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Bacteriological and *ecfX*- gene specific PCR based identification of *Pseudomonas aeruginosa* isolated from Chittagong industrial area and characterization of its extracellular amylase

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Abstract

Amylases are the starch degrading enzymes with applications ranging from food, fermentation, textiles to paper industries. In this study, a soil bacterium from the Chittagong industrial area was screened primarily for amylase activity using starch agar media. An attempt has been made to isolate and identify this amylolytic bacterium, optimization of its amylase production and characterization of the crude enzyme. The isolate was identified as *Pseudomonas aeruginosa* based on microscopic and biochemical tests. Beside this a species-specific gene *ecfX* (146 bp) for *P. aeruginosa* was amplified by PCR to confirm the identification of the isolate. A broad range of parameters for the optimization of enzyme production based on selection of best carbon and nitrogen sources, optimum pH, temperature, incubation period and effect of additional metal ions on culture media were investigated. Best enzyme activity (343.60 U/mL) was found in the presence of glucose as carbon source with yeast extract as nitrogen source at temperature 37°C after 24h at pH 9 in addition of Mg^{++} in submerged fermentation broth culture. The Mg^{++}, SDS and Ca^{++} increased the amylase activity whereas EDTA and Zn^{++} were found as the inhibitory agents for amylase.

Key words: Microbial amylase, *Pseudomonas aeruginosa*, *ecfX* gene, Production optimization, Characterization.

INTRODUCTION

Starch is a polymer of glucose linked to one another through the glycosidic bond. Two types of glucose polymers are present in starch: amylose and amylopectin. Amylose and amylopectin have different structures and properties. Amylose is a linear polymer consisting of up to 6000 glucose units with α-1, 4- glycosidic bonds. Amylopectin consists of short α-1, 4 glycosydic bonds linked to linear chains of 10–60 glucose units and α-1, 6 glycosydic bonds linked to side chains with 15–45 glucose units (Muralikrishna & Nirmala, 2005, Sorensen *et al.*, 2004; Tester *et al.*, 2004). In spite of the large number of plants able to produce starch, only a few plants are important for industrial starch processing. The major industrial sources are maize, tapioca, potato, and wheat. But some limitations such as low shear resistance, thermal resistance, thermal decomposition and high tendency towards retrogradation can limit its use in some industrial food applications (Agrawal *et al.* 2005, Goyal *et al.*, 2005).

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Amylases are widely distributed in microbial, plant and animal kingdom. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes (Aiyer, 2005). Although amylases can be derived from several sources, including plants, animals and microorganisms, microbial enzymes generally meet industrial demands. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry (Pandey *et al*., 2000). Amylases, especially alkaline amylases are used in detergents. Sometimes, amylases are also used as digestive aids to improve digestibility of some of the animal feed ingredients (Aiyer, 2005).

In this study a PCR assay, based on the *ecfX* gene as a species-specific target was used. The *ecfX* gene was chosen since it was previously identified as a reliable genetic marker of *P. aeruginosa* (Lavenir *et al.*, 2007, Anuj *et al.*, 2009, Hillenbrand *et al.*, 2011). *ecfX* encodes an ECF (extracytoplasmic function) sigma factor of *P. aeruginosa*, and might play a role in virulence. Specificity and sensitivity analyses showed the *ecfX* gene based PCR screening to be highly reliable, giving PCR products of the expected size for all *P. aeruginosa* strains tested and not amplifying DNA from any of the other *Pseudomonas* species tested (Lavenir *et al*., 2007).

Optimization of media is very important to maximize the yield and productivity, and minimize the product cost. The present study was mainly focused on the production of amylase from soil isolated *Pseudomonas aeruginosa* by optimizing various parameters such as carbon and nitrogen sources, pH, temperature, incubation time and metal ions. Then the crude amylase was characterized (based on the effect of pH, temperature, chemical agent, reaction time on enzyme-substrate reaction phase.). The identification of *Pseudomonas aeruginosa* was performed using different morphological and biochemical characterization, which was followed by *ecfX* gene specific PCR based identification at species level of the organism.

MATERIALS AND METHODS

Collection of sample and isolation of bacteria: Soil samples were aseptically collected from KDS (Knitting industry), Oxygen, Chittagong. The wastes of the industry are discharged in the soil of the surrounding environment. So the soil of this area may be source of soil was collected from the grassy soil behind the industry. After collection of the samples, serial dilution was performed. For the isolation of bacteria, Luria- Bertani (LB) agar media was used. The media consists of (gL^{-1}): Peptone, 10; Yeast extract, 5.0; NaCl, 5.0; Dextrose anhydrate, 10; and Agar, 15.

Screening of amylase production: The isolate was screened for amylase production using the gram iodine solution on Horikoshi Agar Medium. (Kaur and Vyas, 2012). The media (pH 9) is composed of (gL^{-1}): Yeast extract, 5.0; Peptone, 5.0 ; Soluble starch,10.0; K_2HPO_4,1.0; $MgSO_4.7H_2O$, 0.20; and Agar, 20.0;. The autoclaved media was poured and kept to solidify. After solidification, a loop full of 24 hours old bacterial culture in LB agar media was taken and streak onto the plate in zigzag way and incubated at 37°C for

24-48 hours. After incubation, the plate was flooded by Gram iodine solution for further observation.

Assay of amylase activity and protein estimation: The Horikoshi and Akiba broth (Kaur & Vyas, 2012) was dispensed at the rate of 50 ml per 100 ml conical flask and sterilized at 120ºC and 15 lb pressure for 20 minutes. After cooling, each flask was inoculated with 1 ml of 24 hours old bacterial culture and was incubated at 37ºC for 24-48 hours on a rotary shaker at 150 rpm. After incubation the culture media were centrifuged at 10,000 rpm for 15 minutes at 4ºC in a refrigerated centrifuge. The resulting supernatant was used as crude enzyme for estimation of α-amylase.

