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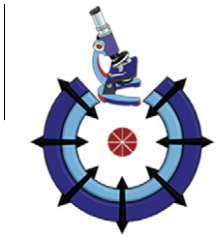

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ARTICLE

Optimization and immobilization of amylase obtained from halotolerant bacteria isolated from solar salterns

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KEYWORDS

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Immobilization

Abstract The objective of the present study was to isolate halotolerant bacteria from the sediment sample collected from Marakanam Solar Salterns, Tamil Nadu, India using NaCl supplemented media and screened for amylase production. Among the 22 isolates recovered, two strains that had immense potential were selected for amylase production and designated as P1 and P2. The phylogenetic analysis revealed that P1 and P2 have highest homology with *Pontibacillus chungwhensis* (99%) and *Bacillus barbaricus* (100%). Their amylase activity was optimized to obtain high yield under various temperature, pH and NaCl concentration. P1 and P2 strain showed respective, amylase activity maximum at 35 °C and 40 °C; pH 7.0 and 8.0; 1.5 M and 1.0 M NaCl concentration. Further under optimized conditions, the amylase activity of P1 strain (49.6 U mL^{-1}) was higher than P2 strain. Therefore, the amylase enzyme isolated from *P. chungwhensis* P1 was immobilized in sodium alginate beads. Compared to the free enzyme form (49.6 U mL^{-1}), the immobilized enzyme showed higher amylase activity as 90.3 U mL^{-1}. The enzyme was further purified partially and the molecular mass was determined as 40 kDa by SDS–PAGE. Thus, high activity of amylase even under increased NaCl concentration would render immense benefits in food processing industries.

1. Introduction

Amylase plays a vital role in the hydrolysis of starch to dextrins and other small polymers constituted of glucose units [40]. This enzyme had been reported to be obtained from various sources such as plants, animals and microorganisms [28]. Amylases have significant applications in numerous industries such as food, feed, detergents, textile, pharmaceutics and paper [14,32]. Despite its widespread applications in industries,

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amylases obtained from mesophilic organisms failed to cope up with the industrial processes that are carried out in the presence of salt solutions, organic solvents, heavy metals, high temperature and extremes of pH that would therefore inhibit the enzymatic activity [26,31,30]. Hence there arises a need for identifying amylases that are functional under such extreme conditions.

One potential source for obtaining amylase that is stable under extreme conditions is to exploit halotolerant bacteria from salterns that are also subjected to higher temperatures in addition to a high concentration of salt [24]. Several halophilic or halotolerant bacteria have been reported as potential amylase producers, that includes *Bacillus dipsosauri*, *Halobacillus* sp, *Bacillus halodurans*, *Halothermothrix orenii*, *Bacillus* sp. strain TSCVKK, *Chromohalobacter* sp. TVSP101, *Rheinheimera aquimaris* [11,2,15,36,20,29,13]. Previously, few studies had standardized various cultural parameters that may increase or decrease the amylase activity. Mevarech et al. [25] has stated that halophilic enzymes have optimal activity and stability in high salt concentrations. Other factors influencing bacterial amylase includes metal cations such as Ca^{2+}, Rb^{+}, Li^{+}, Cs^{+}, Mg^{2+} and Hg^{2+} which increased enzyme activity, whereas the heavy metals like Fe^{2+}, Cu^{2+} and Zn^{2+} decreased. Similarly, chemicals like PMSF (phenylmethylsulphonyl fluoride) and β-mercaptoethanol increased the activity but EDTA (ethylenediaminetetraacetic acid) decreased it. Difference in the type of organic solvents also influenced the activity, like cyclohexanol has not shown any significant effect; however, cyclohexane increased the amylase activity [33].

