Research Article

Optimization of Process Parameters for the Enhanced Biosynthesis of α-Amylase by *Bacillus subtilis*

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Abstract | The α-amylases are primary industrial amylases with the expected global market to grow at a CAGR (Compound Annual Growth rate) of 7 % during 2021-2028. Due to widespread industrial applications, alpha-amylases are of continuous research focus. The present work depicts the enhanced alpha amylase bioproduction by *Bacillus subtilis* by optimizing the solid state fermentation (SSF) parameters. In this context, the fermentation rate for optimal alpha-amylase production was optimized using a solid substrate, wheat bran. Optimal enzyme production (2.1±0.15 U/ml) was achieved with 48 hours fermentation period. A considerable increase in enzyme activity (4.5±0.031 U/ml) was observed when diluent with initial pH of 7 was used. Among different carbon sources (maltose, glucose, starch, galactose, arabinose and sucrose) best results were obtained with soluble starch. Alpha-amylase production is sensitive to the supply of nitrogen sources. In this context, both inorganic and organic nitrogen sources, were evaluated. Optimal enzyme production was obtained with diluent supplemented with $NaNO_3$ (0.25%) and peptone (0.25%) as inorganic and organic sources, respectively. Among the different metal ions evaluated, Ca^{2+} gave the maximum activity of alpha-amylase (25.4± 0.73 U/ml).

Received | July 21, 2022; **Accepted** | October 19, 2022; **Published** | December 12, 2022
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Citation | Hameed, U., A. Liaqat, M.A. Khan, I. Haq and M. Aslam. 2022. Optimization of process parameters for the enhanced biosynthesis of α-amylase by *Bacillus subtilis*. *Journal of Innovative Sciences*, 8(2): 285-292.
DOI | https://dx.doi.org/10.17582/journal.jis/2022.8.2.285.292
Keywords | Amylases, Alpha-amylases, Wheat-bran, Solid state fermentation, *Bacillus*

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1. Introduction

Starch is an important macromolecule and the main source of carbohydrates in food. Chemically, it is a glucose polymer with amylose and amylopectin as structural components. Amylose, which makes up approximately 20% of starch, is made up of linear alpha-1, 4 linked glucose units. Whereas, amylopectin is a highly branched glucose polymer with alpha-1, 4 linked residues and alpha-1, 6 linkage at the branching point. It makes up about 80% of starch. Starch hydrolysis is a key process in many industries that is carried out preferably by amylases (Wang *et al*., 2022; Chorfa *et al*., 2022; Gan and Evers, 2022; Fuentes-Zaragoza *et al*., 2010). Amylases are categorized into beta amylases with EC (Enzyme Commission number) 3.2.1.2, glucoamylases (EC: 3.2.1.3) and alpha amylases with (EC: 3.2.1.1).

Alpha amylases break down alpha-1, 4-glycosidic linkages, thus, resulting in the conversion of glycogen and starch and related polysaccharides into a range of products from monosaccharides (glucose) to low molecular weight oligosaccharides including maltose and maltotriose units, maltodextrin derivatives or cyclodextrins (Gupta *et al*., 2003; Rajagopalan *et al*., 2008).

Starch hydrolyzing enzymes i.e., amylases are considered to be quite versatile and have numerous biotechnological applications. Alpha-amylases are the major shareholder in the world market occupying 25-30% of the market (Chavez-Camarill *et al*., 2022; Hallol *et al*., 2022). It has extensive applications in textile, paper, animal feeds, environment, biofuel, detergent and clinical., health care, pharmaceutical and most importantly the food processing industry (Gavrilescu and Chishti, 2005; Couto and Sanroman, 2006; Ghorai *et al*., 2009; Al-Dhabi *et al*., 2020; Chavez-Camarill *et al*., 2022; Balakrishnan *et al*., 2021; Hallol *et al*., 2022).

Alpha-amylases are produced naturally in plants, insects, animals, and microorganisms (Balakrishnan *et al*., 2021). However, enzymes obtained from microbes (bacterial sources) have significant industrial applications (Kandra, 2003; Gupta *et al*., 2003; Reddy *et al*., 2003; Al-Dhabi *et al*., 2020). Members of genus *Bacillus* such as *B. subtilis, B. circulans, B. amyloliquefaciens, B. megaterium*, *B. stearothermophilus*, and *B. licheniformis* are the leading producer of commercial alpha-amylases (Konsoula and Liakopouloukyriakides, 2007; Prakash and Jaiswal, 2009; Luang-In *et al*., 2019). The rod-shaped *B. subtilis* (hay bacillus) is a catalase-positive and gram-positive soil bacterium. It is capable of producing a rigid, protective endospore which allows it to tolerate unusual environmental conditions. It produces thermostable enzymes that decrease the risk of contamination and exhibit increased the diffusion rate in reaction (Peixoto *et al*., 2003; Madigan and Martinko, 2005; Pant *et al*., 2015).

