Isolation, characterization and cultivation of novel bacteria from Lake Chamo for production of amylase

ABSTRACT
This study reported the isolation, characterization and cultivation of a novel bacterium from Lake Chamo for amylase production. Various methods including biochemical and morphological identification were used in order to obtain high productivity of the enzyme. The amylase production was studied as a function of carbon source, nitrogen source, pH and temperature as influential factor at a time. It was verified that the maximum amylase activity was obtained by Bacillus sp. in the presence of maltose (0.386 U/ml) and peptone (0.761 U/ml) in the cultivation medium. The optimum pH and temperature for enzyme activity was found to be at pH 7 (0.402 U/ml) and temperature at 35°C (0.494 U/ml). The results obtained from this study open doors for amylase production at industrial scale.

Key words: Amylase, optimization, production, characterization, pH, temperature.

INTRODUCTION
The amylolytic enzymes have an important role in biotechnological applications such as food, fermentation, textile, paper and pharmaceutical industries (Gomes and Steiner, 2004). The global market for industrial enzymes was estimated at $2 billion in 2010 and is expected to rise at an average annual rate of 3.3% (Sujata, 2010).

The study of amylase production from microbial diversity in lake environments has attracted the attention of researchers and industrialists in last decades. This microbial diversity plays a significant role in various biotechnology companies for the manufacture of mass market consumer products (Horikoshi, 1999).

Amylase (1,4-α-D-glucan glucanohydrolase, endoamylases) is an enzyme that catalyze the hydrolysis of starch into glucose, maltose and others oligosaccharides by the cleaving of internal α-D-(1-4) glycosidic linkage. This enzyme can be obtained from several sources such as bacteria, fungi, yeast and actinomycetes. However, the amylases from bacterial and fungal sources are the most used in industrial applications (Kathiresan and Manivannan, 2006). Also, bacterial amylases are preferred for application in starch saccharification process and textile industries due to its action at different temperature and neutral pH (Sivaramakrishnan et al., 2006).

In food industry, the amylase is used in the production of glucose syrups, high fructose corn syrups and maltose syrups; applied for the reduction of apparent viscosity of sugar syrups, solubilization and saccharification of starch for fermentation process (Sivaramakrishnan, 2006).

Various microbial diversity studies on bacterial amylase have been reported from various lakes in Tunisian (Baati et al., 2010), Turkey (Birbir et al., 2004), and Spain (Sanchez-Porro, 2003). From scientific literatures there has been also reported from Ethiopian Soda Lake for the isolation of bacteria (Delgado et al., 2006). However, no attention has been given for the screening of the bacteria for the production of amylase enzyme from Lake Chamo for industrial application. Thus, the aim of this study was to isolate and characterize bacteria from Lake Chamo for the production of amylase enzyme.

MATERIALS AND METHODS
Isolation of microorganism
Samples were collected from Chamo Lake, Arba Minch, Ethiopia, in order to find a potential amylolytic enzyme.
Serial dilution followed by enrichment technique was made. Thus, 1 ml of Lake Chamo water sample was transferred to 9 ml of sterilized distilled water in test tube and mixed by vortex shaker for 20 s. The suspension was then diluted up to 10⁻⁵ dilutions. Then, 0.5 ml of each diluted sample was spread on petri plates containing nutrient agar medium (pH 7.0) and inoculated at 30°C for 48 h. Then, the pure colonies were distinguished by size and color (Dipal and Pandey, 2012). Each colony was carefully picked and cultured in liquid medium. Individual bacterial isolates were further subcultured on the respective medium in order to obtain pure culture. Pure isolates were maintained at 4°C in refrigerator for further studies.