In general, immobilized form of amylase has shown enhanced stability compared to free enzymes. It offered the reuse of this biocatalyst for repeated fermentation process and also can be easily separated from the reaction mixture [21]. Among various immobilization methods, entrapment is one of the most preferable method because it prevents excessive loss of enzyme activity, protects enzyme from microbial contamination [8]. Physical entrapment of α-amylase in calcium alginate beads has shown as a relatively easy, rapid and safe technique [12]. Several studies have reported the production of amylases from the halotolerant bacteria and they occupy 25% of the enzyme sold in the market [7]. However, till date there is a high demand for obtaining amylase with properties including high pH stability, thermostability and tolerance to NaCl concentration [28]. In the present study, we isolated halotolerant *Pontibacillus chungwhensis* P1 and *Bacillus barbaricus* P2 and optimized the culture growth parameters like pH, temperature variation and NaCl that resulted in higher yield of amylase and then partially purified the enzyme of *P.chungwhensis* using ammonium sulphate precipitation, further immobilized the enzyme and quantified the amylase activity.

2. Materials and method

2.1. Sample collection, screening bacterial cultures for amylase activity

The soil sediments were collected randomly from the solar saltern region of Marakanam, Tamil Nadu, India. The sediments were collected from the depth of 5 cm, pooled and stored at 5 °C untill further processing. The soil sediment was diluted at ten folds in sterile saline solution and 100 µL aliquots of 10^{-6} dilution was spread on modified nutrient agar (MN), prepared by amending nutrient agar with 1 M sodium chloride. Inoculated samples were incubated at 37 °C for 24 h. Control and triplicates were maintained for all the experiments. To observe production of amylase, aliquots in broth culture of all the isolates were spotted on starch agar medium containing 1 M NaCl. After 24 h of incubation at 37 °C, the plates were flooded with 0.6% iodine solution. A clear halo zone around the colonies was expected for amylase positive strains [18].

2.2. Biochemical and phylogenetic characterization

The strains were analyzed for various biochemical traits along with utilization of different carbon sources. The salt tolerance levels were determined by plating on nutrient agar containing 0.2–4.5 M NaCl [17]. For phylogenetic characterization, genomic DNA was extracted from the isolates as described by Babu et al. [5]. From the purified genomic DNA, the 16S rRNA gene was amplified using polymerase chain reaction (PCR) with universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-ACG GCT ACC TTG TTA CGA CTT-3′). The PCR amplified product was purified and sequenced at SolGent Co., Ltd. (Daejeon, Republic of Korea). neighbour joining method using Jukes and Cantor model with a bootstrap value of 500 was employed for phylogenetic tree reconstruction with the MEGA v5.04 [35]. The 16S rRNA gene sequences were submitted to GenBank under the accession number GQ200826 and GQ200827.

2.3. Growth kinetics and enzyme activity

The growth kinetics and enzyme production of the isolated strains were studied in nutrient broth supplemented with 1 M NaCl for a period of 30 h at 37 °C and 120 rpm on orbital rotary incubator. Growth was monitored in regular intervals up to 30 h, in terms of absorbance at 600 nm using UV visible spectrophotometer (Shimadzu Pvt. Ltd., Japan). Cell free supernatants were checked for the extracellular enzyme activity at regular time intervals up to 30 h.

2.4. Quantification of extracellular amylase activity

Amylase activity was studied in nutrient broth with 1 M NaCl and 1% starch. About 1% inoculum was added and flasks were maintained at 37 °C for 24 h in an incubator shaker at 120 rpm. At every 1 h interval, 1.0 mL of the culture broth was centrifuged at 10,000 rpm for 15 min and the cell free supernatant was used for the estimation of amylase activity. Amylase assay was conducted by incubating 0.5 ml of supernatant with 0.5 ml soluble starch (1% w/v) in 0.1 M sodium phosphate buffer (pH 7.0) for 15 min at 45 °C. The reaction was arrested by adding 1 mL of 3,5-di nitro salicylic acid (DNSA) and incubated at 90 °C for 10 min. The absorbance was measured in a UV/Vis spectrophotometer at 540 nm [6]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol of reducing sugars per minute under the standard assay conditions.

2.5. Partial purification of the enzyme

2.5.1. Preparation of crude enzyme

The culture was harvested in late log growth phase and centrifuged at 8000 rpm for 10 min at 4 °C to obtain the supernatant as an enzyme source.