1ml of supernatant used as crude enzyme was added to 5ml of 1% soluble starch prepared in 0.1M Glycine-NaOH (pH 9.0) and was taken in a 50 ml test tube. One ml of alkaline copper reagent (freshly prepared 25 ml of reagent 'A' and 1 ml of reagent 'B') was added. The mixture was boiled in a boiling water bath for 20 minutes. After cooling in running water 1 ml of arsenomolybdate color reagent was added to this tube, mixed well (by vortex machine) and diluted to 25 ml. After 15 minutes the color intensity was measured at 500 nm in a spectrophotometer (SHIMADZU UV Spectrophotometer) and compared with standerd curve prepared with D-glucose. OD values were plotted in a standard graph prepared with different concentration of glucose. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 µmol of glucose/min under the assay condition (Nelson, 1944). Control was maintained without addition of enzyme.

Identification by bacteriological analysis: Identification was carried out according to the methods described in Bergey's manual of systemic bacteriology. All isolates were cultured well on LB media at 37°C for 24 hours for the growing of bacterial isolates. From appropriate dilutions one representative colony was picked and tentatively identified after Gram stain reaction, colony appearance, cell morphology, catalase test, oxidase test, indole test, methyl red test, voges-proskauer test, citrate utilization test and carbohydrate fermentation patterns as delineated by Bergey's manual (Hensyl 1994). After performing oxidase test, cetrimide agar base media was used to grow the organism. After 24 hours of incubation at 37º C, the plate with organism was visualized under UV radiation.

Molecular identification of bacterial isolate

DNA extraction from isolates: DNA was extracted from the sample isolated from soil samples according to the classical heat-thaw method (Salehi *et al*., 2005). Pure bacterial culture from LB agar slant was subcultured in LB broth medium from which 1.5 ml broth culture was taken in eppendorf tube and centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and remaining liquids were removed by soaking and the pellet was collected. 200 µl of autoclaved deionized water was added to the pellet and dissolved by shaking. The cap of the eppendorf tube was pierced by sterile needle and then the eppendorf tube was boiled in water bath at 100°C for 10 minutes. Just after boiling, the eppendorf tube was kept in ice for 10 minutes and then centrifuged at 10,000 rpm for 10 minutes. Then 100-150 µl supernatant containing bacterial chromosomal DNA was collected.

Species specific PCR amplification: To determine the affiliation to species level of bacterial isolate, species-specific PCR assay was carried out with *ecfX* gene-specific primer set, Primer 1: 5´- AAGCGTTCGTCCTGCACAA- 3´ and Primer 2: 5´- TCATCCTTCGCCTCCCTG- 3´, (Colinon *et al*., 2013). The PCR reaction mixture and amplification conditions were applied under the conditions described by Colinon *et al*., 2013.

The polymerase chain reaction was carried out using thermal cycler (GeneAmpR PCR System 9700, Applied Biosystems). The reaction mixture (20 µl) contained 1µl (100 ng/µl) of each primer added with 10µl 10× PCR Master Mix, 6µl of PCR water and 2µl of template. DNA fragments were amplified as follows: initial denaturation at 95°C for 5min, followed by 50 cycles consisting of denaturation at 98°C for 10s, annealing a 63°C for 20s, extension at 63°C for 20s, and a 8 min final extension step at 72°C. The products were stored at 4°C until analysis. Aliquotes of the amplified products were subjected to electrophoresis in 1.5% agarose gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2). Gels were stained with ethidium bromide (5 µg/ml) and visualized under UV light.

Optimization of enzyme production: Optimization of the amylase production condition is very important for getting good yield of enzyme. By maintaining all the optimized condition, maximum amylase was produced by the bacteria. Optimization of amylase production was performed by optimizing the carbon and nitrogen sources, maintaining temperature and pH, observing the effect of incubation time on enzyme production and optimizing the effect of metal ions on enzyme production.

Effect of carbon sources on amylase production: Effects of various carbon sources were studied by using sucrose, soluble starch, lactose, glucose at a concentration of 1% with the basal Horikoshi and Akiba broth medium (pH 9.0). *Pseudomonas aeruginosa* was cultured in a 100 ml flasks containing 50 ml medium. Each flask contained each of the carbon sources. After inoculation, the flasks were incubated at 37ºC±2ºC for 2 days. After incubation the media were centrifuged at 10,000 rpm for 15 minutes at 4ºC in a refrigerated centrifuge. The resulting supernatant was used as crude enzyme for estimation of α-amylase (Kaur & Vyas, 2012). A control medium was used without addition of carbon sources.

Effect of nitrogen sources on amylase production: Enzyme production was optimized by using different organic nitrogen sources such as Yeast extract, Peptone, Beef extract and inorganic nitrogen such as Ammonium sulphate, Potassium nitrate, Sodium nitrate at a concentration of 1% (w/v) added in Horikoshi and Akiba broth (pH 9.0) media. Each flask contained each of the nitrogen sources. After inoculation, the flasks were incubated at 37ºC±2ºC for 2 days. The optimum nitrogen source was found by analyzing the results of amylase production. A control medium was used without addition of nitrogen sources.

Effect of temperature on amylase production: In order to determine the effective temperature for optimum amylase production by the *P. aeruginosa*, culture was carried out in the range of 27ºC to 47ºC±2ºC. The optimized media with carbon and nitrogen

source (pH 9.0) were incubated for 2 days at the temperatures of 27ºC±2ºC, 32ºC±2ºC, 37ºC±2ºC, 42ºC±2ºC and 47ºC±2ºC. After the incubation, the enzyme assay was carried out.