Two main fermentation setups are SmF (Submerged fermentation) and SSF (solid-state fermentation) which are utilized for alpha-amylase production. SmF has long been used for enzymes production because the different reaction parameters such as pH, temperature, aeration, moisture and oxygen transfer are easily controlled in SmF (Couto *et al*., 2006; Gangadharan *et al*., 2008; Balakrishnan *et al*., 2021; Hallol *et al*., 2022). Yet, many researchers prefer SSF for enzyme production (Tanyildizi *et al*., 2007; Couto *et al*., 2006). SSF has the natural advantages of high volumetric productivity, better product recovery, reduced level of catabolite repression, low capital investment, less usage of water, and the utilization of agricultural industrial wastes which reduces pollution problems and less effluent generation. Moreover, SSF also resembles the native habitat of microorganisms and thus, is a preferable choice for the growth of fungal, yeast and bacteria as well as the biosynthesis of valuable products (Pandey *et al*., 1999, 2000; Sivaramakrishnan *et al*., 2006; Singhania *et al*., 2009; Chimata *et al*., 2010).

The development of a successful fermentation process is directly dependent on the optimization of fermentation conditions. Thus, much attention has been shifted towards the optimization of culture medium as it is one of the main strategies for achieving maximum enzyme yield. Enzyme yield is also dependent on microbial strain, incubation time, cultivation technique, optimal pH and temperature, nutrients and cultivation temperature (Al-Dhabi *et al*., 2020). As these conditions and requirements vary from strain to strain so, it is crucial to optimize these process parameters to evaluate the amylase-producing potential of the individual strains.

Pakistan has a wide range of agricultural resources such as rice straw, rice husk, soybean meal, wheat straw and wheat bran, etc. which have tremendous potential as a solid substrate for commercially significant enzymes bioproduction. Therefore, the present project is focused on optimizing batch fermentation parameters of *B. subtilis* for the utilization of the locally available and cheap substrate to synthesize alpha-amylase via SSF.

2. Materials and Methods

2.1 Bacterial strains

Bacillus subtilis was obtained from stock culture of PCSIR labs, Lahore and was maintained on the nutrient agar medium.

2.2 Solid state fermentation (SSF)

2.2.1 Inoculum preparation

Overnight grown inoculum of *B. subtilis* (at 37°C and 200 rpms) in nutrient broth (pH 6.8) was used.

2.3 SSF technique

The batch fermentation was carried out in a 250 ml flask for the bioproduction of alpha-amylase. Diluent (10 ml) was transferred into the flask containing wheat bran (10 g), was mixed well and sterilized using an autoclave at 121 ºC and lbs/in^2 for 15 minutes. Inoculated the flasks aseptically with 1.0 ml of vegetative inoculum after cooling to 30 ºC and incubated at 37±1 ºC for two days. All the Experiments were run in duplicate.

Diluent composition: (%, w/v) glucose 0.5, $(NH)_2SO_4$ 0.25, Urea 0.25 and KH_2PO_4 0.5 pH 6.5.

2.4 Enzyme extraction

Distilled water (50 ml) was added to each flask after an incubation period of 48 h followed by rotation on a rotary shaker for 2 h set at 150 rpm and 37±1ºC. The slurry was filtered and centrifuged at 4ºC at 8000 rpm for 10 minutes to separate fermented substrate, spores, cell mass, and small particles. The supernatant obtained was for enzyme assay.

2.5 Enzyme analysis

For analysis of alpha-amylase was performed by Rick and Stegbauer's Method (1974). The reaction mixture containing 0.5 ml of extracted enzyme and 0.5 ml of starch solution (1.0% solution prepared in acetate buffer of pH 5.0) was incubated for 30 minutes at 50°C. Reducing sugar estimation was carried out by the dinitrosalicylic acid (DNS) method after Miller (1959) using a standard curve of maltose.

One enzyme unit is the protein amount which in 10 minutes hydrolyses 1.0 µM reducing sugar (maltose) from 1.0% Lintner's soluble starch per min per ml under standard assay conditions.

2.6 Statistical analysis

Computer software Costat was used for statistical analysis of the results of all the experiments that were run in duplicate.