2.5.2. Ammonium sulphate precipitation

Ammonium sulphate was added to the crude culture supernatant to 40% saturation at 4 °C and left over for 2 h. After that, the precipitate was recovered by centrifugation at 10,000 rpm for 20 min at 4 °C and dissolved in a minimum volume of 50 mM phosphate buffer (pH 7.4) and dialyzed overnight against 10 mM phosphate buffer [34].

2.5.3. Determination of Molecular mass by SDS–PAGE

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out for the determination of purity and molecular weight of the amylase as described by Sundararajan et al. [34] using a 4% (w/v) stacking gel and a 10% (w/v) separating gel. After electrophoresis, gels were stained with Coomassie Brilliant Blue R250. The molecular weight of the amylase was estimated using standard protein molecular weight markers (Genei, Bangalore) consisting of: phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and lysozyme (14 k Da).

2.6. Optimization of temperature, pH and NaCl concentration for amylase production

The effect of various abiotic stresses on amylase production was assessed by growing the strains in the nutrient broth subjected to the variation in the parameters. Temperature tolerance was determined by incubating the bacterial strains at a temperature range of 25–45 °C with 5 °C difference incrementally. For pH optimization studies, the pH of the medium was adjusted from 5.0 to 9.0 at 1.0 unit variation. Similarly, for the determination of optimal NaCl concentration for the production of amylase, the bacterial strains were cultured in the presence of NaCl from 0 to 2 M NaCl at 0.5 M variation. Finally, the bacterial strains were cultured under optimized parameters (Temperature, pH and NaCl) and their enzyme production was quantified according to Bernfeld method [6].

2.7. Enzyme immobilization and its assay

The enzyme was mixed with sodium alginate solution (4%) at 1:1 ratio and the mixture was eluted drop wise through a syringe into 0.25 M $CaCl_2.2H_2O$ solution and left for 2 h in order to obtain enzyme immobilized bead with the size of 2 mm. The synthesized beads were thoroughly washed with sterile distilled water to remove excess material and used for enzyme assay [12]. Two grams of calcium alginate beads were made to interact with 20 mL of 2% (w/v) starch solution prepared in acetate buffer (0.1 M, pH 4.5) and incubated at 35 °C in an orbital shaker to facilitate the enzyme reaction. The product (0.5 ml) was assayed for amylase activity using DNSA method according to Bernfeld [6]. One unit of enzyme was defined as the amount of amylase that produced 1 μmole of reducing sugar under assay condition per gram of bead. The immobilization efficiency was determined from the difference in enzyme activity in the solution before and after the immobilization according to Dey et al. [12].

3. Results and discussion

3.1. Isolation and screening of amylase producing bacteria

In this study, 22 bacterial isolates exhibiting distinct colony morphologies like pigmentation, texture, elevation, size and margin surface [41] were selected and maintained over the MN plates. These isolates were capable of tolerating 1 M NaCl indicated that they are halotolerant. Previous studies showed that halotolerant bacteria have greater ability to adapt and survive in a wide range of salinities from 0.1 M to 4.5 M [39,19]. The isolates obtained were maintained in glycerol stocks and stored at −80 °C. Based on the starch agar plate assay conducted, 2 strains (P1 and P2) showed significant amylase producing zone around the colonies. Further these two strains were biochemically and phylogenetically characterized and used for enzyme optimization studies.

3.2. Biochemical and phylogenetic characterization of bacterial strains

Both strains were found to be Gram positive, rod-shaped bacteria. Strain P1 produced yellow pigment and required NaCl for growth. For both the isolates the growth pattern was observed in media containing 0.2–4.5 M NaCl with maximum growth at 1 M NaCl. Strain P1 and P2 showed NaCl tolerance up to 4 M and 2 M, respectively. Their morphological and biochemical characteristics were listed in Table 1. The results showed a typical characteristic of *Bacillus* sp.

