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Optimization of Cultural Conditions for Production of Alpha-amylase from *Bacillus sp.* under Submerged Fermentation (SmF)

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A B S T R A C T

Current study was designed to study to screen the most efficient amylase producing strains in submerged fermentation, and optimization of the cultural conditions for the optimum production of alpha amylase. The soil sample was collected from the Chikkaballapura district, Karnataka, and screened for amylase producing bacterial strains on nutrient agar plates. Amylase positive bacterial strains were identified and recorded based on the clear zone formation around the bacterial growth. Amylase producing bacteria was grown on the starch production media in submerged fermentation method. Optimization of cultural conditions like incubation period, pH, temperature, salt concentration, carbon & nitrogen sources was carried out. DNS method of enzyme assay was followed and activity was expressed in IU. Study findings delineated that newly isolated bacterial strain *Bacillus sp.* yields maximum enzyme production at 40°C & pH 8.0 when incubated for 48 h under submerged fermentation. Furthermore, optimum amylase enzyme production from *Bacillus sp.* was obtained when starch was used as carbon source and yeast extract as nitrogen source under submerged fermentation. At NaCl concentration of 3.0% highest amylase production was observed under submerged fermentation using newly isolated *Bacillus sp.* Therefore, newly isolated *Bacillus sp.* having potentials of industrial biotechnological applications and hence, it could be recommended for large scale production to exploit industrial purposes.

Introduction

Microbial amylases are of enormous value because of characteristics like bulk production and easy genetic manipulation (Souza and Magalhaes, 2010). Amylases obtained from bacteria are known for greater stability, high productivity and reduced cost of production. The genus *Bacillus* is a major producer of many extracellular enzymes, including amylases. *Bacillus sp.* is a preferred choice for industrial production of microbial enzymes due to attributes such as short fermentation cycle, safe-handling, easy manipulation, consistency, efficient enzyme activity under stress conditions, and eco-friendly characteristics (John and Elangovan, 2013).

The amylase production from different *Bacillus sp.* shows a great deal of variation because the production of amylase depends on the composition of medium and other physical parameters. There is a

growing need for increasing amylase titre without enhancing the overall cost of production. With these background the present study was designed with the main aim of screening the most efficient amylase producing bacterial strains in submerged fermentation, and optimization of the cultural conditions for the optimum production of alpha amylase.

Materials and Methods

Sample Collection and Isolation of Bacteria

Soil sample was collected from the Chikkaballapura district headquarter of Karnataka state. The samples were inoculated onto nutrient agar plates and incubated at 37ºC for 24 h. The colonies obtained after incubation were further sub cultured and preserved under 4ºC for further use (Keshavamurthy *et al.,* 2018).

Screening and Selection of Strain for Optimization

The isolates were screened for alpha-amylase production; bacterial colonies were screened on starch agar medium (g/L: Starch -10.00, Peptone - 5.00, NaCl - 2.00, MgSO4 - 0.20, Agar – 20.00, and pH 9.0). Inoculated plates were incubated at 37°C for 24 hrs. After incubation, the plates were flooded with iodine solution (g/L: Potassium iodide - 2.50, Gram's iodine - 0.125). Amylase positive bacterial strains were identified and recorded based on the clear zone formation around the bacterial growth.

Production of Alpha-amylase by Submerged Fermentation (SmF)

Amylase producing bacteria was grown on the starch production media (g/L: Peptone – 5.00, Starch 10.00, NaCl-2.00, MgSO4-0.20, pH 9.00), and incubated on a rotary shaker for 48 hours at 37°C.

Enzyme was extracted by centrifuging the incubated broth at 5,000 rpm at 4°C for 5 minutes. Supernatant was used as crude enzyme source of amylase.

The experiment was carried out in 250 mL plugged Erlenmeyer flasks, each containing 100 mL sterile starch broth medium and inoculated with 1% of standard inoculum (2.30 × 106 CFU ml^{-1}) for the tested bacterial isolate which was incubated at 50°C on rotary shaker at 150 rpm for 48 h. The fermented medium was centrifuged at 10,000 rpm for 10 min in order to determine periodically the cell dry weight and amylases activity in the precipitate and supernatant respectively (Fossi *et al.,* 2011).

