westh *et al.*1995). Expression of the three related factors; ermA, ermB and ermC, are responsible to make the bactria resistance to macrolides and other related antibiotic groups (Kayode *et al*. 2006; Westh *et al*. 1995).

In this research, among the three erythromycin resistance determinants, *erm*B (33.3%) resistance gene was the most frequently identified followed by *erm*C (13.3%) and *erm*A (2.2%). Bahraminia *et al.* (2017) reported similar pattern of *erm* genes distribution while studying bovine mastitis caused by *S. aurues*. A research done to determine types of *S. auerues* linages affecting human showed that *erm A* as the most frequently found resistance gene (Bahraminia *et al*. 2017; Westh *et al.* 1995) although there are some findings supporting higher prevalence of *ermC* among the three determinants (Ross *et al.*1990). Lina *et al.* (1999) correlate the distribution of erm genes with methicillin resistance and susceptibility in coagulase positive or negative streptococci species. The *ermA* gene found more common in methicillin resistant *S. aureus* stains (57.6%) compared to the sensitive ones (5.6%) (Westh *et al*. 1995). The contribution of each of the *erm* determinants (*erm A, erm B* and *erm C*) towards phenotypic macrolide resistance is essential. Westh *et al*. (1995) determined that *ermA* and *ermC* are responsible for erythromycin resistance in more than 98% of *S. aureus* strains. In another report, *ermC* was the dominant *erm* gene in *S.aureus* and was responsible for erythromycin resistance in 72% of the strains in the years 1983 to 1988 (Duran *et al*. 2012).

In the current study, despite the high prevalence of *ermB* gene, we have observed only a single erythromycin resistance isolate (1.6%). This finding agrees with Westh *et al.* (1995) which reported that *erm B* is less responsible for phenotypic macrolide resistance. However, we have identified a single isolate harboring this gene together with *erm* genes (2.2%). Our finding showed similar results of previous works by Lina *et al.* (1999) and Ross *et al.* (1990) which identify prevalence of 3.3% and 3.6% respectively for similar combination.

Staphylococcal resistance to penicillin is attained by the gene *blaZ* (beta lactamase). It encodes for extracellular enzyme bate lactamase, which hydrolyse the beta-lactam ring rendering the β-lactam inactive (Franklin, 2003). In our study, the gene was widely spread among the isolates (80%) which are in line with the report by Duran et al. (2012). According to Franklin (2003), More than 90% of staphylococcal isolates produce penicillinase (beta lactamase enzyme). This indicates the wide distribution of the resistance gene globally through spread of resistance strains. Although *blaZ* is the primary key player for penicillin resistance among staphylococcus isolates, it is not the sole factor. In this study, not all isolates which show penicillin resistance by disk diffusion test harbor *blaZ* gene. This finding agree with previous studies by Yang *et al*. (2015) and Goa *et al.* (2012) who identified staphylococcus and streptococcus isolates showed resistance to penicillin but not carrying *blaZ* gene. Point mutation rather than gene acquisition could be another factor for only phenotypic resistance. Biofilm

production and multi-drug efflux development are also other possible protection mechanism of the bacteria (Katayama *et al.* 2005; Wielders *et al.* 2002).

In this study, no isolate containing *mecA* gene was found. Similarly, another study mentioned that *mecA* is rarely found in many Staphylococcus isolates originated from animal infections (Ross *et al*. 1990). The limited distribution of staphylococcus chromosomal cassette mec (SCC mec) which carries *mecA* gene by nature may be considered for its rare occurrence. The occurrence of *S. aureus* resistance to antimicrobial agents is growing in alarming rate. Therefore, the community should be aware by responsible bodies about the risk of consuming of raw dairy products. The veterinary service delivery should be improved in order to avoid subjective treatment of animals by non-professionals. Besides, further studies should be conducted to obtain full figure of phenotypic as well as genotypic antimicrobial resistance pattern.

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Bovine Mastitis: Prevalence, Major Bacterial Pathogens and Associated Risk Factors in Central Highlands of Ethiopia

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Abstract

A cross sectional study was carried out from September 2015 to July 2016 to determine the prevalence of ***mastitis,*** *major causative pathogens and its associated risk factors in lactating dairy cows in five selected districts of central highland of Ethiopia (Holetta, Menagesha, Burayu, Sululta and Sebeta). From 303 cross breed dairy cows, 214 cows were positive using California Mastitis Test (CMT) and of which, 187 cows showed strong positive reaction and samples were taken from these strong positive cows. Over all prevalence of mastitis at animal level were 70. 62% (214/303) by CMT screening. Out of 187samples strong positive by CMT test, the most prevalent isolate were Staphylococcus aureus 79(42.25%) followed by Streptococcus agalactiae 27 (14.43%). Other bacterial isolates included CNS (Coagulase Negative staphylococcus species) 24 (12.83%), Streptococcus dysgalactiae11 (5.88%), Escherichia coli 25 (13.38%) and Entrococcus feacalis 21(11.23%) were also isolated. Generally, Age, parity number, visible teat abnormalities, husbandry practice, barn floor status and milking hygiene were considered as risk factors of* ***mastitis.*** *And they were found significantly associated with the occurrence of* ***mastitis*** *(P<0.05).This study revealed the importance of* ***mastitis*** *and associated bacterial pathogen in the study area ,which indicates appropriate control measures should be taken to reduce its impact on dairy production in the study area.*

Introduction

Ethiopia is believed to have the largest livestock population in Africa. This livestock population has been contributing significantly to the national economy of the country, and still promising to rally round the economic development of the country. It is eminent that livestock products and by products in the form of meat, milk, honey and eggs serves as a source of animal protein contributing to the improvement of the nutritional status of the people (CSA, 2009). Whereas Ethiopia, in spite of its huge livestock population; the per capita milk consumption is far below (16 kg) other sub Saharan African countries (Asfaw, 1997). This is partly due to the low genetic milk production potential of the indigenous zebu cattle. To increase milk production, cross breeding of indigenous zebu with exotic breeds particularly with Holstein Friesian is widely practiced and this has resulted in a larger portion of the dairy cattle population especially in urban areas to be with a high level of exotic blood. However, this market

oriented dairy production, a rapidly growing system in Ethiopia, is subjected to diseases of intensification including mastitis and reproductive disorders (Lemma *et al.* 2001).

Mastitis is one of the most important diseases affecting dairy cows and is a multi-factorial disease with worldwide distribution, which incurs serious economic losses to dairy industry (DeGrave and Fetrow, 1993). It is considered one of the most common and substantial production diseases of dairy cattle worldwide. The disease results in decreased production, discarded milk and medical treatments as well as a higher level of premature culling of affected animals. Milk and milk products contribute direclty to the social and economic development of rural areas where the dairy cattle production is one of the major sources of income in many households. Dairy products also provide essential food and nutrition for people in these areas (Singh, 2013). The economic loss due to the disease is considerable and can be crucial, especially for small-scale dairy farmers in Ethiopia. Therefore, the objectives of this study were to determine the prevalence of bovine mastitis, identify the major bacterial pathogens causing bovine mastitis and to determine the various risk factors associated with the occurrence of mastitis in selected districts of Central Highland of Ethiopia.