Effect of incubation period on amylase production: To find out the effect of incubation period on optimum enzyme production the Horikoshi and Akiba broth (pH 9.0) media containing best carbon and nitrogen source were kept in 1, 2, 4, 6 and 8 days intervals for incubation at optimum temperature.

Effect of pH on amylase production: To determine optimal pH, *P. aeruginosa* was cultured in a 100 ml flask containing 50 ml of optimized media with different pH ranges from 5.0 to 12.0. The pH of the media was adjusted to 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 by using 1N HCl or 1N NaOH. The flasks were kept at optimum temperature for the optimum periods of time derived from earlier experiments. Amylase assay was then performed as per described protocol.

Effect of Metal Ion on Amylase Production: The effect of different metal ions on amylase production was determined by inoculating medium with different metal ions such as Zn^{++}, Mg^{++}, Fe^{++}, K^{+} in their sulfate form except Ca^{++} (Calcium chloride). These metal ions were added in optimized Horikoshi and Akiba basal media at optimum pH at a concentration of 0.02%. The optimum incubation temperature, incubation period and optimum carbon, nitrogen sources were used for conducting the reaction. Amylase assay was then performed as per described protocol.

Characterization of crude amylase

Effect of reaction pH: The effect of pH on the enzyme susstrate reaction was determined in four different buffer solutions covering the pH range 8-13 such as Phosphate buffer (pH 8.0), Glycine- NaOH buffer (pH 9.0 to 10.0), Sodium hydrogen orthophosphate-Sodium hydroxide buffer (pH 11.0), Potassium chloride- Sodium hydroxide buffer (pH 12.0 to 13.0). The crude amylase collected from optimized medium by maintaining optimized condition was kept at different pH 8.0 to 13.0 for the determination of the effect of reaction pH on enzyme activity. The standard assay condition was performrd at 500 nm.

Effect of reaction temperature: The enzymatic reaction was carried out at different temperatures ranging from 40°C to 100°C (40°C, 50°C, 60°C, 70°C, 80°C, 90°C and 100°C) in water bath. Reaction mixtures were prepared in Glycine-Sodium Hydroxide buffer (pH 10.0) and kept at respective temperatures. After that enzyme activity was determined using standard assay conditions at 500 nm to find out the effect of temperature on enzyme-substrate reaction.

Effect of reaction time: The enzymatic reaction was carried out at different reaction time ranging from 20 minutes to 2.5 hour (20 min, 40 min, 1hr, 1.5 hr, 2 hr and 2.5 hr) in water bath. Reaction mixtures were prepared in Glycine-Sodium Hydroxide buffer (pH 10.0) and kept at optimum temperature. After that enzyme activity was measured using standard assay conditions at 500 nm to determine the effect of reaction time on enzyme- substrate reaction.

Effect of chemical agents and surfactant on enzyme –substrate reaction: The chemical agents used were Sodium dodecyl sulfate (SDS), Ethylene diamine tetra-acetic acid (EDTA), $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $ZnSO_4.7H_2O$ at 5 mM and 10 mM concentration. Reaction mixtures were prepared in Glycine-Sodium Hydroxide buffer (pH 10.0). All the chemical agents were added in the reaction mixture at 5mM and 10mM concentration separately and kept at optimum reaction temperature for 20 minutes. The enzyme assay was carried out using standard assay conditions at 500 nm. A control reaction mixture was performed using all solutions except the chemical agents.

RESULTS AND DISCUSSION

Screening for the amylase production: The bacteria isolated from Industrial soil of Chittagong were screened for amylase production on starch agar medium. After 24 hours of incubation of *P. aeruginosa*, when Gram iodine solution was applied on the starch agar media, 2 minutes after, a white zone was observed on the plate. This whitish zone indicates the starch hydrolysis activity that caused by the amylase production by *P. aeruginosa*. The control plate without any bacterial colony did not show the hydrolyzed zone confirmed that the starch hydrolysis activity was caused by the isolates.

From the soil samples 5 bacterial strains were isolated. But later during screening it was found that only one strain showed amylase activity. After that, morphological characterization of bacterial isolate, identification of bacteria through bacteriological analysis, molecular identification through species specific PCR and optimization, characterization of amylase were performed.

Characterization of bacterial isolate

Morphological characterization of bacterial isolate: The bacterial isolate produced small, round shape and whitish colony in LB medium. Then it was examined under bright field microscope to observe (40X) their morphological features. The isolate was found Gram negative, short and rod shaped (Tripathi *et al*., 2011).

Identification of bacteria through bacteriological analysis: A series of biochemical test including catalase test, oxidase test, gelatin hydrolysis test, indole test, methyl red (MR) test, voges-proskauer (VP) test, citrate utilization test and carbohydrate fermentation patterns were tested as delineated by Bergey's manual systematic Bacteriology and the summarized results are shown in Table 1. The isolate was oxidase and catalase positive and in Indole, Methyl-red, Voges proskauar, tests, isolate was found negative. As the organism gave positive result in gelatin hydrolysis and citrate utilization tests, thereby these might confirm the isolate was *Pseudomonas aeruginosa* (Freitas & Barth, 2004).