Optimization Studies

Optimization of cultural conditions for the optimum production of alpha-amylase under SmF using newly isolated bacterial strain *Bacillus sp.* was conducted for the following parameters.

Incubation period

To study the effect of incubation period on alpha-amylase production by newly isolated *Bacillus sp.* under SmF was carried out at different incubation periods *viz.* 24 h, 48 h, 72 h, 96 h and 120 h. The optimum incubation time achieved by this step was fixed for subsequent for experiments.

pH

In order to optimize the pH of the fermentation medium, the alpha-amylase activity produced from newly isolated *Bacillus sp.* was measured at different pH of fermentation medium *viz.* 6.00, 7.00, 8.00, 9.00 and 10.0.

Temperature

To study the effect of temperature on alpha-amylase production using newly isolated *Bacillus sp.* in SmF was carried out at different temperatures *viz.* 30°C, 40°C, 50°C and 60°C.

Salt concentration

The effect of sodium chloride (NaCl) on alpha amylase enzyme production using newly isolated

Bacillus sp. under SmF was carried out by adding varied concentrations of NaCl viz. 0.50%, 1.00%, 1.50%, 2.00%, 3.0% to the production media.

Carbon sources

The fermentation medium was prepared with different carbon sources such as arabinose, fructose, glucose, lactose, maltose and sucrose at 1.0% concentration, and assessed for alpha-amylase production.

Nitrogen sources

Different organic nitrogen sources such as ammonium chloride, beef extract, peptone and yeast extract at concentration of 1.0% were incorporated in to the fermentation medium and assessed for their effect on alpha-amylase enzyme production.

Enzyme Assay

The alpha-amylase assay was measured as described by Bernfeld (Bernfeld, 1955). Alpha-amylase activity was assayed in the reaction mixture containing 0.50 mL enzyme, 1mL of 1% starch as substrate, 1mL phosphate buffer and incubated at room temperature for 15 minutes. The reaction was arrested by adding 1mL DNS reagent.

The inactivated reaction mixture was incubated on water bath for 10 minutes and, made up to 10 mL and absorbance was measured at 540 nm. Blank was prepared by immediate addition of 1mL DNS reagent to 0.5 mL enzyme, followed by the addition of 1mL starch and 1mL phosphate buffer. One unit of enzyme activity (IU) was defined as the amount of enzyme that releases 1µmol of reducing sugar (maltose) in 1 min under the assay conditions.

Results and Discussion

Out of five isolates, three bacteria showed the zone of clearance on starch agar media and among three, one isolate was identified as *Bacillus sp.* showed the maximum zone of clearance on the starch agar medium (Figure 1), and hence, it was selected for the further study.

Literature reports evidenced that efficient amylase production in enriched media from *Bacillus subtilis* LKS87 (Park *et al.*, 2014), *Bacillus subtilis* KIBGE HAS (Bano *et al.*, 2011), *Bacillus amyloliquefaciens* P-001 (Deb *et al.*, 2013), *Bacillus amyloliquefaciens* (Abd-Elhahlem *et al.*, 2015) *Bacillus sp.* (Khusro *et al.*, 2017), *Bacillus methylotrophicus* strain PII-2 (Xie *et al.*, 2014), *Bacillus sp.* TM1 (Sajedi *et al.*, 2004), *Bacillus Sp.* YX-1 (Liu and Xu, 2008), *Bacillus cereus* (Kuddus and Ahmad, 2012), *Bacillus sp.* strain TSCVKK (Kiran and Chandra, 2008), alkaliphlic *Bacillus sp.* TS-23 (Chi *et al.*, 2010), and *Bacillus licheniformis* SKB4 (Samanta *et al.*, 2014).