Both the strains P1 and P2 were phylogenetically identified using 16S rRNA gene sequences that were aligned using ClustalW. Using BLAST (NCBI) search the closest relatives were identified. Thus identification of the strains by 16S rRNA gene analysis revealed that they belonged to *Bacillaceae* family (Fig. 1). Strain P1 displayed 99% similarity with its closest relative *P. chungwhensis* (AY553296), a moderately halophilic bacterium isolated from a solar saltern in Korea [22]. Strain P2 showed higher similarity (100%) with *B. barbaricus* (AJ422145), isolated from an experimental wall painting [38].

3.3. Growth and enzyme production

The growth kinetics study of *P. chungwhensis* and *B. barbaricus* in the modified nutrient (1 M) medium had indicated that the lag phase of the bacteria was extended for 4–6 h and the exponential phase up to 24 h, which was followed by the stationary phase. The growth and enzyme production followed a similar pattern, thus indicating that the enzyme production has a positive correlation with the growth rate of the organism, however, enzyme activity was detected highest in the final stages of the log phase. Production of amylase by *P. chungwhensis* started during log phase and reached its maximum during late exponential phase after 20 h (25 U mL^{-1}) of incubation (Fig. 2 A)

Table 1 Morphological, biochemical characteristics and phylogenetic identification of strain P1 and P2.

Characteristic features	Strain P1	Strain P2
Morphological		
Pigment	Yellow	–
Gram stain	+	+
Shape	Rod	Rod
Biochemical		
Catalase	+	+
Oxidase	–	–
Methyl red test	–	–
Voges-proskauer test	–	–
Indole	–	–
Urease	–	–
Nitrate reduction	–	–
Citrate	–	–
Gelatin hydrolysis	–	–
Tolerance to NaCl	22%	12%
Glucose	–	–
Lactose	–	–
Maltose	–	–
Sucrose	–	–
Arabinose	–	–
Phylogenetic		
16S rRNA based phylogenetic identification	*Pontibacillus chungwhensis*	*Bacillus barbaricus*

+ Positive, – negative.
P1-*Pontibacillus chungwhensis*.
P2-*Bacillus barbaricus*.

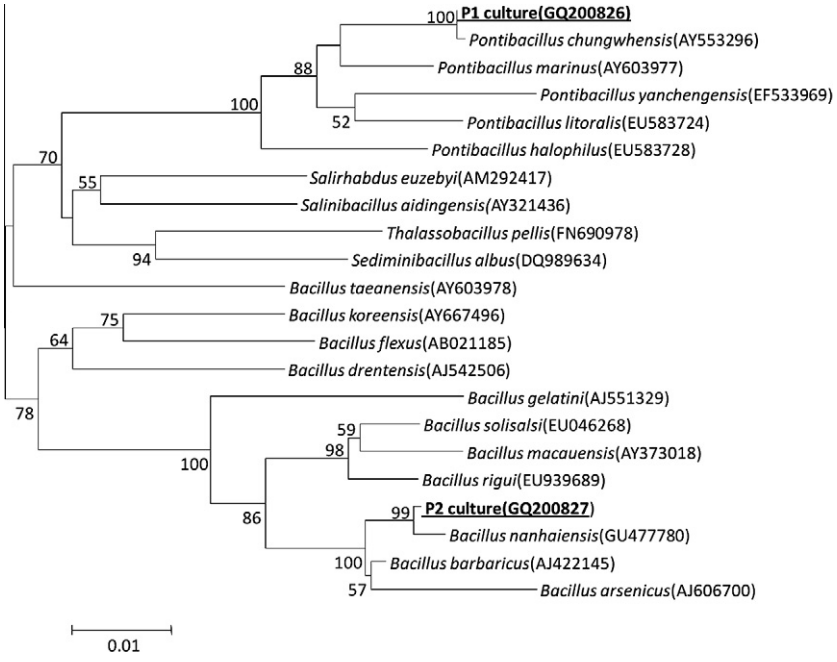

Figure 1 Phylogenetic tree of P1 and P2 cultures (bold and underlined) recovered from Solar saltern was re-constructed with the 16S rRNA gene sequence using the Neighbour-joining method. Note the clustering of both the isolates within the phylum *Firmicutes*. The numbers at the nodes are percentage indicating the levels of bootstrap support, based on analysis of 1000 resampled datasets. The scale bar represents 0.01 substitutions per nucleotide position.