Materials and methods

The study area

The research was conducted in Finfinne surrounding special zone of Oromia Regional state. The zone has an estimated total area of 4,800 km^2. For the purpose of this study, five (Holetta, Menagesha, Burayu, Sululta and Sebeta) major Towns were selected based on number of commercial dairy farm.

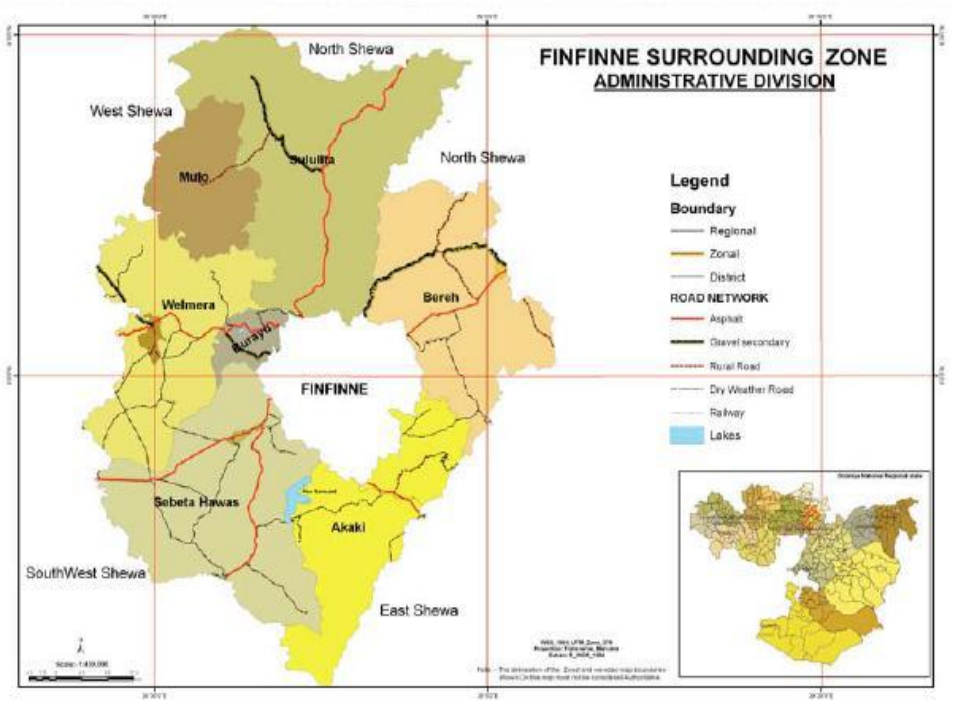

Figure 1. Finfinne surrounding Oromia special Zone central highland Ethiopia

Study design and sampling methodology

Cross-sectional study was conducted from September 2015 to June 2016 in lactating cross breed dairy cows owned by state and other commercial dairy farms found in the study area. The animals were often managed under both semi-intensive and intensive management system. The sample size was determined at 95% confidence interval, 5% precision and from previous studies in the study area by Girma D. (2010), with an expected prevalence of 34.4%. Thus, the sample size value was calculated based on Thrusfield M. (2007) and the sample size was 346 animals. However, due to the lack of cooperation in some farm owners, 303 lactating cows were used for this study. Milk samples were collected by standard milk sampling techniques from all lactating cows with clinical and sub clinical mastitis. To reduce contamination of the teat ends during sample collection, the near teats were sampled first followed by the far once. Approximately 10 ml of paired milk was collected from each quarter (one for CMT and one for bacteriological examination) into labeled sterile screwed cap universal bottle after discarding the first three milking streams. The CMT was carried out as screening test for selections of samples for culture following the method described by Quinn *et al.* (1994). CMT positive samples were placed in icebox and immediately transported to the National Agricultural Biotechnology Research Center (NABRAC) of EIAR for further processing and analysis.

Bacteriological isolation and identification

Milk samples from both clinical and sub clinical quarters were bacteriologically examined according to the procedures used by (Quinn *et al*. 1994). Each culture was subjected to gram staining and different biochemical test such as catalase test, mannitol salt agar (Oxiod,UK), purple base agar (Difco), Tube coagulase test, SIM medium (Oxiod,UK), TSI (Triple Sugar Iron) (Oxiod,UK), MacConkey agar (Oxiod,UK), Simmon's citrate agar (Oxiod,UK) and Edward Medium was used to differentiate the mastitis causing organisms.

Data entry and analysis

Data were coded, cleaned and entered into Microsoft Excel. Statistical analysis was carried out using SPSS version 20 and was analyzed descriptively using descriptive statistics in the first step; thereafter association of the different variables with interest of outcome was analyzed using a Chi-squared ($\chi 2$) test.

Results and Discussion

Prevalence and associated risk factors

From 303 dairy cows, which were screened by CMT, an overall mastitis prevalence rate of 70.62% (214/303) was observed. The prevalence of mastitis was significantly higher (81.97%) in cows older than 10 years followed by cows in the age range of 6 to 10 years and lowest in cows younger than 6 years. The statistical analysis also showed that there existed highly significant differences among the three age groups ($p<0.05$) in the occurrences of mastitis. Both parity number and visible teat abnormalities were found to be significantly ($P<0.05$) associated with the occurrence of mastitis. The highest prevalence of mastitis was observed in 90% (45/50.) cows with parity of more than six followed by less than 3 and 3-6 parity and among the visible teat abnormalities. The highest prevalence of mastitis was observed 91.66% (99/108.) in cows with two teat blinds followed by teat laceration/ lesion and one teat blind (as indicated Table1). Cows managed intensively ($p<0.05$) was highly susceptible to mastitis when compared to those managed under semi intensive management systems. With an overall mastitis prevalence rate of 84.04% (79/94) and 64.59% (135/209) respectively.The prevalence of mastitis was higher 96.07% (196/204) in cows on farms with poor barn floor status condition and lower 8.08% (8/99) in farms with good conditions barn floor status condition. Statistically, highly significant association was observed between mastitis and barn floor status of the farms visited ($P<0.05$) (Table1).

Table 1. Association between some of the selected extrinsic factors with the occurrence of bovine mastitis in the study area

Risk factor	Category	Number of examined	Number of positive	Prevalence (%)	X^2	P-Value
Age group (Years)	< 6	50	28	56		
	6-10	87	50	57.47	22.63	0.000
	>10	166	136	81.97		
Parity Number	<3	67	48	71.64		
	3-6	186	121	65.05	11.86	0.003
	>6	50	45	90		
Visible teat abnormalities	Normal	135	74	54.81		
	One teat blind	46	31	67.39		
	Two teat blind	108	99	91.66		0.000
	Teat laceration	14	10	71.42	39.55	
Husbandry practice	Intensive	94	79	84.04		
	Semi-Intensive	209	135	64.59	11.82	0.000
Barn floor status	Poor	204	196	96.07		
	Good	99	8	8.08	194.95	0.000
Milk Hygiene	Only washed	187	174	93.04	118.36	0.000
	Washed and dried with towel	116	40	34.48		

Furthermore, mastitis prevalence was found to be higher 93.04% (174/187) in only washed during milking and lower 34.48 % (40/116) in washed and dried with towel. Statistical analysis showed the existence of significant ($P<0.05$) association between the occurrence of mastitis and milking hygiene. Generally, in the present study, age, parity number, visible teat abnormalities, husbandry practice, barn floor status and milking hygiene were considered as risk factors for the occurrence of mastitis. Moreover, they were found significantly associated with the occurrence of mastitis ($P<0.05$). Among the study area, mastitis prevalence was found to be higher 174 (93.04%) in Burayu and lower 40 (34.48%) in washed and dried with towel (Table 2).