Optimization Studies

Incubation period

The results of the current study revealed that the increase in the alpha-amylase enzyme production was observed from 24 h to 48 h. However, alpha-amylase enzyme production by the newly isolated *Bacillus sp.* was found to be maximum at 48 hrs *i.e.* 153 IU (Figure 2). The results of our study are in accordance with the study conducted by Kumar *et al.*, on α-amylase production by *B. altitudinis* in shake flask for different intervals of time. The production of enzyme was reached maximum at 48 h after inoculation. Further increase in incubation period however, did not show any significant increase in enzyme production rather it was decreased.

This is because the cells would have reached decline phase with lowered enzyme synthesis. It might be also due to the depletion of the nutrients, death phase of organism or due to the production of amylase in the medium (Kumar *et al.*, 2014). This result was also supported by Oyeleke *et al.*, where the optimum incubation period on the yield of amylase enzyme was found at 48 h (Oyeleke *et al.*, 2009).

pH

In the present study, the peak amylase activity was reached at pH 8.0 i.e., 265 IU (Figure 3). Literature reports evidenced that the effect of pH of medium on amylase production by different species of *Bacillus* showed maximum activity by *Bacillus* SMIA-2 at pH 8.5 (de-Carvalho *et al.,* 2008), by *Bacillus subtilis* at pH 7.5 (El-Banna *et al.,* 2007).

Temperature

In our study at 40°C optimum production of alpha-amylase was estimated as 365 IU (Figure 4). These findings were in accordance with various research findings reported by various other researchers. Ashwini *et al.,* found optimum production of amylase by *B. marini* at 40°C (Ashwini *et al.,* 2011).

Oyeleke *et al.,* reported that activity of amylase produced by *B. megaterium* was found to be maximum at 40°C followed by a sharp decrease in amylase activity at 50°C (Oyeleke *et al.,* 2009). This was further supported by Jogezai *et al.,* and Liu and Xu (Jogezai *et al.,* 2011; Liu and Xu, 2008).

Salt concentration

In our study the maximum alpha-amylase production i.e., 610 IU was induced when the media was supplemented with 3.0 % NaCl (Figure 5). This is similar to the results of Vijayabaskar *et al.,* wherein authors also revealed 3% NaCl concentration was suitable for the alpha-amylase production (Vijayabaskar *et al.,* 2012). While Ashwini *et al.,* reported optimum alpha-amylase enzyme yield at 4.5% NaCl concentration (Ashwini *et al.,* 2011). Kokab *et al.,* produced amylase from *B. subtilis* having medium containing 2.0 % concentration of NaCl (Kokab *et al.,* 2003). Furthermore, de-Carvalho *et al.,* delineated that a thermophilic *Bacillus sp.* strain SMIA-2 retained 63.4% enzyme yield in 2.0% NaCl (de-Carvalho *et al.,* 2008).

Carbon sources

In the current study maximum alpha-amylase production from newly isolated *Bacillus sp.* was measured when production medium was supplemented with maltose *i.e.,* 688 IU (Figure 6). Literature findings evidenced that amylase is an inducible enzyme and is generally induced in the presence of carbon sources such as starch, its hydrolytic product, or maltose (Ashwini *et al.,* 2011). Similar finding was observed by Ashwini *et al.,* when amylase production was optimized using different sugars at 1% (w/v) concentration. *B. marini* showed the maximum enzyme activity in the presence of starch as carbon source. Whereas, the minimum enzyme activity was observed in the presence of dextrose (Ashwini *et al.,* 2011). The decrease in the production of amylase enzyme with other carbon sources may be due to catabolite repression. The findings in our study were also in agreement with Rameshkumar and Sivasudha, wherein starch was observed as the best carbon source utilized by the organism (Rameshkumar and Sivasudha, 2011). These findings were further supported by various other others reported in the literature (Goyal *et al.,* 2005; Varalakshmi *et al.,* 2008).

Nitrogen sources

Literature reports evidenced that high yield of enzyme production was obtained when organic nitrogen source compounds were used (Lin *et al.,* 1998). In our study the supplementation of nitrogen sources on amylase production showed that yeast extract was found to be a better nitrogen source for the production of amylase i.e., 665 IU (Figure 7). These findings were in agreement with Guerra and Pastrana and Roohi *et al.,* wherein authors delineated that yeast extract was the best nitrogen source for amylase production, probably due to its high content in minerals, vitamins, coenzymes and nitrogen components (Guerra and Pastrana, 2002; Roohi *et al.,* 2011).