Table1. Results of morphological characteristics and biochemical tests of Isolate [Here '+' = positive result; '-' = negative result]

Characteristics/ Tests	Result
Colony Morphology	Whitish and round
Microscopic Characteristics	Short and rod shaped
Gram Staining	-
Oxidase test	+
Culture on Cetrimide agar base media	Grown
Visualization of Culture on Cetrimide agar base media plate under UV radiation	Production of blue- green pigment
Catalase test	+
Gelatin hydrolysis test	+
Citrate utilization test	+
Indole test	-
MR test	-
VP test	-
Glucose	+
Dextrose	+
D(-) Fructose	-
Sucrose	-
Lactose	-
Maltose	-
D(-)Ribose	-
D(+)Xylose	-
L(+)Rhamnose	-
D(-) Mannitol	-

In this study, the bacterial isolate was able to ferment glucose and dextrose whereas other 8 different carbohydrates, i.e. sucrose, fructose, lactose, xylose, ribose, maltose, mannitol, rhamnose could not be fermented by the organism. This report was found relevant to the findings of Hossain *et al. (*2013).

Molecular identification through *ecfX* gene specific PCR: Bacteriological and biochemical tests are time consuming and may give false negative or false positive results. Polymerase Chain Reaction (PCR) is a powerful tool where DNA templates and specific primers are used for molecular identification of any specific bacteria. So the bacterial identity was confirmed specifically by performing PCR using *P. aeruginosa* specific *ecfX* gene primer. In this study after visualizing the PCR product by 1.5% agarose gel electrophoresis, an expected sharp DNA band of 146 bp of species specific *ecfX* gene was observed which was expected to confirm the organism as *P. aeruginosa*. The result was compared with the specific DNA size of standard or known *Pseudomonas aeruginosa* as positive control / PC. This observation was similar with that of Deredjian *et al.* (2014) and Colinon *et al.* (2013). The negative control without any template DNA did not give any band (Fig. 1).

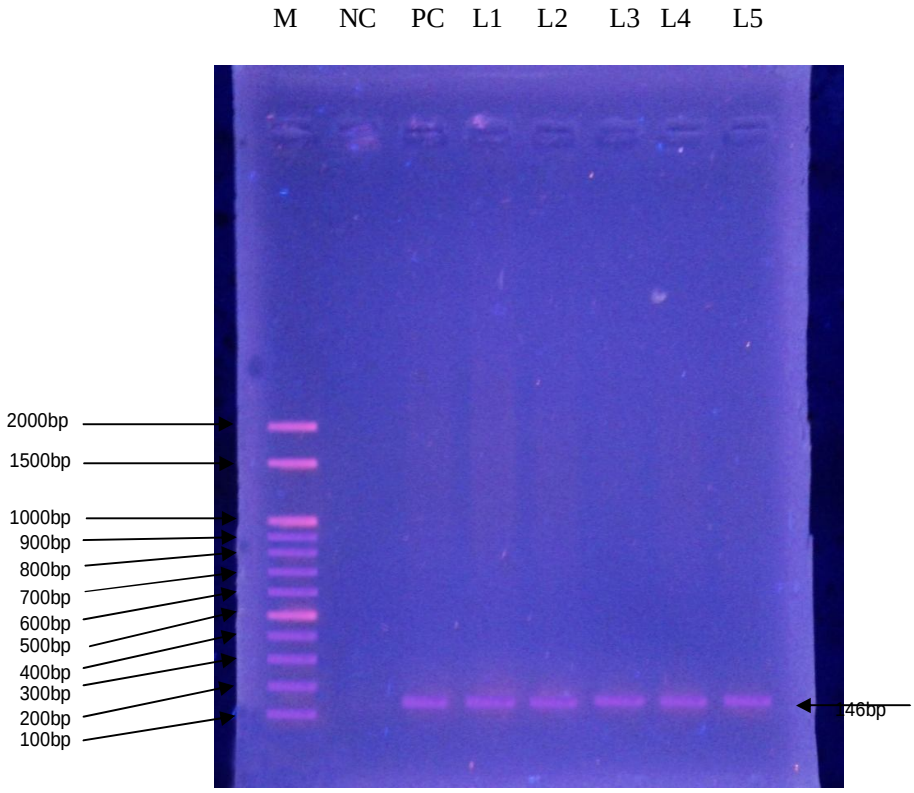

Fig. 1. Results of the PCR that have amplified a 146 bp gene fragment correspond to the *ecfX* gene of *P. aeruginosa* on 1.5% agarose. Here, Lane M: Marker, Lane NC: Negative Control (without any template DNA), Lane PC: Positive Control (DNA isolated from standard *P. aeruginosa*) , Lane L1- L5: different PCR products of isolated sample of *P. aeruginosa)*

Optimization of enzyme production

Effect of carbon sources on amylase production: The addition of carbon sources in the form of either monosaccharide or polysaccharide may influence the production of amylase enzyme. The effect of carbon sources on the production of enzyme by *P. aeruginosa* was investigated by using different carbon sources such as sucrose, soluble starch, lactose and glucose in the production of amylase. Glucose was found to be the best carbon source for the enzyme production (32.07 U/mL). Lactose gave the lowest activity. Among the carbon sources used, the soluble starch was the second best carbon source for amylase production (Fig. 2). A similar report went on to by Alariya *et al.* (2013) that glucose acted as an optimum carbon source for the production of amylase by *P. aeruginosa.*