Table 2.Prevalence of Mastitis at cow level in different districts in central highland Ethiopia

District	Number of examined cows	Number of positive cows	Prevalence (%) P value
Holetta	154	91	59.09
Menagesha	13	11	84.61
Burayu	27	27	100
Sululta	79	57	72.15
Sebeta	30	28	93.33

Bacteriological examination result

Analysis of bacteriological examination of milk samples was made to identify the main etiological agents involved in the disease. The organisms were identified based on their cultural, staining characteristics and different biochemical test. Contagious pathogens like Staphylococcus bacterial species and environmental pathogens like *Escherichiacoli* were identified. From 187 samples, the highest prevalent bacteria were found to be *Staphylococcus aureus* 79/187(42.25%) followed by *Streptococcus agalactiae* 27/187(14.43%), *Escherichia coli* 25/187(13.38%), *Coagulase Negative staphylococcus species* 24/187(12.83%), E*ntrococcus feacalis*21/187 (11.23%) and Streptococcus *dysgalactiae* (5.88%) in that order (Table 3).

Table 3. The frequency of bacteria isolated from bovine mastitis in selected districts of central Ethiopia.

Bacterial species	Total number of isolates	Prevalence (%)
Staphylococcus aureus	79	42.2
CNS	24	12.83
Streptococcus agalactiae	27	14.43
Streptococcus dysgalactiae	11	5.88
E*ntrococcus feacalis*	21	11.23
Escherichia coli	25	13.38
Total	187	100

CNS = Coagulase Negative staphylococcus species

The study was conducted in selected districts of central highlands of Ethiopia with objectives to determine the prevalence, its major causative agents, and risk factors associated with bovine mastitis. The overall prevalence of mastitis was 70.62 % at cow level. The present findings was comparable to the findings of Bishi (1998), Regasa *et al.* (2010) , Mekibib *et al.* (2010) and Zeryehun *et al.* (2013) who reported 69.8% in dairy farms of Addis Ababa and its vicinity, Ethiopia, 71% in dairy farms of Holetta town , 71.1% from Holetta, and 74.7% around Addis Ababa, respectively. The finding was higher than previous reports of Kerro and Tareke (2003), Workineh *et al.* (2002), Mungube *et al.* (2004) and Fekadu (1995) who reported 40.4% in southern Ethiopia, 38.2% in Adami-Tulu central Ethiopia, 39.8% in and around Addis Ababa and 39.7% in Chaffa valley in Northeastern Ethiopia, respectively.

However, it is lower than the reports of Ararsa *et al.* (2014) who indicated that 81.0 % were positive for mastitis at cows' level in Holetta, central highland of Ethiopia. The variation in prevalence and incidence of mastitis between the different studies might be partly due to different types of diagnostic tests, sampling procedures and associated risk factors such as age, number of parity, visible teat abnormalities, husbandry practice, barn floor status, milking hygiene, and breed of the animals included in the studies. Among the risk factors considered, age, number of parity, visible teat abnormalities, husbandry practice, barn floor status and milking hygiene were found to be statistically significant ($P<0.05$) with the occurrence of mastitis.

The result showed that the prevalence of mastitis was significantly higher (81.97%) in cows older than 10 years followed by cows in the age range of 6 to 10 years and lowest in cows younger than 6 years. There was a statistically significant difference among different age groups. This finding is in agreement with reports made by different authors (reference) in different parts of the country. Regassa *et al*. (2010b) and Mungube *et al*. (2001) who reported age considered as potential risk factor to mastitis and older cows were more affected by mastitis than younger cows. This finding is also agreed with previous reports on mastitis in Southern Ethiopia (Kerro and Tareke, 2003) and (Mekibib *et al.* 2010) in Holetta area. The increase in prevalence rate with the advancing age may be due to gradual suppression of immune system of the body, structural changes in udder and teats and repeated exposure to milking practices.

The highest prevalence of mastitis was observed (90.00%) in cows with parity of more than six, followed by less than 3 and 3-6 parity. The highest prevalence of mastitis was observed (91.66%) in cows with two teat blind, followed by teat laceration/ lesion (71.42%) and one teat blind (67.39%). This is in agreement with Mungube *et al*. (2004), Biffa *et al*. (2005), Gizat *et al*. (2008), Girma (2010), Lakewu *et al.* (2009) and Molalegn *et al*. (2010) who identified parity as risk factor to mastitis in the study conducted at different parts of Ethiopia. This might be due to the increased opportunity of infection with time and the prolonged duration of infection, especially in a herd without mastitis control program and associate with the ability of the immune system of an animal to defend infection-causing agents.

Intensively managed cows present a higher risk for the development of mastitis (84.04%), followed by semi-intensive (64.59%), with least risk among extensively managed animals. This result was in line with previous reports on mastitis in (Sori *et al*. 2005). Housing increases the risk of mastitis because of the confinement of the animals and the multiplication of pathogens in the litters elevates teat challenge and consequently mastitis. Mastitis prevalence increases in herds housed under poor stable and drainage conditions. This is much more evident for coliform mastitis (Sudhan and Sharma, 2010). Several factors in the environment affect the exposure of a cow to microorganisms. Sources of environmental exposure are manure, bedding, feeds, dirt, mud and water. A good example of this is *E.coli,* which is present in the environment of the cow. Several studies have indeed linked the cleanliness of the barn, and the colony count in the bedding with the incidence of clinical mastitis (Bramley and Neave 1975). Prevalence of mastitis was significantly ($p<0.05$) associated with milking hygienic practice. Cows at farms with poor milking hygiene standard (96.07%) are severely affected than those with good milking hygiene practices (8.08%). This result agreed with the report of (Junaidu *et al.* 2011; Lakew *et al*. 2009; Sori *et al*. 2005). This might be due to high stocking density, dirty bedding or ground, infected utensils, poor ventilation and high humidity.

In this study, the predominant organisms isolated from mastitis to be *Staphylococcus aureus* followed by *Streptococcus agalactiae.* The predominance and primary role of *S. aureus* isolate in bovine mastitis has also been reported in other studies (Atyaib *et al.* 2006; Fadlelmoula *et al.* 2007; Mekbib *et al.* 2010) and Radostitis *et al.* (2007). Different researches finding indicate that, *Staphylococcus aureus* was the most frequently isolated bacteria as per the reports of Regassa *et al*. (2010b), Matios *et al*. (2009), Getahun *et al*. (2008) in dairy farms of Holetta, Asella and Selalle Towns, respectively.