3. Results and Discussion

3.1 Time course study for the bioproduction of alpha-amylase

Optimizing incubation time is quite critical for the optimal growth of bacterial culture (Lulko *et al.*, 2007). The alpha-amylase production takes place only in cells in which normal cellular multiplication no longer occurs (Yamaguchi *et al.*, 1974; Nomura *et al.*, 1956). Therefore, a research study consisting of 24-96 hours was carried out for alpha-amylase production by *B. subtilis*. As shown in Figure 1, enzyme productivity increased with increasing incubation time while the maximum productivity (2.1±0.15 U/ml) was obtained at an incubation time of 48 hours after inoculation. This is because *B. subtilis* enters the stationary phase after 48 hours and produce maximum alpha-amylase. After 48h of incubation, a rapid decrease in alpha-amylase production was observed due to nutrient depletion and accumulation of metabolites and toxic substances in the fermented bran (Chamber *et al.*, 1999; Gangadharan *et al.*, 2006). These findings are similar to the work of other researchers (Riaz *et al.*, 2003; Haq *et al.*, 2005). Hence, the incubation period of 48 hours was considered optimal for alpha-amylase production by *B. subtilis*.

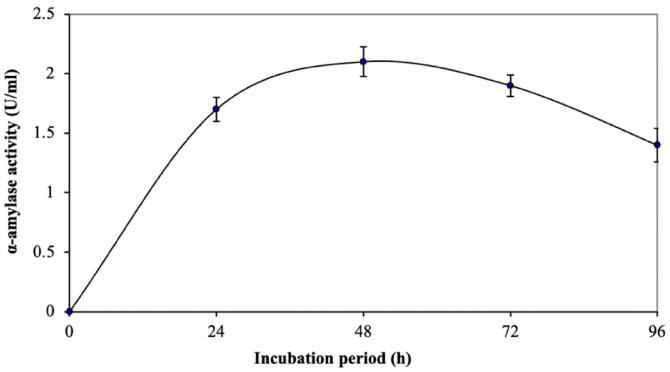

Figure 1: Time course study for α-amylase bioproduction.
The standard deviation (±SD) among replicates is represented as Y-error.

3.2 Selection of diluent's initial pH of diluent for the bioproduction of alpha-amylase

Figure 2 shows the effect of the diluent pH (5.5-8.5) on alpha-amylase bioproduction by *B. subtilis*. It was noted that with increasing pH, alpha-amylase production increased gradually. The maximum enzyme production (4.5±0.31 U/ml) was obtained at neutral pH. Further increase in pH declined the enzyme production by *B. subtilis*. It is because the optimum pH requirement for the growth of bacteria is neutral pH (7). At acidic pH, enzyme activity was less because it resulted in poor bacterial growth. Similar findings are reported by others as well (Haq *et al.*, 2005; Syu and Chen, 1997; Tanyildizi *et al.*, 2005).

3.3 Selection of carbon sources for the bioproduction of alpha-amylase

In addition to the cultivation method, alpha-amylase

production is greatly affected by essential nutrients among which carbon source is most noticeable. In the present work different mono and polysaccharides; glucose, soluble starch, maltose, sucrose and arabinose were used for optimal alpha-amylase production by *B. subtilis* (Figure 3) and the maximum production of alpha-amylase (9.5±0.43 U/ml) was obtained with soluble starch (0.25%, w/v). It is because starch as the substrate of alpha-amylase is the best inducer. Similar results are reported by Rajagopalan *et al.* (2010). However, glucose and arabinose gave the least production i.e., 3.5±0.6 and 3.1±0.23 U/ml, respectively. While contradictory results are reported by Anto *et al.* (2006) who discussed maximum amylase production in the glucose-supplemented culture medium.

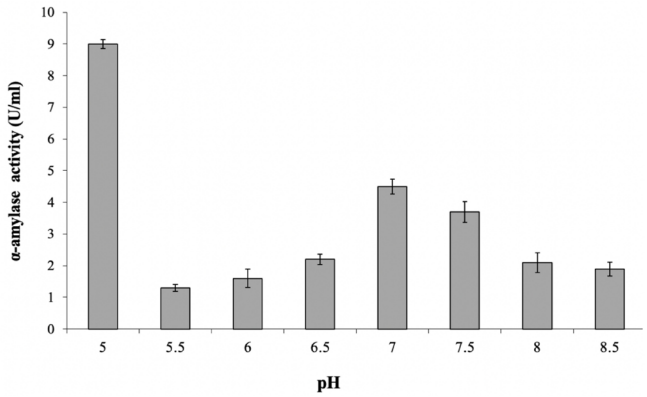

Figure 2: Selection of diluents initial pH for α-amylase bioproduction.
The standard deviation (±SD) among replicates is represented as Y-error.