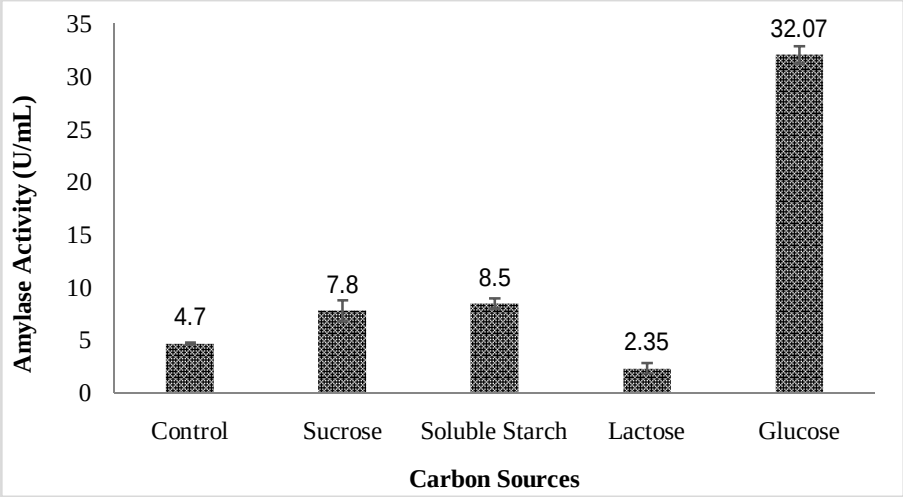

Fig. 2. Effect of carbon sources on amylase production by *P. aeruginosa*

Effect of nitrogen sources on amylase production: The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the production. A remarkable result was observed when optimizing the nitrogen sources. Organic nitrogen sources produce enough amylase activity whereas the inorganic sources show minute activity. Among the tested organic and inorganic nitrogen sources, yeast extract was the best candidate for the maximum production of amylase (55.43U/ml). There were no remarkable differences found between the control (without any nitrogen source) and the presence of inorganic nitrogen sources in the media in relation to production of amylase (Fig. 3). The best nitrogen source was yeast extract for maximum amylase production was also reported by Raju & Divakar, (2013).

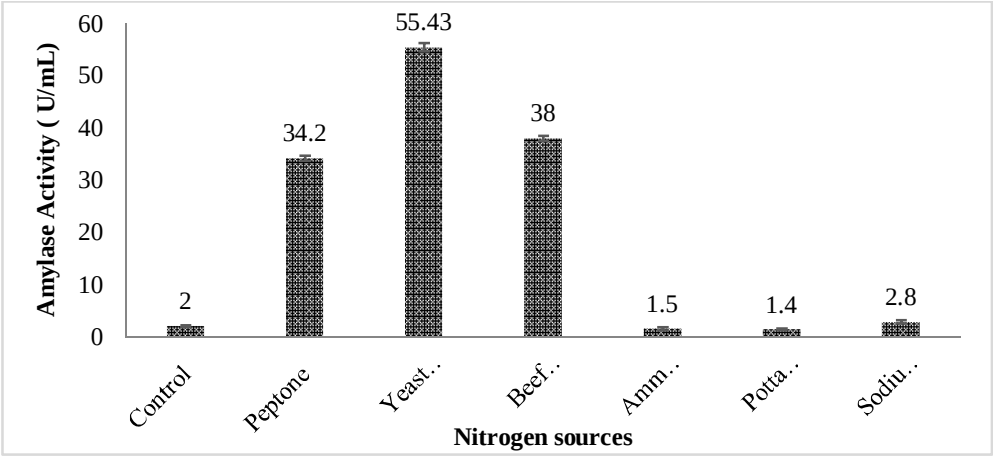

Fig. 3. Effect of nitrogen sources on amylase production by *P. aeruginosa*

Effect of temperature on amylase production: Temperature is a vital factor that controls the amylase activity. Optimum temperature was found to be 37˚C showing amylase activity of 88.14 U/mL thereby indicating its mesophilic nature of the enzyme. This result resembles as the report by Raju & Divakar, (2013); Viswanathan *et al.* (2014); Haq *et al.* (2005).

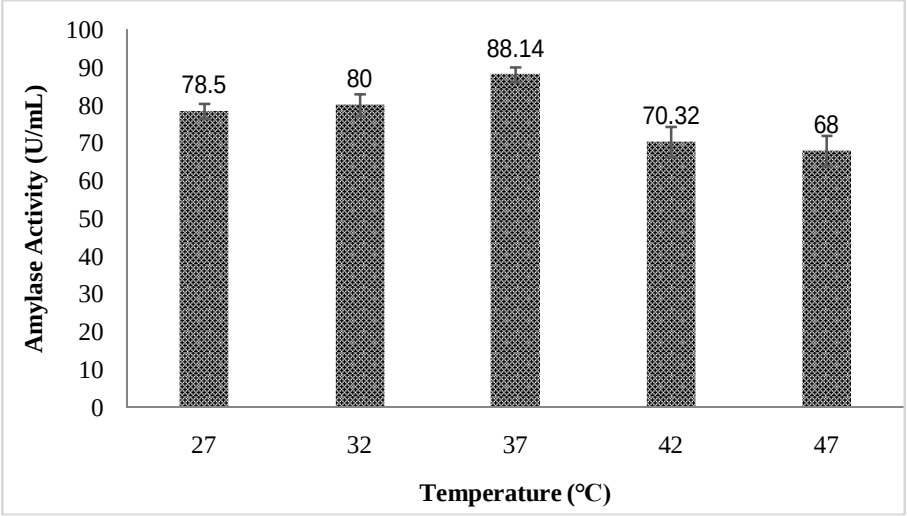

Fig. 4. Effect of temperature on amylase production by *P. aeruginosa*

The enzyme activity was found gradually decreased when the temperature increased above 37°C (Fig. 4). Similar observation was also reported by Alariya *et al*. (2013). Moreover, Viswanathan *et al*. (2014) reported the same result with *Bacillus spp*.

Effect of incubation period on amylase production: *P. aeruginosa* produced appreciable amount of amylase (30 U/mL) on the first day of incubation (Fig. 5). The amount of production of amylase reduced according to the increasing days of incubation. Minimum amount of amylase production was observed on the 8th day of incubation. Viswanathan *et al*. (2014) has evaluated the production of amylase at 24, 48 and 72 hours and obtained more production at 24 hours with *Bacillus spp*.

