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RESEARCH ARTICLE

OPTIMIZATION OF CULTURAL CONDITIONS FOR α-AMYLASES PRODUCTION FROM *BACILLUS CLAUSII* MCC 233-50

[1]Imrana Khushk, [1]Safia Lashari, [1]Muhammad Aqeel Bhutto, [2]Abdul Nabi Jatt and [1,*]Abdul Sattar Qureshi

[1]Institute of Biotechnology and Genetic Engineering, University of Sindh Jamshoro (76080), Pakistan
[2]Institute of Microbiology, University of Sindh, Jamshoro (76080), Pakistan

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ABSTRACT

In this study, several bacterial strains were isolated from soil samples of Jamshoro district and screened for α-amylase production, *Bacillus clausii* MCC 233-50 was found best amylase producing strain. Fermentation conditions were optimized for amylase production in shake flask using different carbon sources, nitrogen sources, initial pH, and temperature and time period. Amylase production of 593 U/mL was observed after 36 h in mineral medium containing glucose and this increased to 760 U/mL when starch was used as energy source at initial pH 7.0 and 37ºC.Urea was found to be best nitrogen source. Further improvement in final enzyme titer was observed when initial pH and temperature were optimized. Maximal amylase concentration of 1137 U/mL was obtained under optimized cultural conditions. Strain is alkalophilic and thermophilic which makes it suitable for amylase production.

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INTRODUCTION

Amylase is second important hydrolytic enzyme that is used in detergent formulations for both laundry and dishwashing detergents. Amylase breaks down the glucosidic linkage of polysaccharides especially starch and convert it into amylose and amylopectin and finally into maltose and glucose (Hmidet *et al.,* 2009; Carvalho *et al.,* 2008). Amylase (EC 3.2.1.1) is commercially important enzyme used in various industries including detergents, textile, food, conversion of starchy materials to glucose and in paper (Simiar *et al.,* 2017, Bhutto and Dahot, 2010, Gupta *et al.,* 2003). Amylase accounts for 25% of the enzyme market (Carvalho *et al.,* 2008; Burhan *et al.,* 2003). Amylase are classified into two broad classes on the basis of its mode of action as endo-amylase and exo-amylase. Endo-amylase acts in an unsystematic manner at interior of the starch molecules and produces linear and branched oligosacchrides of different chain lengths. However, exo-amylase acts from the non-reducing end and resulting produced the short end product (Gupta *et al.,* 2003). Amylases could be produced from plants, animals, fungi and bacteria (Aquino *et al.,* 2003; Van Der Maarel *et al.,* 2002; Qureshi *et al.,* 2004). Microbial amylase are preferred over other sources and commonly produced from bacteria belonging to the genus *Bacillus* such as *Bacillus megaterium* (Aqeel and Dahot, 2010), Bacillus sp. BCC-01-50 (Simiar *et al.,* 2017), Bacillus sp. BBXS-2 (Qureshi *et al.,* 2016) *B. amyloliquefaciens*, *B.stearothermophilus*, *B. subtilis* and *Bacillus licheniformis* (Sajedi *et al.,* 2005). Characteristics of individual amylase, for example, thermostability, pH stability, stability in presence of organic solvents and detergents varies with fermentation conditions and microbial strain used. Properties of amylase enzyme produced for specific application must match in terms of stability and activity to its real reaction conditions. For example, α-amylases for saccharification of starchy materials should be active and stable in acidic pH (4-5) and high temperature (above 70 °C) but in detergent industry stability and activity at alkaline pH and moderate temperature could be sufficient (Qureshi *et al.,* 2016, Simiar *et al.,* 2017). In our previous study, we have tested crude amylase enzyme in the removal of starchy stains from piece of cloth that suggested suitability of enzyme in local detergent formulation (Qureshi *et al.,* 2016). In another study, amylase enzyme of Bacillus sp was tested for its application in detergents to remove starch stains and conversion of starchy materials to fermentable sugars (Simiar *et al.,* 2017). In this study, fermentation parameters for amylase production were optimized to achieve high amylase titer from *Bacillus clausii* MCC-233-50.

Corresponding autour: **Abdul Sattar Qureshi**
Institute of Biotechnology and Genetic Engineering, University of Sindh Jamshoro (76080), Pakistan.