**Screening of Amylase producing isolates**

Bacterial isolates were screened for amylolytic properties by starch hydrolysis test on starch agar plate. The microbial isolates were streaked as a line on the starch agar plate and plates were incubated at 30°C for 24 h (Anjea, 2003). After incubation 1% (v/v) of iodine solution (freshly prepared) was flooded on the starch agar plate. The presence of a clear zone of hydrolysis around the colony indicates a positive result (Gupta et al., 2003). The isolate producing highest clear zone of hydrolysis was considered for further investigation. The isolates were examined for their morphology, gram characteristics, motility and biochemical tests were performed according to Bergey's Manual of Systemic Bacteriology (Bergey, 1994).

**Enzyme extraction and enzymatic activity assay**

The bacteria were grown in 50 ml of cultivation medium with shaking of 150 rpm overnight. Then, the cultures were collected and subjected to centrifugation (Hermel z300) at 5,000 rpm for 20 min. The supernatants were collected and check for the enzyme activity using UV spectrophotometer (UV Vis spectrophotometer RS-290). The remaining supernatant was kept at 4°C for further study.

Amylase activity was determined by spectrophotometric method (Miller, 1959). For this, 1.0 ml of crude enzyme was added in the test tube followed by addition of 1 ml of 1% (w/v) soluble starch in phosphate buffer (pH 7.0).

The test tubes were covered and incubate at 35°C for 10 min (Miller, 1959). Then 2.0 ml of 3, 5-dinitrosalicylic acid (DNS) reagent was added in each tube and they were kept in boiling water bath for 10 min. After cooling at room temperature, the final volume of tubes test were completed to 10 ml with distilled water and the absorbance was measured at 540 nm by spectrophotometer. One unit (U) of amylase activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute under the assay conditions.

The concentration of the enzyme produced and the kinetics was evaluated against the standard amylase enzyme. One unit (U) of α-amylase activity was described as the amount of enzyme that released 1 µmol of reducing sugar per minute, under assay conditions.

**Experimental design and data analysis**

Isolation and cultivation experiments for amylase production were studied as a function of carbon source, nitrogen source, pH and temperature, varying one-factor at a time. All the experiments were done in duplicates and the data analysis was done using SPSS software version 16.

**RESULTS AND DISCUSSION**

**Isolation and characterization of the potential bacteria**

The samples were collected from Lake Chamo, Arbaminch, Ethiopia, in order to isolate and characterize the potential amylolytic enzymes. The isolated bacteria were screened for its potential to produce amylase using starch as a substrate. The clear zone around the bacterial colony indicated the amylase positive bacteria. Hence the positive colonies were considered for further study. The various morphological and biochemical study were carried out and the result is shown in (Figure 1A-H). Hence, the bacteria were identified as an amylase producer and it was taken for further studies. The bacteria found to be *bacillus* species is shown Table 1.

**Media optimization**

Different carbon sources can greatly influence the production of amylase. Effect of different carbon sources such as lactose, sucrose, glucose, fructose, maltose and starch in the basal media (Maltose (2%), KHPO₄, H₂O-0.2 gm, NaCl-0.05 g, MgSO₄, 7H₂O-0.05 g, FeSO₄.7H₂O-0.01 g, MnSO₄.7H₂O-0.007 g, CaCl₂.2H₂O-0.07 g) incubated for 48 h at 35°C for amylase activity and enzyme production was studied. The maximum enzyme production was obtained in the presence of maltose (0.386 U/ml) in the production media (Figure 2).

Our finding is in agreement with the reports on maltose as a source for amylase production from *Bacillus* sp. (Gurudeeban et al., 2011).

In the studies of Ashwini et al. (2011), *Bacillus mairini* presented the maximum enzyme activity in the presence of starch as carbon source, whereas the minimum enzyme activity was observed in the presence of dextrose. These results indicated that the disaccharides were more suitable carbon source than polysaccharides.

Different nitrogen sources such as peptone, tryptone,
Figure 1. Morphological and biochemical test of isolate A) Bacteria isolated; B) Starch hydrolysis test; C and D) Casein hydrolysis test; E) Gram staining; F) Simmon citrate test; G) Voges-Proskauer test and H) Indole test.
Table 1. Morphological and biochemical features of bacteria.