and *B. barbaricus* reached its maximum level of production after 24 h (13 U mL^{-1}) of incubation (Fig. 2 B). Similar pattern of α- amylase production was reported earlier for *Halomonas meridiana* [10] but most of the halophilic bacteria has been reported to produce amylase during stationary phase [20,29]. The growth patterns of *P. chungwhensis* and *B. barbaricus* were quite similar but their enzyme producing efficiency was clearly different.

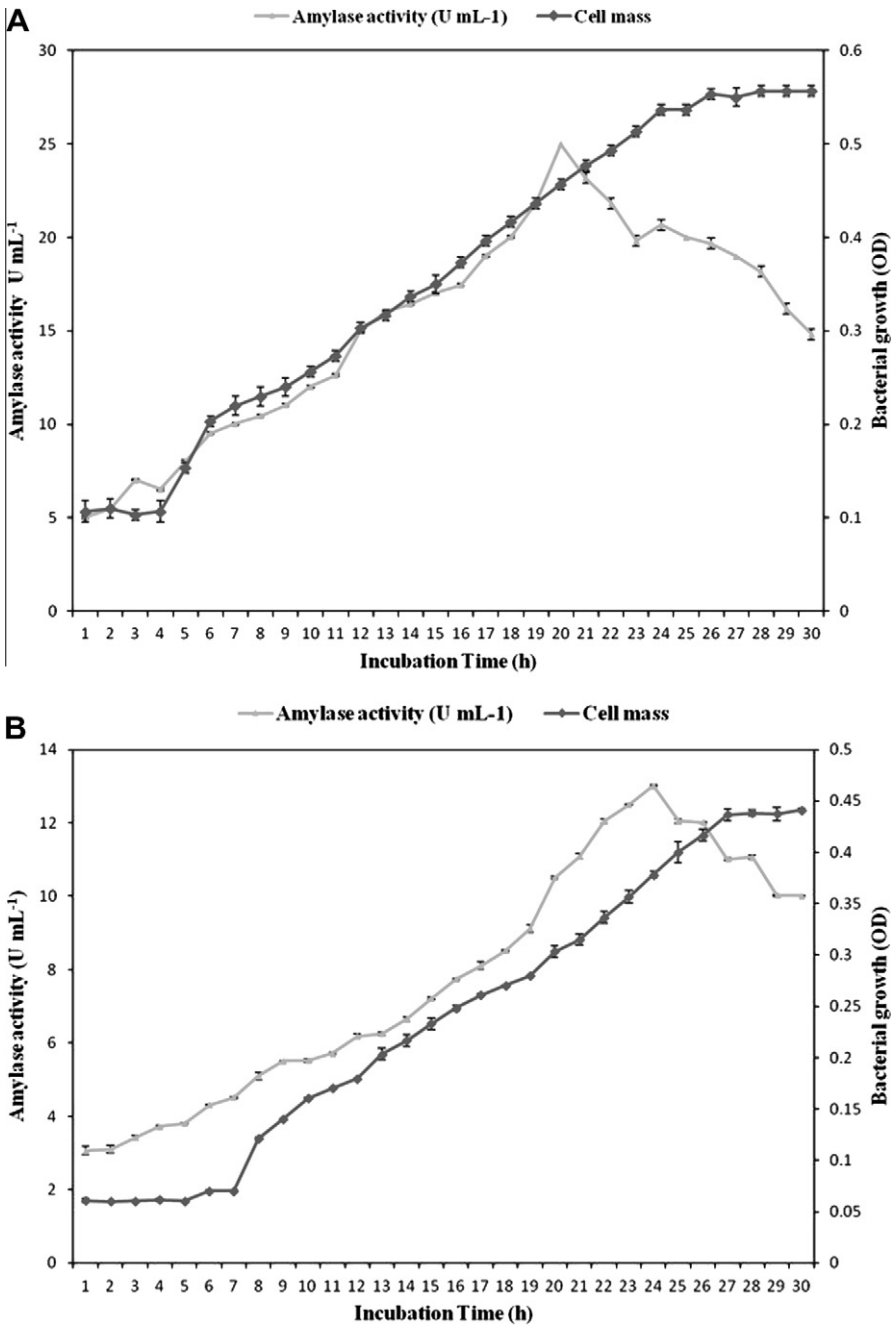

Figure 2 The bacterial growth and enzyme activity was measured in nutrient broth supplemented with 7% NaCl, it was incubated for a period of 30 h. (A) Effect of Incubation time on growth and Amylase production of *Pontibacillus chungwhensis* (B) Effect of Incubation time on growth and Amylase production of *Bacillus barbaricus*.

3.4. Partial purification of the enzyme

The enzyme was partially purified by ammonium sulphate precipitation and the results were summarized in the Table 2. The extracellular amylase exhibited increased specific activity values at each level of purification, leading to an increased enzyme activity with purification fold of 10.1. Using SDS–PAGE, the molecular mass of *P. chungwhensis* was approximately calculated to be 40 kDa via molecular markers (Fig. 3). In general, molecular weight of amylases from *Bacillus* sp. was reported between 21–60 kDa [16].

3.5. Effect of pH, temperature, salt concentration and optimized parameters on amylase production