MATERIALS AND METHODS

Microorganisms: Screening and Identification

Bacterial strains were isolated from soil and grown in synthetic medium containing glucose 20 g/L, peptone 5 g/L, yeast extract 5g/L and NaCl 5 g/L for 24 h. Each strain was sub cultured to obtain pure culture. Pure cultures were screened on starch agar medium to observe amylase production. Inoculated plates were incubated in static condition 37 °C for 48 h. After 48 h, gram iodine solution was flooded on the plates to observe clear zone around microbial colonies. Best culture was examined for various morphological and biochemical characteristics as per Berger's Manual of Determinative Bacteriology. Microorganism was identified as Bacillus clausii that was best amylase producer on starch agar plate and fermentation experiments. This strain was used in all fermentation experiments.

Cultivation conditions

Bacillus clausii MCC 233-50 was activated by growing on synthetic medium containing glucose 20 g/L, peptone 5 g/L, yeast extract 5 g/L and NaCl 5 g/L incubated for 24 h in shaking incubator at 37 °C. Then, 5.0 % v/v culture was transferred to fresh medium and inoculated for 15 h at 37 °C. 1.0 % v/v of culture was used as inoculum in final fermentation step, fermentation medium composition was slightly modified as reported in Simiar *et al.,* 2017. Fermentation medium contained 20 g/L of glucose, 10 g/L of peptone, 1 g/L of $MgSO_4 \cdot 7H_2O$ and 2 g/L of KH_2PO_4. Flasks were incubated for 72 h at 37 °C initial pH was adjusted to 7. The culture broth was centrifuged at 12,100g for 10min to collect supernatant and was used for biochemical analysis of α-amylase activity, total protein, reducing sugars, and total sugars. All media were sterilized at 121 °C, 15 lb/inch^2 pressure for 20 minutes prior to inoculation.

Optimization of culture conditions

Fermentation conditions including carbon and nitrogen source, pH and temperature were optimized to maximize the amylase concentration. Effect of different sugars (starch, sucrose, glucose, galactose, lactose and molasses) was checked on bacterial growth and amylase production. The inoculated flasks were incubated in shaker incubator at 37 ± 2ºC for 36 h. Effect of different nitrogen sources by replacing peptone with urea (NH_2–CO–NH_2), sodium nitrate ($NaNO_3$), ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) was evaluated. Effect of pH was evaluated by changing initial pH of synthetic medium with 1 M H_2SO_4 or 2 M NaOH from 5-10. Effect of temperature (30-60 °C) was tested for bacterial growth and amylase production. Culture was incubated for 36 h in shaking incubator.

Assay of α-amylase activity

α-amylase was determined by spectrophotometric method adopted from Simiar *et al.,* 2017. Briefly, 1 mL of crude enzyme solution was mixed with 1 mL of substrate (prepared by dissolving 1% w/v of soluble starch in distilled water). Reaction was allowed at 37 °C for 15 min. Then 2 mL of dinitrosalicylic acid (DNS) was added in all test tubes and placed in boiling water bath for 10 min. Then, tubes were cooled and read against control at 540 nm. Control were prepared by replacing sample or substrate with 1 mL of distilled water and 2 mL of DNS was added (same as for test sample). One unit of α-amylase activity was defined as amount of enzyme required to release 1 µ mol of reducing sugars under the assay conditions.

RESULTS AND DISCUSSION

Amylase is industrially important enzymes that contributes to 25% of enzyme market. Its increasing demands could be fulfilled from microbial amylase production. Hyper amylase producing strains those could grow at alkaline pH and high temperature could certainly produce enzymes with industrial characteristics. In our previous studies, we have reported amylase production from bacterial and fungal strains. Aqeel and Dahot (2010) have evaluated amylase production from locally isolated *Bacillus megaterium*. Qureshi *et al.,* (2004) evaluated submerged fermentation conditions for amylase production from fungal strains. We tested amylase production from *Mucor geophilus*, *Mucor lilicinum*, *Aspergillus niger* and *Aspergillus fumigates* alone and mixture of different strains. Results suggested that amylase production rate was higher when *Aspergillus niger* and *Aspergillus fumigatus* were mixed.In another study, Simiar *et al.,* 2017 tested amylase production from Bacillus sp BCC 01-50 and suggested that optimization of fermentation conditions plays significant role in enhancing the enzyme titer.