Effect of pH on amylase Production: pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. The bacterium showed a broad range of pH on the production of amylase. The optimum production was observed on pH 9.0 with maximum 43.5 U/mL amylase activity. The enzyme activity gradually deceased when increasing the pH from the optimum value (Fig. 6). Kaur & Vyas, (2012) also evaluated the similar optimization of pH on amylase production with *Bacillus sp*.

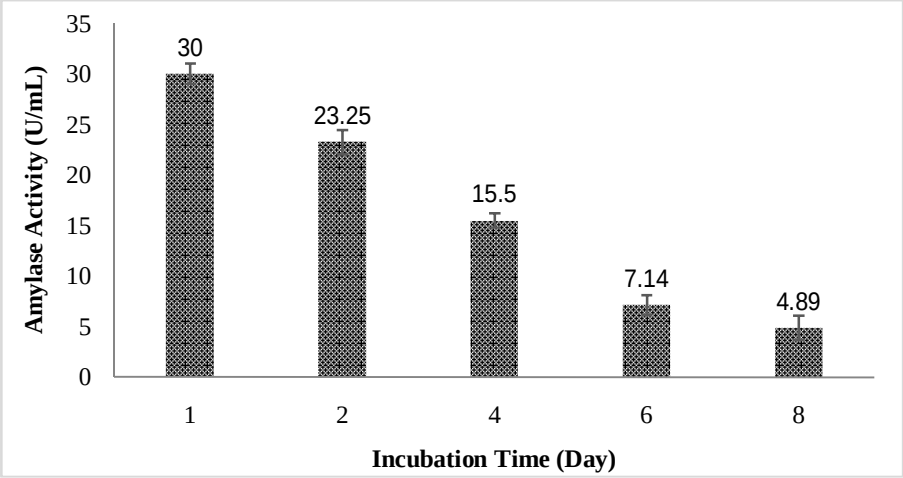

Fig. 5. Effect of incubation period on amylase production by *P. aeruginosa*

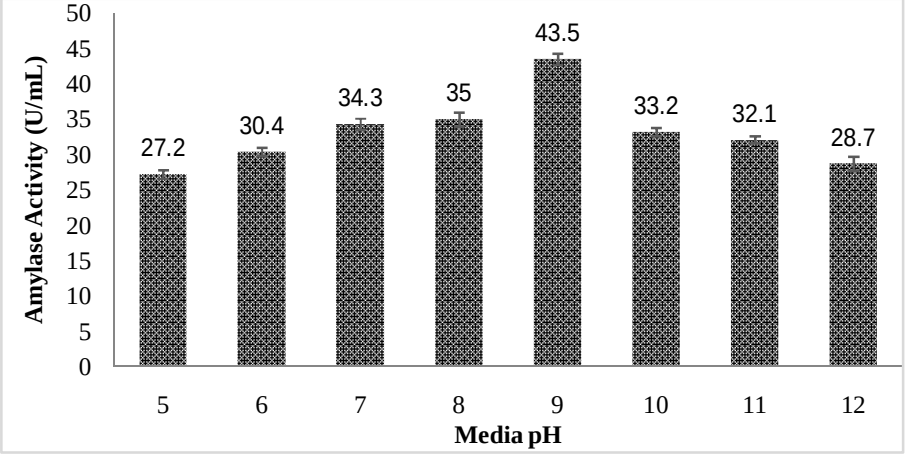

Fig. 6. Effect of pH on amylase production by *P. aeruginosa*

Effect of metal ions on Amylase Production: Some of the metal ions and trace elements are often facilitate the fermentation media for increasing the amylase production. In this experiment, as all culture flasks after inoculation were maintained at previous standard optimum parameters (carbon-nitrogen source, pH, temperature, incubation period etc.) found in the present study, the control one (without adding any metalions) showed enzyme activity (250 U/mL). This one is the highest enzyme activity at all optimizing condition by the isolate. But the results showed that magnesium and potassium ion increases the amylase enzyme production (343.60 U/mL and 275 U/mL respectively) compare with the control one (without adding any metal ions in media). Beside these, calcium, zinc and iron were found to reduce the production of amylase by *Pseudomonas aeruginosa* (Fig. 7). The same findings were also reported by Alariya *et al.* (2013).