Growth of the strains was observed over a wide pH range of 6.0–9.0. In *P. chungwhensis*, the amylase production was found to be high at a pH 7 with maximum activity of 26 U mL^{-1} (Fig. 4A). Even a *Bacillus* sp. KCA102 has been previously reported to have similar optimum pH for maximum enzyme activity [1,4]. In *B. barbaricus* inoculated culture flask, the optimum pH for the production of amylase was 8.0 with the activity of 12 U mL^{-1} (Fig. 4A). Therefore, the media adjusted

Table 2 Purification of amylase from *Pontibacillus chungwhensis* strain P1.

Step	Volume(ml)	Amylase activity(U/ml)	Total activity(U/ml)	Total protein(mg)	Specific activity(U/mg)	Purification fold
Crude	200	25	5000	208	24.03	1
Ammonium sulphate	2	34	68	1.8	37.7	1.56
Dialysis	3	52.8	158.4	0.65	243.6	10.1

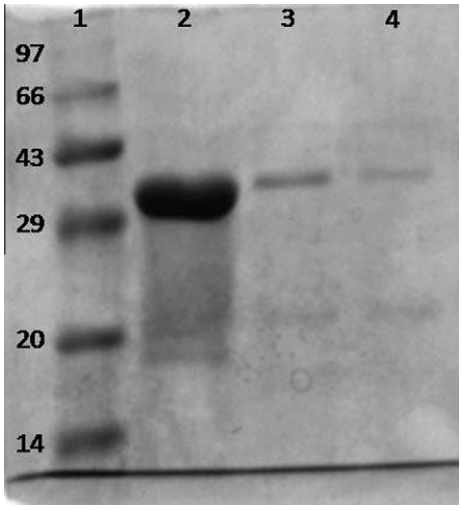

Figure 3 SDS–PAGE illustrates the presence of purified amylase enzyme. Lane 1 shows the position of molecular markers (kDa), lane 2 shows the ammonium sulphate precipitated amylase, lane 3 depicts the crude amylase enzyme, lane 4 illustrates the amylase obtained after dialysis.

to the pH 8 supported both the optimum growth and the amylase activity of *B. barbaricus* culture.

Most of the amylases producing bacterial strains were reported to grow on a wide range of temperatures ranging from 25 to 60 °C [14,9,3]. In the present study, at 35 °C, optimum growth and maximum production of amylase was recorded for *P. chungwhensis* with the activity of 27 U mL^{-1} (Fig. 4B), however, for *B. barbaricus* maximum enzyme activity of 14 U mL^{-1} was recorded at 40 °C (Fig. 4B).