The preponderance of contagious mastitis in this study may be ascribed to the lack of proper milking procedure before milking, during the time of milking and post milking. For instance, absence of pre and post teat dipping using antiseptics, washing of milker's hands and using teats secretion as a lubricant of teats at the time of milking which is often practiced in the study area might contributed to the spread of these pathogens from infected teats to healthy ones.

The present study indicated that environmental bacteria like *Escherichia coli* was isolated in high proportion (9.4%). This is in congruent with the reports of Mekonnen and Tesfaye (2010), Matios *et al*. (2009) who found 7.5% of the total isolates. In contrast, this figure is higher than isolates reported by Regassa *et al*. (2010b), Sori *et al*. (2005) and Getahun *et al*. (2008) who reported 4.57%, 0.75% and 0.5% in different parts of Ethiopia, respectively. The presence of environmental bacteria might be an implication of unhygienic milking and contamination of cows' teats and environment with their dung.

Conclusion

The current study showed that an overall prevalence of 214 (70.62%) from 303 cows examined bovine mastitis was recorded. This indicates that mastitis is a serious problem across herds in these districts. Therefore, it is a problem for individual particular and the country in general. In line with above facts, the following recommendations are forwarded:

- Awareness creation should be given to the dairy herds on the impacts of bovine mastitis and its associated risk factor ;
- All quarters of the udder of each cow should be periodically checked for the timely treatment and prevention ;
- Good record keeping practice on the general herd health of dairy farms ;
- Adequate housing with proper sanitation and ventilation should be regularly maintained and
- Since the bacteria isolated from cows' milk samples in the present study are types that cause both contagious and environmental mastitis; correct and good milking techniques should be applied.

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Prevalence and Antimicrobial Susceptibility of Salmonella Isolates from Meat Swabs in Addis Ababa and Debrezeit

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Abstract

A cross-sectional study was conducted from September 2015 to march 2016 to determine the prevalence and antibiotic susceptibility of salmonella isolates in Addis Ababa and Debrezeit abattoir, Ethiopia. Swab samples were taken from 288 meat samples (72 bovine, 72 ovine, 72 caprine and 72 chickens) from abattoirs and were subjected to microbiological examination. Based on the microbiological examination, ***Salmonella*** *is wide spread with overall prevalence rate of 5.21% (n=15). Species-specific prevalence rate were found 5.56% (n=4) for bovine, 6.94% (n=5) caprine, 6.94% (n=5) ovine and 1.39% (n=1) chicken. There was no significance difference (P>0.05) on the prevalence of* ***salmonella*** *among species. The isolates were resistant to ampicillin (66.67%) and streptomycin (53.33%). Fifteen* ***salmonella*** *isolates were tested for susceptibility to seven selected antimicrobials. Out of the 15 isolates tested, 8 (53.33%) isolates were multiple resistant. The overall result of this study indicates that meat samples were contaminated with* ***Salmonella*** *and isolates were resistance to commonly used antibiotics.*

Introduction

Salmonella is a major food borne pathogens associated with diseases in animals and humans (Olsen *et al*. 1997). Meat samples are considered as one of the main sources of Salmonella infections. This is because Salmonella is known to colonize the gastrointestinal tract of animals without producing any clinical signs. Therefore, carcasses can become contaminated with Salmonella at the time of slaughter (Stolle, 1986). Meat can be contaminated by Salmonella at pre-harvest level, slaughtering, dressing, cutting and deboning operations and also it might even be spread by abattoir workers (Gomes-Neves *et al*. 2012; Heyndrickx *et al*. 2002). Some studies (Skov *et al*. 2008) indicate that Salmonella can also be transmitted from infected herds of production animals (cattle and pigs) to wildlife that lived amongst or in close proximity to them.

With the emergence of drug resistant strains of salmonella, the disease has become a serious public health concerns globally. The reason for this is mainly the irrational use of drugs in food animals (Ahmed *et al.* 2014; Thai and Yamaguchi, 2012). This suggests that meat could be a source for multidrug-resistant salmonellosis in humans (M'Ikanatha N *et al.* 2010). Those *salmonella* strains adapted to the antibiotics used in

animals can infect humans. There is also existing evidence that drug resistance genes can be transferred horizontally with the possibility of their transfer to humans. This may lead to therapeutic failure in humans and animals (Ahmed *et al*. 2014; Oloya *et al.* 2009; Sinwat *et al.* 2015).

Consequently, the survey of *salmonella* contamination in meat samples is of outmost importance for the control and prevention of severe diseases. Studies on the prevalence of salmonella and incidence of antibiotic resistant strains help determining future policies regarding antimicrobial drug use in domestic animals and humans (Oloya *et al*. 2009). In Ethiopia, where raw meat consumption is customary, such type of studies will help improve the impact of *salmonella* on the general public health. In Ethiopia, detail information on the prevalence of *salmonella* on meat samples is lacking. Thus, this study is designed to study the prevalence of *salmonella* in meat samples collected from abattoirs and to evaluate the antibiotic resistance level of *salmonella* isolates to commonly used antibiotics.

Materials and Methods

Sample collection and preparation

In a similar study area, Molla *et al*. (2006) reported a prevalence of *salmonella* in sheep (11.3%) and in goats (3%) . These prevalence rates were used to estimate the sample size following the formula given by (Thrusfield, 2005). However, to increase the accuracy, a total of 288 (72 bovine, 72 ovine, 72 caprine and 72 chickens) meat swab samples were taken by systematic random sampling from Addis Ababa abattoir and Debrezeit poultry slaughter house from September 2015 to March 2016. Samples were collected aseptically from thigh muscle using aseptic sterile cotton swab and put into sterile universal bottles with peptone water for transport and enrichment media. The samples were kept in an icebox with ice pack and were brought immediately to National Agricultural Biotechnology Research Center (NABRC) laboratory for microbial examination and antibiotic susceptibility test.

Bacterial isolation and characterization

The collected samples were cultured onto different selective and basal cultural media like:

- Rappaport-Vassiliadis (RV) Medium (OXOID, England);
- nutrient agar (HIMEDIA, India);
- Deoxycolate citrate agar (HIMEDIA, India);
- Salmonella-Shigella (SS) agar (HIMEDIA, India);
- Eosin Methylene Blue (EMB) agar (HIMEDIA, India);
- TSI (OXOID, England) and
- MacConkey (MC) agar (HIMEDIA and India).

Upon bacteriological examination the isolated organism was subjected to Gram stain test and different biochemical tests – catalase test, indole (HIMEDIA, India)

production test, Methyl Red (MR-VP) test (HIMEDIA, India) and Citrate utilization test (Pronadisa, Spain) for confirmation.

Antimicrobial susceptibility pattern of *salmonella* isolates

Salmonella isolates were subjected to antimicrobial susceptibility test using Kirby-Baur disc diffusion method on Mueller-Hinton agar (Sigma-Aldrich Corp., St. Louis, MO, U.S.A.) following the procedures described by Quinn *et al*. (2011). Then antibiotic impregnated paper disc (Oxoid, UK) were applied and pressed onto the plate with forceps. Plates were incubated aerobically at 35°C for 18-24 hrs. The diameters of zones of growth inhibition were measured in millimeter and interpreted as sensitive, intermediate and resistant to different antibiotics as per the procedures of Quinn et al. (2011). The drugs used were erythromycin (10mcg), ampicillin (10µg), streptomycin (10µg), tetracycline (30 µg), Chloramphenicol (30 µg), sulphamethoxazole-trimethoprim (25 µg) and gentamicin (10 µg).