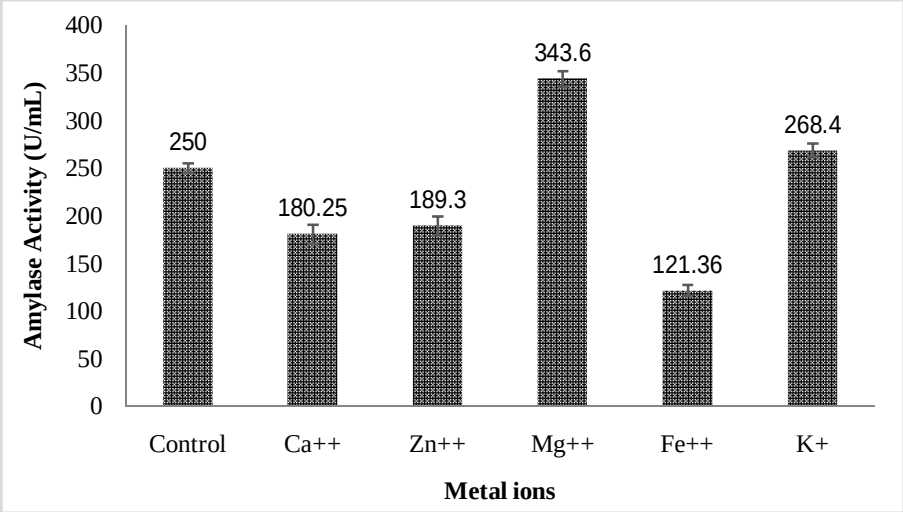

Fig. 7. Effect of metal ions on amylase production by *P. aeruginosa*

Characterization of crude amylase

Effect of reaction pH on amylase Activity: Amylases are known to be active in a wide range of temperature pH (7 - 13). The enzyme was active at alkaline pH of 8.0 to 13.0, with optimum pH 10.0 (471U/mL) prepared in Glycine-NaOH buffer indicating broad pH ranges for enzyme activity. The enzyme activity was found gradually decreased when the pH increased above 10.0 (Fig. 8). Similar finding was also evaluated by Krishnan & Chandra, (1983) with *Bacillus licheniformis* CUMC305 in their study.

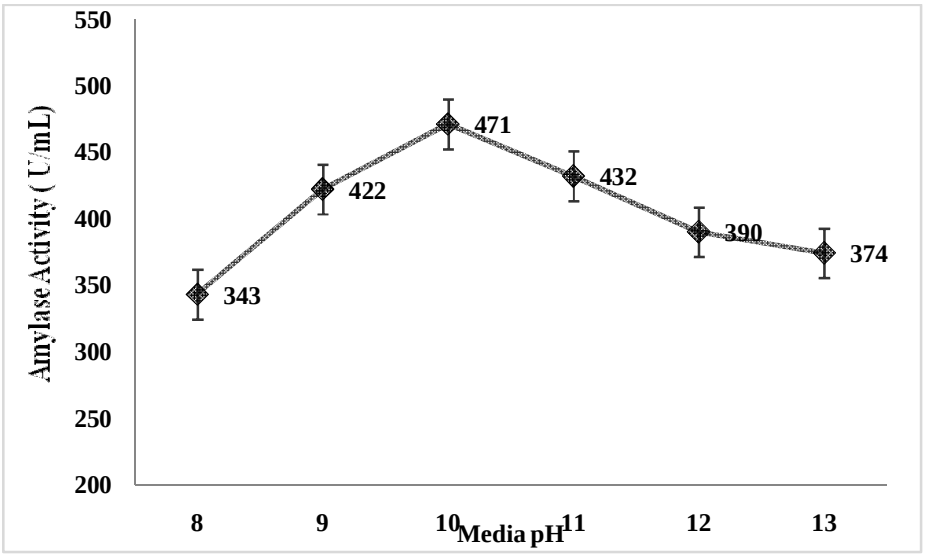

Fig. 8. Effect of reaction pH on amylase activity of *P. aeruginosa*

Effect of reaction temperature on amylase activity: Amylases are known to be active in a wide range of temperature (40- 100°C). The effect of reaction temperature on enzyme activity was measured by incubating the enzyme-substrate reaction phase at different temperatures for 20 minutes. Optimum enzyme substrate reaction temperature was found to be 70°C showing maximum enzymatic activity of 512.38 U/mL and thereby indicating its thermophilic nature (Fig. 9) of this enzyme. The temperature of the stability of amylase was 70°C, which is similar to that reported by Saxena *et al.* (2011). This identifies the unique characteristic of this *P. aeruginosa* that grows at 37°C as a mesophile.