Addition of NaCl into the media strongly influenced the growth and production of amylase in both the cultures. In *P. chungwhensis*, compared to control (10 U mL^{-1}) a 15 times (26.6 U mL^{-1}) increase in activity was observed on addition of NaCl up to 1.5 M. however, the activity gradually decreased on further increase in the NaCl level. (Fig. 4C). In a previous study at a similar NaCl concentration, the amylase activity observed was only 2.4 U mL^{-1}, which was approximately 20 times lower than the activity observed in the present study, however the methodology of estimation was different and even in their study the activity decreased with increased concentration of NaCl [2]. In *B. barbaricus*, the amylase activity was 14.5 U mL^{-1} in 1 M NaCl, and like strain P1 even strain P2 showed decline in activity upon the increase in the concentration of NaCl (Fig. 4C). The amylase activity observed for both the cultures in presence of NaCl, were higher compared to previous studies [2,27]. This variation need to be carefully interpreted since the methodology used in the previous studies (Iodometry) were different from the one used (DNSA) in the present study.

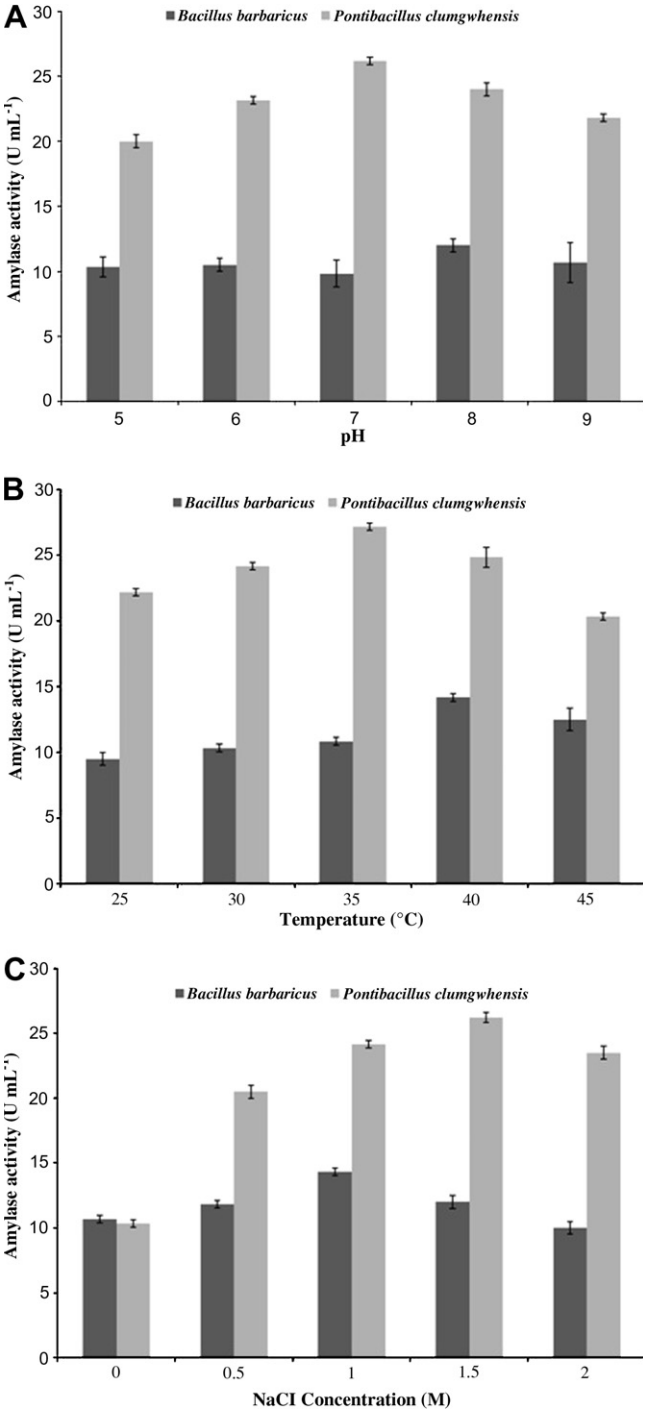

Figure 4 The effect of various stress parameters (temperature, pH and NaCl concentration) on the amylase activity was measured by growing the strains in nutrient broth with appropriate stress factor. The bars indicate the standard deviation of three replicates analysed for each strain. (A) pH (B) Temperature (C) NaCl.