<table>
<thead>
<tr>
<th>Cultural characters</th>
<th>Colony morphology</th>
<th>small, circular, creamy white colonies</th>
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<tbody>
<tr>
<td>Microscopic</td>
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<td>Gram staining</td>
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<td>Gram positive rods</td>
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<td>Motility</td>
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<td>motile</td>
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<td>Indole</td>
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<td>Negative</td>
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<td>Citrate utilization</td>
<td>Positive</td>
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<tr>
<td>Catalase</td>
<td>Positive</td>
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<tr>
<td>Starch hydrolysis</td>
<td>Positive</td>
<td></td>
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<tr>
<td>Casein hydrolysis</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Voges-Proskauer Test</td>
<td>Positive</td>
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Figure 2. Effect of carbon source on amylase activity (U/ml).

ammonium sulphate, ammonium chloride and sodium nitrate were also tested as nitrogen source in growth and production media (Maltose (2%), K₂HPO₄·H₂O-0.2 g, NaCl-0.05 g, MgSO₄·7H₂O-0.05 g, FeSO₄·7H₂O-0.01 g, MnSO₄·7H₂O-0.007 g, CaCl₂·2H₂O-0.07 g) incubated for 48 h at 35°C. Among tested nitrogen sources, organic nitrogen source presented maximum amylase activity (0.761 U/ml) when compared to inorganic nitrogen source.

In this study, the highest level of amylase activity (0.761 U/ml) was obtained for peptone and tryptone (0.673 U/ml) as nitrogen source (Figure 3). Our study found similar results with the reported by Gupta et al. (2003).

Effect of incubation period

The effect of incubation period on amylase activity was considered in this study. The enzyme activity obtained at 48 h of incubation time was 0.271 U/ml (Figure 4). Above this period the amylase activity started to decrease. This result was similar to the results observed by Aiyer (2004) for Bacillus sp. This may be due to the fact that cells reached the decline phase, displaying low amylase synthesis.

Inoculum size

The size of inoculum plays an important role in the fermentation of enzymes (Lin et al., 1998). Optimum inoculum size of 3 μl gave highest yield of enzyme production (Figure 5). The production of enzyme was increased with increase in the size of inoculum. As, the inoculum level was further increased, the production of the enzyme was gradually increased. However, the production
Figure 3. Effect of nitrogen source on amylase activity (U/ml).

Figure 4. Effect of incubation time on amylase activity (U/ml).

Figure 5. Effect of inoculum size on amylase activity (U/ml).
Effect of pH and temperature

The selection of optimum pH and temperature is essential for the production of amylase. In this study, the optimum amylase activity (0.402 U/ml) was found to be at pH 7.0, although the enzyme was active in the pH range of 5.0 to 9.0 (Figure 6). The results suggest that maximum amylase activity was observed at neutral pH. This result is in agreement with Oyeleke and Oduwole (2009). Further, increase in the pH resulted decrease in the activity of amylase. When pH is altered below or above the optimum the activity might be inactivated.

In Figure 7, the effect of different temperature on the production of amylase was shown. For this study, the maximum amylase activity was observed at temperature 35°C (0.494 U/ml). Similarly, according to Krishna and Chandrasekaran (1996) for the production of amylase from banana stalk using *B. subtilis* have been agreed with our finding.

Conclusion

In conclusion, Starch degrading enzymes like amylase have
received a great deal of attention because of their perceived technological significance and economic benefits. This study reports isolation of potential bacterium from aquatic biome for the production of amylase enzymes. Enzyme production was found to be maximum in the presence of 0.2% peptone (0.761 U/ml) as nitrogen source. Hence, we recommend further study on isolation of microbial diversities to explore their importance for production of other industrially important enzyme. In addition, other study like immobilization, scale up and purification of the enzyme is recommended. Characterization of the physicochemical property of the enzyme is also the recommended area for further study.

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