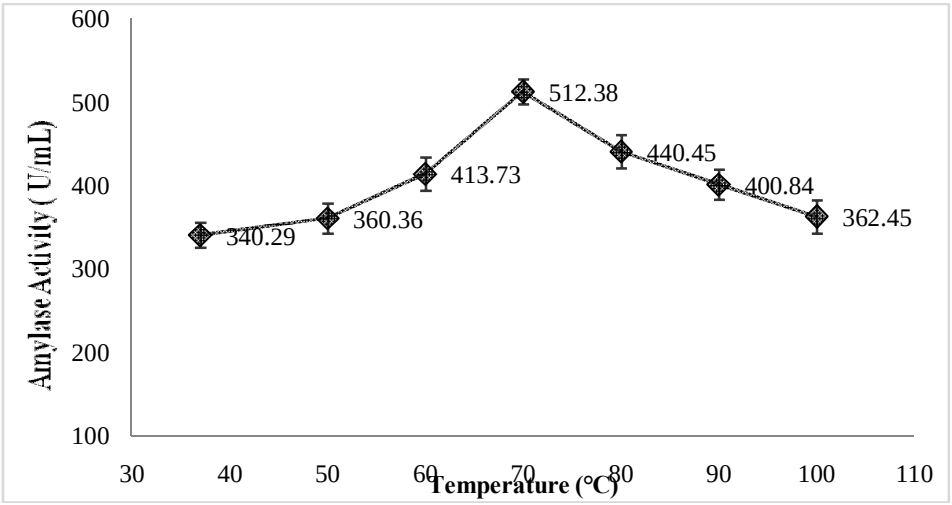

Fig. 9. Effect of reaction temperature on Amylase Activity of *P. aeruginosa*

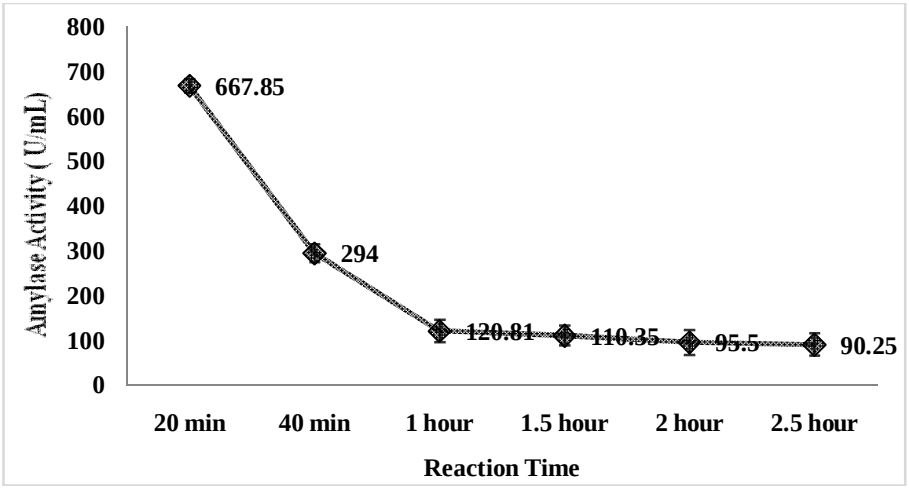

Fig. 10. Effect of reaction time on amylase activity of *P. aeruginosa*

Effect of reaction time on amylase activity: The amylase activity was measured at different reaction time keeping the reaction at 70° C and pH 10.0. The maximum enzyme activity was found at 20 minutes of incubation and it was 667.85 U/ml (Fig. 10). When the reaction time became increased, then the amylase activity was gradually decreased.

Effect of chemical agents and surfactant on amylase activity: Activities of enzyme were stimulated at the presence of $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and SDS. On the other hand, a strong inhibitory effect was observed in the presence of $ZnSO_4.7H_2O$ and EDTA (Fig. 11). This type of effect on amylase activity was reported by Deb *et al*. (2013) with *Bacillus amyloliquefaciens*. In all cases amylase activity at a concentration of 10mM was higher than the 5mM concentration. Inhibition of the amylase enzyme by EDTA suggests that it is a metalloenzyme as reasoned by Aygan *et al*. (2008). SDS has stimulatory effect on amylase activity was reported by De Oliveira *et al*. (2010).

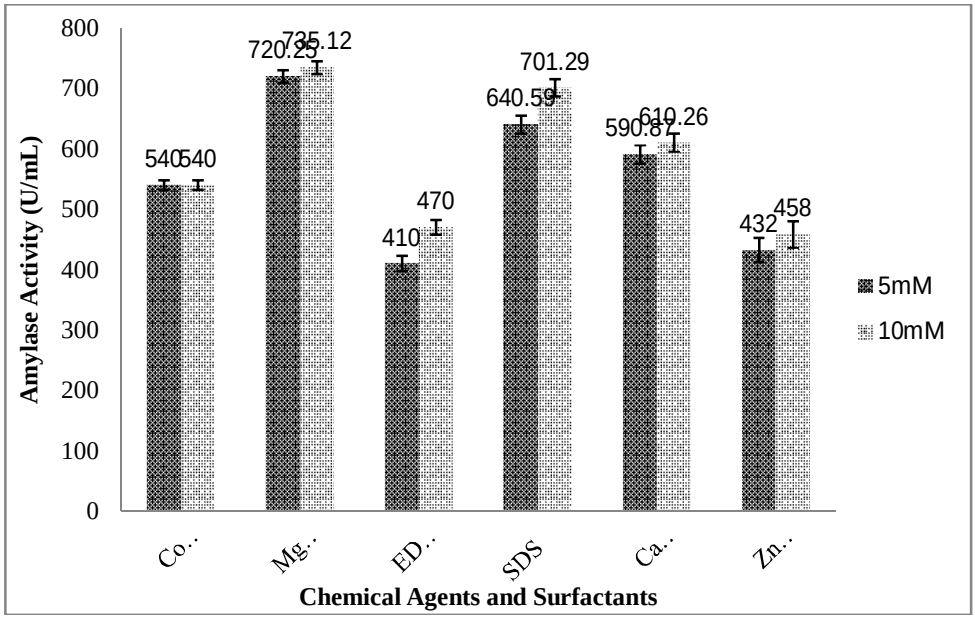

Fig. 11. Effect of chemical agents and surfactants on amylase activity of *P. aeruginosa*

In this study, a soil bacteria *P. aeruginosa* was isolated from the industrial area of Chittagong and the isolated bacterial strain was identified by several morphological, bacteriological and biochemical tests. As PCR is a rapid, sensitive and specific method for detecting any organism, so in this study, molecular detection using *ecfX* gene specific PCR was also performed to confirm the isolate beside bacteriological method. Best enzyme activity (343.60 U/mL) was found in the presence of glucose as carbon source with yeast extract as nitrogen source at temperature 37°C after 24h at pH 9 in addition of Mg^{++} in submerged fermentation broth culture. By maintaining this production conditions in case of this soil bacteria large amount of enzyme can be produced industrially using

bioreactor or fermentor as the enzyme have different industrial applications. Characterization of enzyme was done by keeping the enzyme-substrate reaction at different pH, temperature and time. Effect of different chemical agents (5 mM and 10 mM concentration) on amylase activity was also investigated. The best amylase activity was obtained at pH 10.0, 70° C after 20 minutes. The amylase activity was increased by adding Mg^{++}, SDS and Ca^{++} whereas EDTA, Zn^{++}were acted as inhibitor for this enzyme-substrate reaction as they decreased the amylase activity. In near future we hope to perform the total purification of enzyme. This will clarify the specific activity as well as the other features (molecular weight, protein structure and function for correlation, reaction mechanism etc). The study revealed that *Pseudomonas aeruginosa* could be a good source for the production of amylase. Subsequently, genetic improvement of the strain could be led mass production of the enzyme for different industrial purposes.

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