Data analysis

The collected data were analyzed using SPSS version 20 software. Descriptive statistics was used to determine the prevalence of antimicrobial resistant *salmonella isolates* and Chi-square test ($\chi 2$) was used to assess associated risk factors. In all the analyses, confidence level was held at 95% and $P<0.05$ was set for significance.

Results and Discussion

Bacterial isolation and characterization

From 288 thigh swab samples collected from September 2015 to March 2016, *salmonella* isolates were detected in15 swab samples with an overall prevalence of 5.21%. Prevalence of *salmonella* varied among species of meat swab samples. Highest prevalence was observed in caprine and ovine (6.94%). Meanwhile, low prevalence was observed in chicken with the value of (1.39%). However, this difference was not statistically significant ($p>0.05$) (Table 1).

Table 1. Prevalence of *Salmonella* isolates according to species level

Species	Number of examined	Number of positives	Prevalence (%)	X^2 (P-value)
Bovine	72	4	5.56	.000 (1.000)
Caprine	72	5	6.94	
Ovine	72	5	6.94	
Chicken	72	1	1.39	
Total	288	15	5.21	

Antimicrobial susceptibility pattern of *salmonella* isolates

The result of the In vitro antibiotic susceptibility test showed that *salmonella* isolates were generally resistance to ampicillin and streptomycin. However, the isolates were

considerably susceptible to tetracycline, gentamicin, sulphamethoxazole-trimethoprim and chloramphenicol respectively (Table 2).

Table 2. Antibiotic susceptibility test result of *Salmonella* isolates

Antibiotic discs	Disc concentration	Resistant	Intermediate	Susceptible
Ampicillin	10µg	10(66.67%)	1(6.67%)	4(26.67%)
Gentamycin	10µg	3(20%)	3(20%)	9(60%)
Streptomycin	10µg	8(53.33%)	3(20%)	4(26.67%)
Tetracycline	30µg	1(6.67%)	3(20%)	11(73.33%)
Sulphamethoxazole-Trimethoprim	1.25/23.75 µg	6(40%)	1(6.67%)	8(53.33%)
Chloramphenicol	30µg	5(33.33%)	3(20%)	7(46.67%)
Erythromycin	15 µg	2(13.33)	8(53.33)	5(33.33)

Salmonella isolates from sheep, goat, and bovine meat showed resistance to ampicillin with 60%, 100% and 50 % respectively (Table 3).

Table 3.Antibiotic susceptibility pattern of all *Salmonella* isolates from meat swab samples

Antibiotic discs	Ovine(n=5)			Caprine(n=5)			Bovine(n=4)			Chicken(n=1)		
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
E	1(20)	1(20)	3(60)	0(0)	4(80)	1(20)	1(25)	2(50)	1(25)	0(0)	1(100)	0(0)
Amp	3(60)	1(20)	1(20)	5(100)	0(0)	0(0)	2(50)	0(0)	2(50)	0(0)	0(0)	1(100)
CN	0(0)	3(60)	2(40)	0(0)	0(0)	5(100)	2(50)	1(25)	1(25)	1(100)	0(0)	0(0)
S	2(40)	1(20)	2(40)	3(60)	1(20)	1(20)	2(50)	1(25)	1(25)	0(0)	1(100)	0(0)
TE	0(0)	3(60)	2(40)	5(100)	0(0)	0(0)	1(25)	0(0)	3(75)	0(0)	0(0)	1(100)
Sxt	1(20)	0(0)	4(80)	2(40)	0(0)	3(60)	3(75)	0(0)	1(25)	0(0)	1(100)	0(0)
C	1(20)	2(40)	2(40)	3(60)	1(20)	1(20)	1(25)	1(25)	2(50)	0(0)	0(0)	1(100)

N=numbers R=Resistance I=Intermediate S=Streptomycin, E=Erythromycin, Amp=Ampicillin CN=Gentamycin, S=Streptomycin, TE=Tetracycline, Sxt= Sulphamethoxazole-trimethoprim C= Chloramphenicol

In this study, only one isolate (6.67%) showed resistance to two drugs; four isolates (26.67%) showed resistance to three drugs, two isolates (13.33%) showed resistance to four drugs and one isolate (6.67%) to six drugs with overall multidrug resistance of 53.33% (Table 4).

Table 4. Multidrug-resistance pattern of *Salmonella* isolates

Species	ID Number of Bacterial isolates	Number of antibiotics	Antibiotics	Total (%)
Bovine	B-1	MDR-2	CN*S	1(6.67%)
Sheep	S-8	MDR-3	E*S*Sxt	3(26.67%)
Sheep	S-61	MDR-3	Amp*S*C	3(26.67%)
Goat	G-9	MDR-3	Amp*S*C	3(26.67%)
Bovine	B-31	MDR-3	Amp*S*Sxt	3(26.67%)
Goat	G-12	MDR-4	Amp*S*Sxt*C	4(13.33%)
Goat	G-17	MDR-4	Amp*S*Sxt*C	4(13.33%)
Bovine	B-67	MDR-6	E*Amp*CN*TTC*Sxt*C	6(6.67%)

MDR-2: Multi Drug Resistance for two antibiotics, MDR-3: Multi Drug Resistance for three antibiotics, MDR-4: Multi Drug Resistance for four antibiotics, MDR-6: Multi Drug Resistance for six antibiotics, CN: Gentamycin, S: Streptomycin, E: Erythromycin, Sxt: Sulphamethoxazole-trimethoprim, Amp: Ampicillin, C: Chloramphenicol, TTC: Tetracycline.

This study was designed to determine the prevalence rate and antimicrobial susceptibility pattern of *salmonella* isolates from meat samples collected from abattoirs. In the current study, the prevalence of salmonella isolates among bovine (5.56%), caprine (6.94%), ovine (6.94%) and chicken (1.39%) did not vary significantly ($p>0.05$). This might be because the slaughterhouses of both species are found in similar hygienic status, similar pre-slaughter management of these animals and sampling methodologies. The current prevalence of bovine *salmonellosis* was found to be 5.56%. This finding is lower compared with previous reports from Ethiopia (Nyeleti *et al*. 2000) and Ghana (Adzitey *et al*. 2012) but higher than other reports from Ethiopia (Alemayehu *et al.* 2003a; Sibhat *et al.* 2011), Namibia (Renatus *et al*., 2015) and in Germany (Meyer *et al.* 2010) with prevalence rates of 2.79%, 2%, 2.67% and 1.4% respectively. The variations might be due to differences in pre-slaughter management, presence or absence of animal stressors during transport to market or abattoirs, slaughtering technique and abattoirs' hygienic condition.