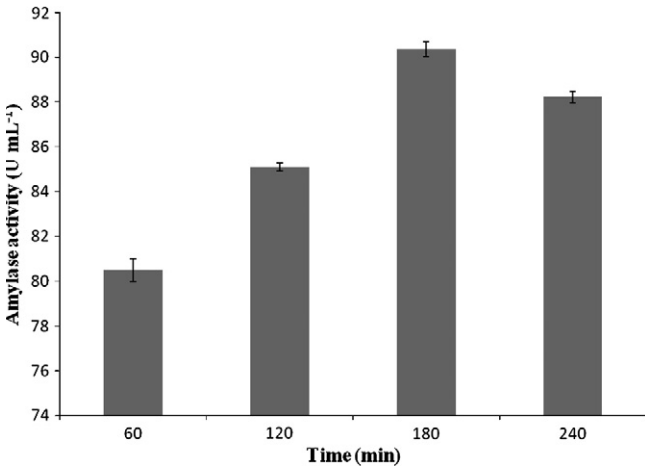

Figure 5 The activity of immobilized amylase enzyme obtained from *Pontibacillus chungwhensis*. The bars indicate the standard deviation of three replicates analyzed.

Finally the amylase activity was estimated under optimized parameters (pH, temperature and NaCl concentration). In the *P. chungwhensis* inoculated flasks, on comparison with the initial activity (25 U mL^{-1}), the amylase activity was approximately doubled (49.6 U mL^{-1}). However, at optimized conditions, there was no significant increase in amylase activity for *B. barbaricus* culture. Previous authors have also reported that optimized cultural conditions highly influenced the growth rate and productivity of amylase [1,37]. Compared to *B. barbaricus*, *P. chungwhensis* was found to be an efficient amylase producing strain.

3.6. Immobilized enzyme assay

The amylase enzyme obtained from the *P. chungwhensis* was immobilized by entrapment in calcium alginate beads and incubated with the substrate solution. The amylase activity was found to be increased with the interaction time of immobilized enzyme and substrate solution. After 180 min of incubation, the maximum amylase activity was found as 90.3 U mL^{-1} was recorded which accounts for 69.6% immobilization efficiency (Fig. 5). In the previous report of Mamo and Gessesse [23] the immobilized cell form showed an increase in amylase activity by 2 U mL^{-1} compared to the free cell , whereas, *P. chungwhensis* immobilized amylase enzyme had shown an increase of 40.7 U mL^{-1} compared to the free enzyme form. This clearly indicated that the direct contact of biocatalyst with substrate increases the activity of amylase. Thus this study raises the possibility of using this immobilized enzyme for potential applications in food, detergent and textile industries.

4. Conclusion

The NaCl tolerant potential differed between the two bacteria. *P. chungwhensis* was able to grow up to the salt concentration of 4 M NaCl, whereas, *B. barbaricus* was observed to grow until 2 M. Through optimization studies *P. chungwhensis* was identified as efficient amylase producer. Significant enzyme activity was observed when enzyme from *P. chungwhensis* was immobilized in 4% sodium alginate beads of 2 mm size. This was the first report on optimization, purification and immobilization of amylase from halotolerant *P. chungwhensis*. Hence we conclude that *P. chungwhensis* can be exploited as a potential source of amylase for commercial production and industrial application. However, optimization of other physicochemical parameters is also necessary to increase efficiency of *in vitro* production of amylase from this bacterium.

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