Fig.1 Zone of clearance on starch agar plate *Bacillus sp.*

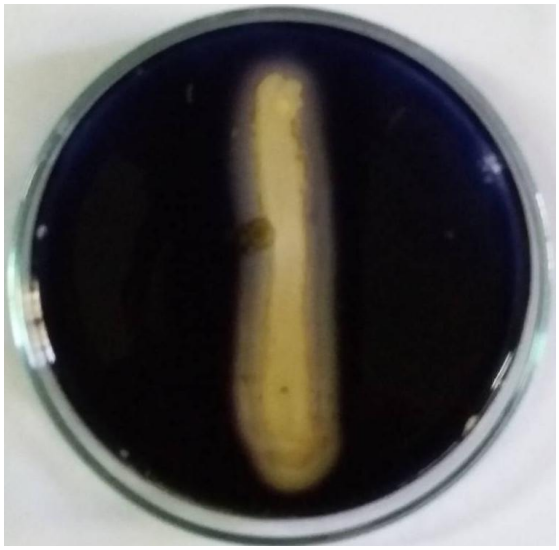


Fig.2 Effect of incubation period on alpha-amylase production

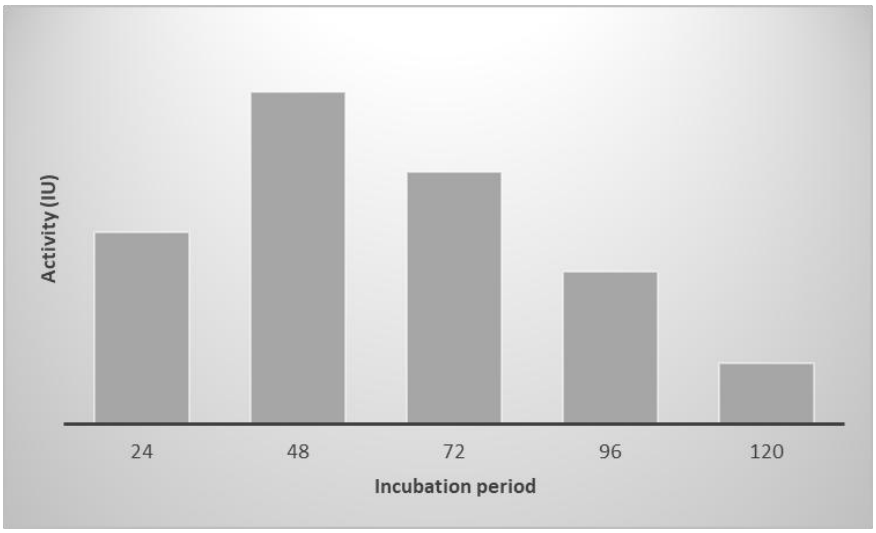


Fig.3 Effect of pH on alpha-amylase production

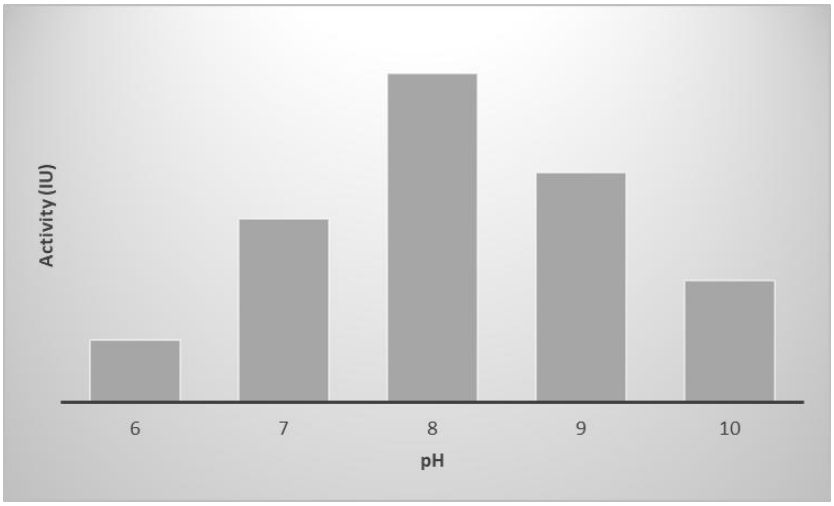


Fig.4 Effect of temperature on alpha-amylase production

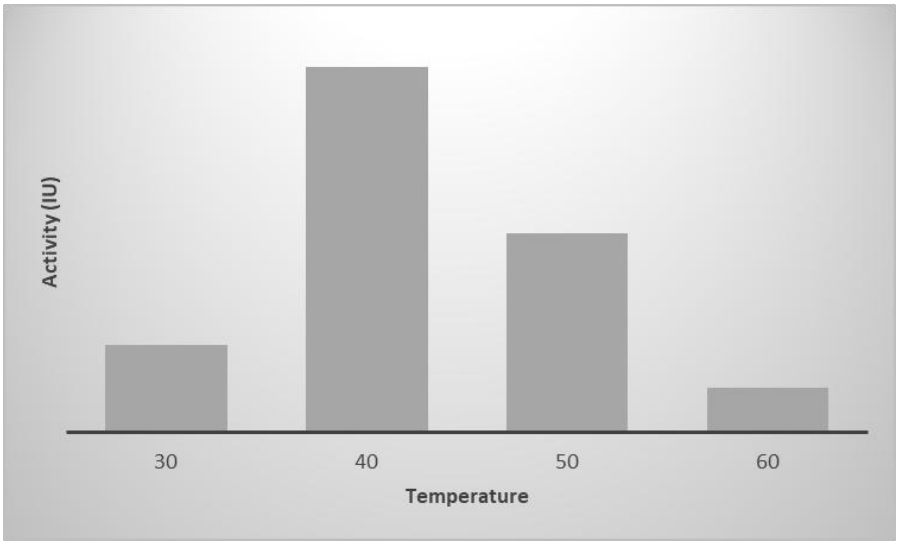


Fig.5 Effect of NaCl on alpha-amylase production

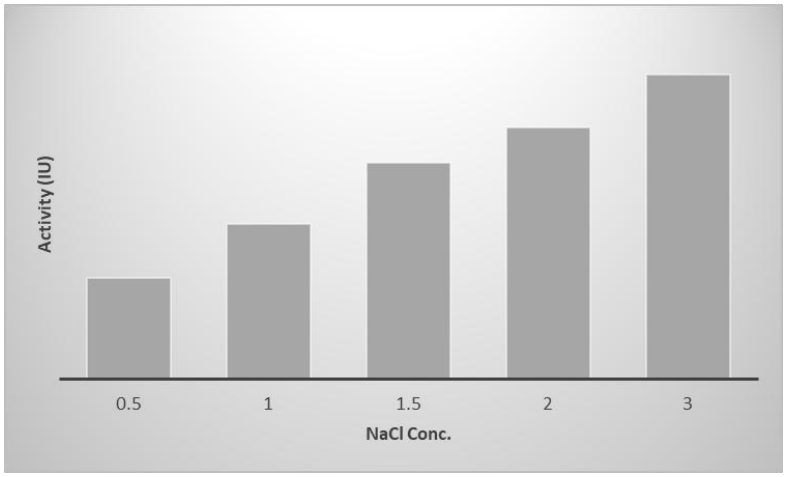


Fig.6 Effect of carbon sources on alpha-amylase production

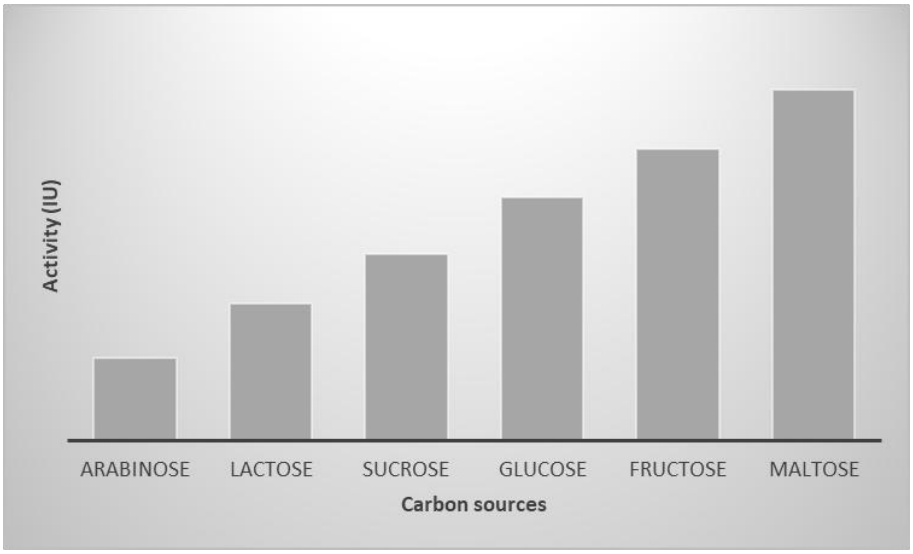
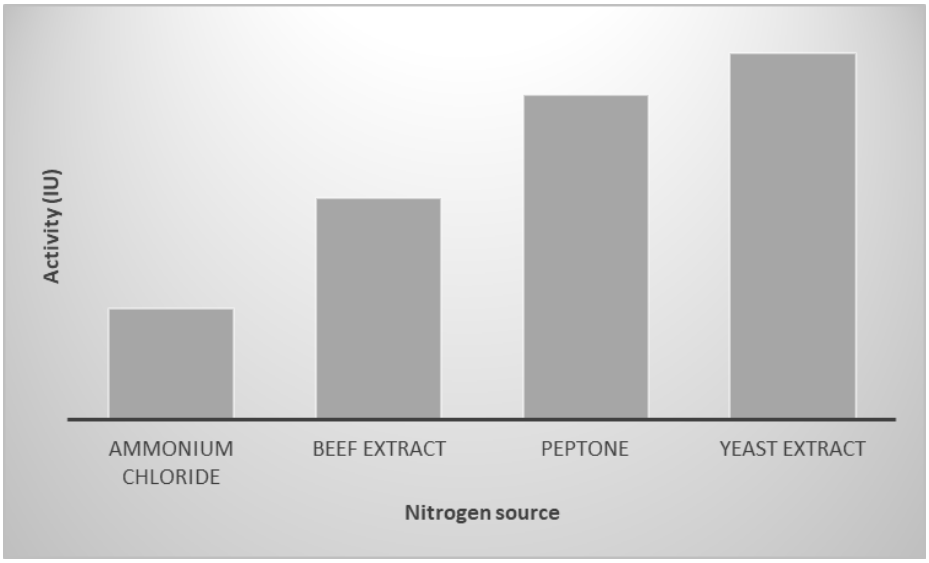


Fig.7 Effect of nitrogen sources on alpha-amylase production



Furthermore, findings of our study are in accordance with the literature reports wherein Narayana and Vijyalakshmi reported maximum yield of amylase activity when yeast extract was used as nitrogen source for *B. stearothermophilus* and *S. albidoflavus* (Narayana and Vijyalakshmi, 2008).

Moreover, Bhattacharya *et al.*, and Santos and Martin also reported the optimum production of amylase by *Bacillus sp.* when yeast extract was used as nitrogen sources (Sourav *et al.*, 2011; Santos and Martin, 2003).

The newly isolated bacterial strain *Bacillus sp.* yields maximum alpha-amylase production at 40°C & pH 8.0 when incubated for 48 h under SmF. Furthermore, optimum alpha-amylase enzyme production from newly isolated *Bacillus sp.* was obtained when starch was used as carbon source and yeast extract as nitrogen source under SmF. At NaCl concentration of 3.0% highest alpha-amylase production was observed under SmF using newly isolated *Bacillus sp.*

Therefore, newly isolated *Bacillus sp.* having potentials of industrial biotechnological applications and hence, it could be recommended for large scale production to exploit industrial purposes.

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