In the present study, the prevalence of caprine *salmonella* was 6.94% which is relatively higher than previous reports from Debrezeit (2.33%) (Woldemariam *et al*. 2005), mojo 11.7% (Akafete and Haileleul, 2011) and Addis Ababa (0.54% (Bedaso *et al*. 2015). Whereas lower than other reports from Diredawa (17.7%) (Ferede *et al*. 2015). The current prevalence of Ovine *salmonella* (6.94) is lower than previous reports from Ethiopia which was 7.7-14.12% (Akafete and Haileleul, 2011; Ejeta *et al.* 2004; Zewdu and Cornelius, 2009). This might be due to difference in variability of carriage of bacteria among animals, sample type and origin of the animal.

The current observed prevalence of *salmonella* in chicken meat (1.39) is lower than other reports from Addis Ababa and Debrezeit (12.3-15.38%) (Molla and Mesfin, 2003; Zewdu and Cornelius, 2009) and in Romania 13.2% (Tirziu *et al*. 2015). This may be attributed to differences in the sample size and sampling condition, chickens management prior to slaughter and slaughtering condition, differences in sanitation of the abattoirs and their premises, difference in meat contamination by abattoir personnel during slaughtering process, carcass handling and personal hygiene.

Widely use of antibiotics in animals and humans as well as in agriculture has resulted to development of resistant microbes to commonly used antibiotics (Meervenne *et al*. 2012). Routine monitoring and assessment of such antibiotic resistant microbes like *salmonella* isolates from foodstuffs helps to design mitigation strategies and policies. In the current study, the overall antibiotic susceptibility patterns of the isolates were, sensitive (33.33 %), intermediate (53.33%) and resistant (13.33%). Of the seven antimicrobial compounds used in susceptibility testing in this study, all of the compounds showed incidents of resistance or decreased susceptibility against *salmonella* isolates collected from meat of bovine and ovine species. Whereas against *salmonella* isolates collected from chicken and caprine meat samples, 4 and 6 of the antimicrobial compounds showed incidents of resistance or decreased susceptibility respectively (Table 3). This indicates the magnitude and challenge of curbing antimicrobial resistant microbes from foods of animal origin emanating from the existing misuse of antibiotics in both human and animals. However, further studies are needed to better understand the patterns and dynamics of antimicrobial use in the country.

Our study showed that, high resistance rate was found to ampicillin (66.67%) and streptomycin (53.33%). Similarly, Ferede *et al.* (2015) reported that *salmonella* isolated from goat raw meat slaughtered at Diredawa-Ethiopia were found resistant to ampicillin (54.5%) and streptomycin (81.8%). Higher resistance rate of ampicillin and streptomycin against *salmonella* isolates were also reported in Egypt (Ahmed *et al*. 2016), Romania (Tirziu *et al.* 2015) and elsewhere (Sinwat *et a*l., 2015; Sodagari *et al.2015)*. In Ethiopia, ampicillin and streptomycin are among the widely used antibiotics in both animals and humans. In addition, they have developed resistance to *salmonella* isolates. This poses a potential public health risks to meat consumption. On the other hand, the isolates showed that 73.33% susceptible to tetracycline, 60% gentamycin, 53.33% sulphamethaxazole- trimethoprim and 46.67% chloramphenicol respectively. Similar to this report, other studies have been reported the susceptibility of gentamycin and tetracycline (Thung *et al*. 2016) and chloramphenicol (Tirziu *et al.* 2015). Relative to the other antimicrobials, these antimicrobials can be used effectively to treat *Salmonella* infections.

The majority of *Salmonella* isolates were resistant to multiple drugs (53.33%); with different resistance patterns. Similarly, multidrug resistance rate of 31.8%-52% (Alemayehu *et al.* 2003b; Molla *et al.* 2006) were reported in Ethiopian for *Salmonella* isolates from apparently healthy slaughtered sheep, goat and cattle. Resistant

salmonella isolates were detected from chicken meat with a multidrug a resistance rate of 29% from republic of Korea, 30% from Romania, (Tirziu *et al*. 2015) and 92.8% (Abd-Elghany *et al.* 2015) from Egypt. These all show that multidrug resistant *Salmonella* serotypes are widespread and the extent and magnitude of distribution of such serotypes may depend on prudent use of antimicrobials in humans and animals, the type of animal handling practices prior to slaughter and meat management practices. Nonetheless, there is a need to relate the type and amount of antimicrobial drugs used in animals with data from history of resistant *Salmonella* infection to monitor changing resistance and to determine if change in the frequency and pattern of resistance are related to specific pattern of antimicrobial usage (Lee *et al.* 1993).

Conclusion

In this study, higher incidence rate of contamination of meat by *salmonella* isolates and higher antibiotic resistance rates by *Salmonella* isolates to the commonly used antibiotics both in humans and animals have been reported. This all indicates the existence of an eminent public health threat from consumption of meat products especially those of uncooked ones. Therefore, a more prudent use of antibiotics in both humans and animals is required.

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Isolation and Identification Of *E. Coli* from Calf Diarrhea from Selected Dairy Farm in Holetta

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Abstract

Calf diarrhea is a multi-factorial disease entity that can have serious financial and animal welfare implication in dairy farm and is one of the most common diseases reported in calves up to 3 months old. Among the bacterial causes of diarrhea in neonatal animals, ***E. coli*** *is the most common and economically important one. The current cross sectional study was undertaken from November 2015 to April 2016 to isolate and identify* ***E. coli*** *from diarrheic calves aged up to 3 months from two selected dairy farm located in Holetta, Oromiya Special Zone, Ethiopia. Purposive type of sampling was made and 99 diarrheic calf samples were used in this study. Standard isolation techniques were used to identify the* ***E. coli*** *from diarrheic cases samples. Out of 99 fecal samples collected from diarrheic calves of different farm, 89 were positive for* ***E. coli*** *with an isolation rate of 89.9%. The potential risk factors sex, body condition, blood level and sites were found not significantly associated with risk of occurrence of* ***E. coli*** *in diarrhea. Further epidemiological and microbiological studies on enteropathogens causing calf diarrhea to identify the serotype and virulent gene of the bacteria strongly recommended.*

Introduction

Livestock production consists one of the principal means of achieving improved living standards in many regions of developing world. In sub Saharan African countries, livestock plays a crucial role both for the national economics and the livelihood of rural communities (ILCA, 1998). Livestock systems in developing countries are characterized by rapid change, driven by factors such as population growth, increases in the demand for livestock products as incomes rise and urbanization. Livestock currently contribute about 30 percent of agricultural gross domestic product in developing countries and is becoming the fastest-growing sub-sector of agriculture (FAO, 2010; Kefyalew and Tegegne, 2012).

Ethiopia lies within the tropical latitudes of Africa and has an extremely diverse topography, a wide range of climatic features and a multitude of agro-ecological zones,which makes the country suitable for different agricultural production systems. This in turn has contributed to the existence of a large diversity of farm animal genetic resources in the country. The country production systems are broadly characterized as low input, mixed crop-livestock, agro-pastoral and pastoral systems as well as medium input, peri-urban and urban enterprises (Annon, 2004).