In this study, several bacterial strains were isolated from soil samples of Jamshoro district and screened for α-amylase production. On the basis of clear zone formation some strains were selected and tested for ferment ability. *Bacillus clausii* MCC 233-50 was found best amylase producer on starch agar plates and submerged fermentation conditions (data not shown). *Bacillus clausii* was used as amylase fermenting strain in this research. Figure 1 shows the fermentation profile of amylase from *B. clausii*, amylase concentration increased with fermentation time and reached maximum (593 U/mL) after 36 h on further incubation, amylase yield decreased probably due to depletion of nutrients, decrease in pH and accumulation of waste products (Simiar *et al.,* 2017). In the next phase, effect of different carbon sources was evaluated. Amylase concentration was highest when starch was added in mineral medium as carbon source (760 U/mL), this happened probably due to inducing effect of starch (Figure 2).

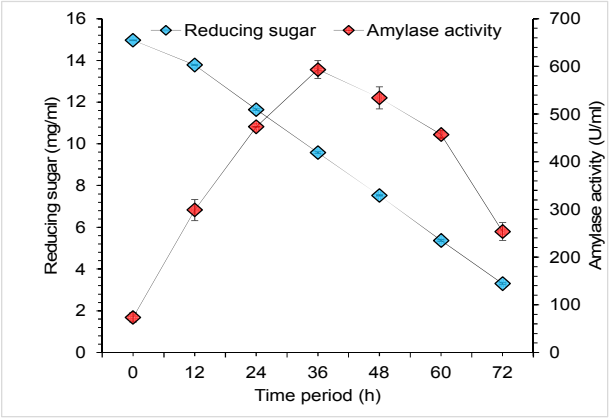

Figure 1. Effect of time period on production of α-amylase and consumption of reducing sugars by *Bacillus clausii* MCC-233-50 in synthetic medium containing glucose 20 g/L, peptone 10 g/L, $MgSO_4 \cdot 7H_2O$ 1.0 g/L, KH_2PO_4 2 g/L and incubated in shaking incubator at 37 °C with initial pH 7.0. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation

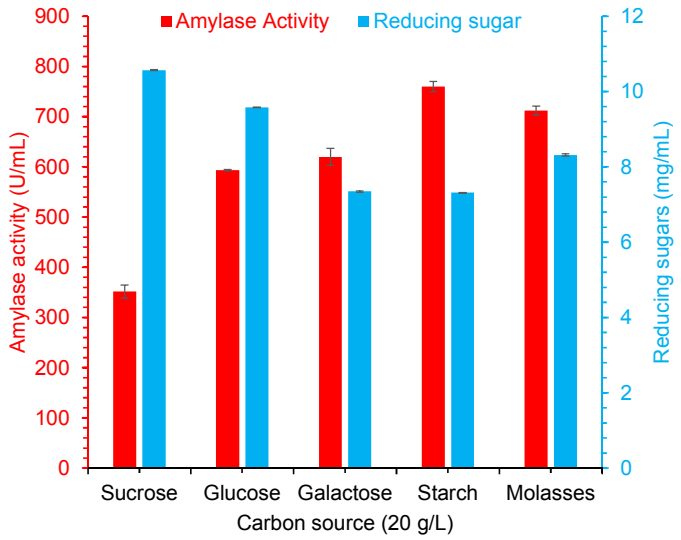

Figure 2. Effect of carbon sources (20 g/L initial concentration) on amylase production from *Bacillus clausii* (37 °C, initial pH of 7.0 for 36 h). The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation

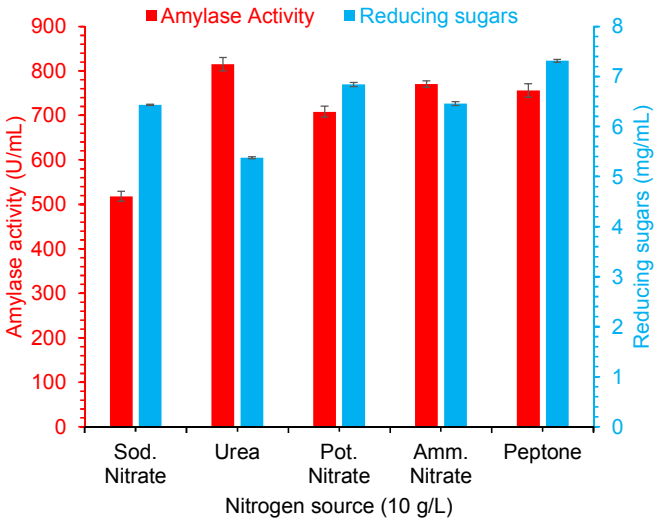

Figure 3. Effect of nitrogen source (10 g/L initial concentration) on cultivation of *Bacillus clausii* for amylase production. Experiments were performed in a mineral medium containing starch (20 g/L), and culture was incubated at 37 °C for 36 h. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation

Effect of nitrogen sources on amylase production is shown in Figure 3. Amylase production varied with nitrogen source and maximum amylase concentration of 815 U/mL was observed when urea was supplemented as nitrogen source. Initial pH of medium is most important parameter for microbial growth and enzyme production. Amylase yield increased with increase in pH upto 8 pH and then declined. Maximal amylase production of 911 U/mL at pH 8 suggest alkalophilic nature of strain (Figure 4). Similar results are reported in literature for amylase production in alkaline pH condition. Simiar *et al.,* 2017 carried out amylase production in synthetic medium at pH 8. Saleem and Ebrahim 2014 also report amylase production from fungal strain in alkaline pH condition (pH 8.0). Amylase of our strain could be added in detergent formulations, further experiments are required to understand the stability ans suitability of crude enzyme.

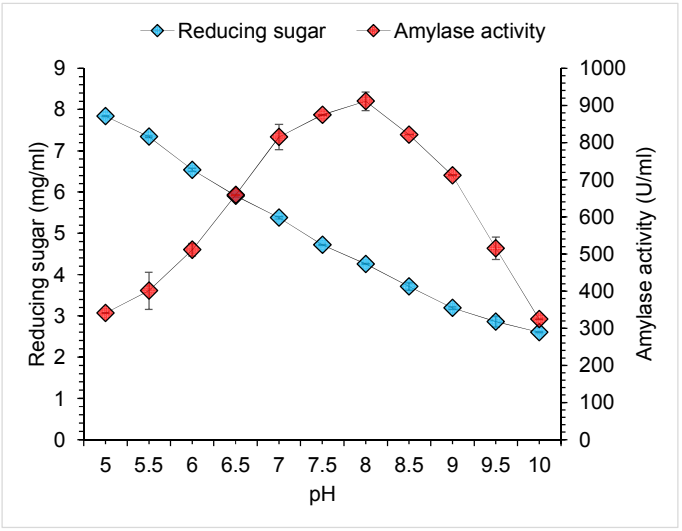

Figure 4. Effect of initial pH on amylase production at 37 °C for 36 h in a mineral medium containing starch (20 g/L) and urea (10 g/L) as carbon and nitrogen sources, respectively. The experiments were performed in triplicate and data presented in figures are average of three parallel experiments. Error bars are shown for standard deviation

Fermentation temperature was optimized for amylase production, amylase concentration increased with temperature upto 45 °C then decreased probably due to denaturation of enzyme and decrease in bacterial growth at higher temperature Simiar *et al.,* 2017; Vijayaraghavan *et al.,* 2015). Maximal amylase concentration of 1137 U/mL was obtained under optimized fermentation conditions. Results suggest great potential of strain for amylase production from locally isolated strains.

Conclusion

In this study, several bacterial strains were isolated from soil samples of Jamshoro district and screened for α-amylase production, *Bacillus clausii* MCC 233-50 was found best amylase producing strain. Fermentation conditions were optimized for amylase production in shake flask using different carbon sources, nitrogen sources, initial pH, and temperature and time period. Amylase production of 593 U/mL was observed after 36 h in mineral medium containing glucose this increased to 760 U/mL when starch was used as energy source at initial pH 7.0 and 37 ºC. Urea was found to be best nitrogen source. Further improvement in final enzyme titer was observed when initial pH and temperature were optimized. Maximal amylase concentration of 1137 U/mL was obtained when cultural conditions were optimized. Strain is alkalophilic and thermophilic which makes it suitable for amylase production.

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