Ethiopia has the largest livestock in Africa and ranks 10th in the world. Livestock is the source of protein, fuel and animal products and by products in general. Currently, a number of urban and peri-urban dairy farms are major suppliers of milk and milk products to the consumers (Lema *et al.* 2001). The intensification of dairy production, especially under hot and humid condition, presents new disease problems (Shiferaw *et al.* 2003). For cattle farmers, there is a strong demand in raising healthy calves as a basis for a persistent and highly productive cow. Calf hood diseases have a major impact on the economic viability of cattle operations. Calf survival is mainly influenced by management factors. However, there is also a genetic background for the mortality of young cattle implying its inclusion in future breeding strategies (Olesen *et al.* 2000).

Diarrhea is one of the most important constraints in food animal production (Lema *et al.* 2001). Its prevalence appears to be m, anagement related especially when calves are housed in unhygienic conditions (Wudu *et al*.2008). Diarrhoea in neonatal calves is a syndrome of great etiological complexity that causes economic losses directly and indirectly from poor growth (Schumann *et al*.1990; Bendali *et al*.1999). A number of infectious and non-infectious factors cause diarrhoea in neonatal calves. Previous studies show that the most important infectious agents are *Rotavirus*, *Coronavirus*, enterotoxigenic *Escherichia coli* (*E. coli). Salmonella species* and *Cryptosporidia* either singly or in combination (Delafuente *et al.* 1998). Numerous infectious pathogens capable of causing diarrhoea among food animals have been associated with foodbome disease and zoonoses in humans (enterohaemorrhagic *E. coli***,** *Salmonella, Rotavirus and C. parvum*) (Trevajo *et al.* 2005).

E. coli is one of the most intensively intensive species. It is part of the microbiota of the lower gastrointestinal tract of mammals including humans and usually exists as a harmless commensal. However, there also exist pathogenic strains of *E. coli* that can cause of diseases in both humans and animals (Elena *et al.* 2005).

Clinical disease due to *E. coli* in calves may be present as enteric or septicemic illness and is one of the most important causes of neonatal mortality in dairy calves (Lofstedt *et al*. 1999). Calf diarrhea is a commonly reported disease in young animals, and still a major cause of productivity and economic loss to cattle producers worldwide. Multiple pathogens are known to cause calf diarrhea. The multi-factorial nature of calf diarrhea makes this disease hard to control effectively in modern cow-calf operations. However, there is paucity of information on the occurrence of *E. coli* in calf diarrhea in current study area. Therefore, the objectives of this study wereto isolate and identify *E. coli* from the fecal samples of calves suffering from diarrhea and todetermine the occurrence level of *E.coli* in calf diarrhea in the selected dairy cow in the study area.

Material and Methods

The study area

The study was undertaken in the Holetta Town of Walmara District of Oromia Special zone around Finfine Town in central highland part of Ethiopia from November 2015 to March 2016. The District has 23 peasant associations (PAs) and one Town with four urban administrative kebele. Its total land area is 80,927 hectares and has 83,784 total human populations. This District is located at 25kms to the West of Finfine Town (8.5°-9.5°N and 38.4°-39.2°E) with altitude of 2000-3380m.a.s.l. It has annual rainfall and temperature ranging from 334-1350mm and 0.1C°-27C°, respectively. It is bordered by Finfine Town to the East, Ejere District to the West, Sululta District to the North, Sebeta Hawas District from the South and its weather condition is classified as 39% Woinadega and 61% Dega. Walmara District has 188,221 cattle, 108,652 sheep, 15,420 goats 365,294 poultry, 8,062 horses, 1,406 donkeys, 229 mule and 1,853 traditional, 870 transitional, beehives (CSA, 2014).

Study animals

Both exotic (Holstein fresian) and crossbreeds (Borena and Holstein fresian) of dairy calves were included in this study. Also both sex groups of calves up to 3 months of age that were clinically affected with diarrhea were observed during this study i.e. purposive sampling. All diarrheic calves in dairy farms of Holetta Agricultural Research Center (HARC) and Bull-dam Farm were incorporated in the study for a period from November 2015 to March 2016. Body condition scoring of sampled animal was carried out according to the method described by Stockdale (2001) and categorized into three scores as poor, medium and good.

Study design

Cross sectional type of study was conducted in dairy farms of, HARC and Bull-dam in Holetta Town of Welmera District, Oromia Region. Selection of farms was done based on willingness of the owners. The health status of each calf was evaluated by clinical examination. Calves free from diarrhea were classified as healthy where as sick calves shown abnormal fecal consistency.

Sample collection

Fecal samples were collected from 99 calves from both farms. The calves with diarrhea and below 3 months of age were chosen for this study. The samples were collected directly from rectum by digital stimulation using a disposable latex glove and samples were cooled on ice packs and transported to Animal Health Research Center Laboratory which governed by National Agricultural Biotechnology Research Center of Ethiopian Institute of Agricultural Research. The samples were processed as soon as possible after collection. Collected samples were clearly labeled date of sampling, age, sex, breed and tag number of the calves were considered.

Isolation and identification of *E. coli*

All medium used were prepared according to the manufacturer instruction. Isolation of *E. coli* was conducted following standard procedures described in Quinn *et al.* (2002). Fecal samples were inoculated on to MacConkey agar medium, which selectively grows members of the *Enterobacteriaceae* and permit differentiation ofenteric bacteria and incubated at 37°C overnight. Colonies showing characteristiclactose fermenting (having pink colonies) were then picked up and sub cultured on to Eosin methyl blue (EMB) agar plates to see the metallic sheen characteristics. The well-separated isolated colonies were sub cultured and stored on nutrient agar for further identification by biochemical tests. All the isolates were stained by Gram stain todetermine the cell morphology; Gram reaction and purity of the isolates under the oilimmersion objective (x100 magnification). Then *E. coli* isolates were identifiedpreliminarily by using indole, methyl red, gelatin hydrolysis, TSI and citrate utilizationbiochemical tests. *E. coli* isolates were presumptively identified (indolepositive, methyl red positive, gelatin hydrolysis negative and citrate negative.

Data analysis

The data was entered into Microsoft Excel spreadsheet. After validation, it was transferred and processed using SPSS version 20 for analysis. The Chi-square (X^2) and Odds Ratio (OR) was calculated to assess the strength of association of different risk factors with the prevalence of *E. coli* in calf diarrhea. A statistically significant association between variables was said to exist if the calculated P-value was <0.05 and the 95% confidence interval (CI).

Results and Discussion

This current study was aimed to isolate and identify *E. coli* from fecal sample of calf suffering from diarrhea. Out of 99 fecal samples collected from diarrheic calves of different farm, 89 were positive for *E. coli* with an isolation rate of 89.9%.On the other hand, an isolation rate of *E. coli* was observed based on sex groups of animals and out of 51 male and 48 females calves examined, 86.3% and 93.8% were harbored it, respectively. However, there was no significance difference of infection rate between sex groups of animals (P > 0.05). Higher isolation rate was recorded in good body conditioned animals (95 %) as compared with medium (84.4 %) and poor (94.1%).

However, statistically insignificant (p> 0.05) was observed. Comparison was made on *E. coli* infection of calves with a little variation between two farms of Holetta Town of Welmera District selected for the study. Different rate of *E.* coli were recorded in two of them 92.3% and 81% in HARC and Bull- dam farms, respectively. A relatively high *E. coli* record was observed in HARC farm. However, statistically insignificant (p> 0.05) was observed. Analysis of blood level wise isolation rate of *E. coli* infection between three blood groups indicated that there was great difference in prevalence rate between these blood groups that was 63, 90.6% and100% in 100%, 75% and 50%

blood groups respectively. However, there was no significant variation on the occurrence of the disease (P>0.05) among blood groups (Table 1).

Table 1.Percentage of *E. Coli* isolate with the risk factors

Risk factors	Number of animal examined	Number of positive animals	Prevalence (%)	P-value	OR	95% CI	
						Lower	Upper
Sex							
Male	51	44	86.3	0.260	2.308	0.538	9.897
Female	48	45	93.8	-	-	-	-
Body condition							
Poor	34	32	94.1	-	-	-	-
Medium	45	38	84.4	0.272	2.553	0.479	13.606
Good	20	19	95	0.824	1.349	0.097	18.784
Blood level							
100% HF	27	17	63	1.874	0.064	0.006	0.196
75% BHF	64	58	90.6	-	-	-	-
50% BHF	14	14	100	-	-	-	-
Site							
HARC farm	78	72	92.3	0.138	2.824	0.717	11.124
Bull-damfarm	21	17	81	-	-	-	-

A diverse range of biochemical reagents are known for the identification of certain metabolism and to differentiate between bacteria. Conventional biochemical tests are often used to identify microorganisms; the results were observed by color change and confirmation was made based on the reaction of an enzyme with certain substrate. *E. coli* was identified using some biochemical tests and was positive for Indole, Methyl red and Catalase tests. Finally, TSI was used during this activity and *E. coli* showed acid slant (yellow), acid butt (yellow) and gas production (Table 2).

Table 2.Biochemical tests with their respective results

Type of test	*E. coli*
Indole	+
Citrate utilization	_
Methyl red	+
Urease	_
Gelatin hydrolysis	_
Catalase	+
TSI	Acid slant (yellow), acid butt(yellow) and gas production

The current study on the isolation and identification of *E. coli* was conducted in Welmera District, Holetta in selected, HARC and Bull-dam dairy farms from November 2015 to March 2016 with an overall isolation of 89.9% in calves.According to the current results, *E. coli* was the bacterial agent cultured with the highest isolation rate, 89.9% from diarrheic calves. This finding agrees with result of Pourtaghi *et al.* (2013) 86.7% in Iran. Foster and Smith (2009) also found *E. coli* to be the causative agent in majority of calf diarrhea 73.12%. It has been reported to be the first organism,

which rapidly colonize the alimentary tract of newborn in great number. That is why its frequency of isolation is greater (Runnels *et al*. 1986).

Nevertheless, the isolation rate of the current study was higher as compared to the reports by Acha *et al*. (2004) and Ashenafi (2013) with 74% in Kombolcha dairy farms and Dawit (2012) reported 64% in Addis Ababa and Debre Zeit dairy farms. However, the result is much higher than the isolation rates reported by Bendali *et al.* (1999), Caple (1989), Herrera- Luna (2009) and Haschek *et al.* (2006) with 27.8%, 5%, 18.9% and 17.9% prevalence respectively. The higher isolation of *E. coli* in this study might be due to delay in first colostrums feeding in the study area because of poor understanding on the time of colostrums feeding. Matte *et al.* (1982) found that each hour of delay in colostrums ingestion in the first 12 hours of age increased the chance of a calves becoming ill by 10%. He also reported that 61% of colostral immunoglobulin containing 80mg/ml of 43 IgG is absorbed in six hours and decreases sharply subsequently. This variation might be due to the difference in the diagnostic technique used (the use of API 20 E system in the previous study might be increase the accuracy of detection rate).

Moreover, the difference might be due to failures in proper management such as inadequate sanitation, improper hygienic management, which increase the opportunity for exposure to these organisms. In addition, the existence of disease predisposing stress factors such environmental stress and feed shortage in the study might contribute for the high occurrence of *E. coli* infection. Charles *et al.* (2003) indicated that the gap in management includes inadequate nutrition, exposure to severe environment, insufficient attention to the new born calf or a combination of these are often involve on scour outbreaks, but the present study finding was less than the report of Ibrahim (1995) with 100% in Egypt. This difference might be due to the agro-ecological differences that exist between the study areas. In addition, the existence of disease predisposing stress factors such environmental stress and feed shortage in the study might contribute for the high occurrence of *E. coli* infection.

Even if they were not statistically significant with the occurrence of *E. coli*, which is highly responsible to cause calf diarrhea, different risk factors were involved to observe their association during this study. Sex-wise comparisons was also made to observe the isolation rate of *E. coli* in calves and out of 51 male and 48 female calves examined 86.3% and 93.8% were positive for *E. coli*, respectively. However, an infection rate was not statistically significant ($P>0.05$) between sex groups of animals. The result of this finding was similar with the report by Lomdard *et al.* (2007). This is due to equal exposure of both sexes and they were from similar agro-ecology and management systems.

An attempt has also been made to isolate and identify *E. coli* based on body condition of calves and they were classified into three groups as good, medium and poor with prevalence rate of 95 %, 84.4 % and 94.1%, respectively. Even if relatively higher isolation was recorded in good body conditioned animals as compared with others,

there was no significant difference (P>0.05) in the occurrence of *E. coli* among body conditions of the animals. This is due to small number of animals examined with good body condition score. Analysis was made to observe prevalence of *E. coli* infection among the three blood groups and the result was indicated that there was great difference with prevalence rate of 63, 90.6% and 100% in 100% HF, 75% BHF, and 50% BHF blood groups respectively. However, there was no significant variation on the occurrence of the disease (P>0.05) among blood groups.This finding indicate that calves with 50% blood level was more affected than the other calves. This might be due to low productive potential of the indigenous breed, Borena that was used for crossbreeding, and due to calving and postpartum problems associated crossbreeding.

The study was conducted at two different farms and comparison was made to observe *E. coli* infection in calves. The result showed a little variation between two farms of different sites in Holetta Town of Welmera District and different rate of *E.* coli were recorded as 92.3% and 81% in HARC and Bull- dam farms, respectively. A relatively high occurrence was observed in HARC farm as compared with that of Bull-dam farm. However, it was statistically insignificant (p> 0.05). This might be due to high number of sample taken from HARC farm, high number of cattle population exist there and also due to existence of different managemental problems like; lack of hygiene and sanitation, overcrowding and feeding problems.

Conclusion

Calf diarrhea is a major cause of productivity and economic loss to cattle producers in different part of the world. Calf diarrhea causes significant economic loss in the bovine industry due to the treatment costs, labor costs, poor growth performance, high mortality and high morbidity. During the current study, isolation and identification of *E. coli* from calf diarrhea were made. Accordingly, it was found that high rate of E. *coli*, 89.9% were detected in the study area. This result is relatively high which associated different problems are like; Management, nutritional and environmental factors, and/or infectious disease account for the majority of scouring problem. Related to infectious disease accurate diagnosis of etiology at early time is critical for prevention and control.

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