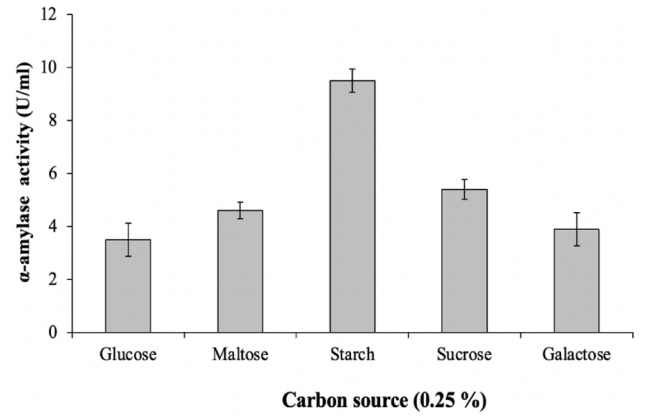

Figure 3: Selection of carbon source for α-amylase bioproduction.
The standard deviation (±SD) among replicates is represented as Y-error.

3.4 Selection of nitrogen sources for the bioproduction of alpha-amylase

Nitrogen sources (organic and inorganic) are known to enhance alpha-amylase production in SSF system (Pederson and Nielson, 2000). Figure 4 depicts the effect of various inorganic nitrogen sources i.e., ammonium citrate, $(NH_4)_2SO_4$, NH_4SO_4, NH_4NO_3, NH_4Cl_2 and $NaNO_3$ on the production of alpha-amylase (Figure 4). The maximum enzyme production (15.4±0.92 U/ml) was achieved when the diluent was supplemented with $NaNO_3$. On the contrary, Aiyer (2004) reported different findings; optimal alpha-amylase production using NH_4Cl_2 as an inorganic nitrogen source. Five organic nitrogen sources (Tryptone, peptone, urea, casein and skimmed milk) were also tested for alpha-amylase fermentation (Figure 5). Among all of them, peptone gave maximum enzyme production (19.7±1.2 U/ml). On the contrary, another study reported a marginal rise in the bioproduction of alpha-amylase with the addition of peptone (Gangadharan *et al.*, 2008).

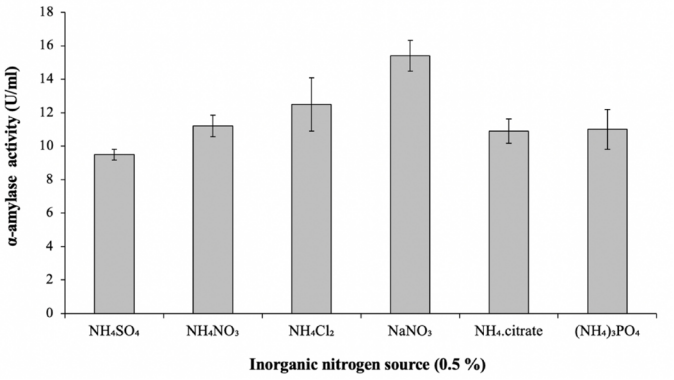

Figure 4: Selection of inorganic nitrogen source for α-amylase bioproduction.
The standard deviation (±SD) among replicates is represented as Y-error.

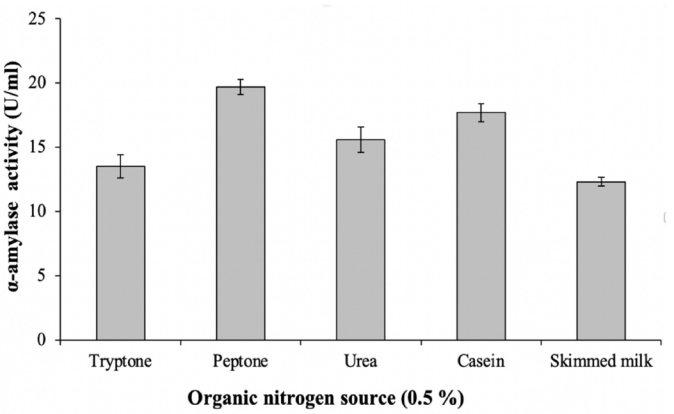

Figure 5: Selection of organic nitrogen source for α-amylase bioproduction.
The standard deviation (±SD) among replicates is represented as Y-error.

3.5 Effect of metal ions on the bioproduction of alpha-amylase

The effect of four different metal ions on alpha-amylase production was studied (Figure 6). In this context, diluent was supplemented with $ZnCl_2$, $CaCl_2$, $MgSO_4$ and $MnCl_2$ individually. The enzyme production was maximum (25.4± 0.73 U/ml) when $CaCl_2$ (0.5 %) was added to the diluent. It is due to the reason that Ca^{2+} ions are essential for the activity and stability of alpha-amylases (Hashemi *et al.*, 2010). Similar findings are also described by others (Kokab *et al.*, 2003; Hashemi *et al.*, 2010).

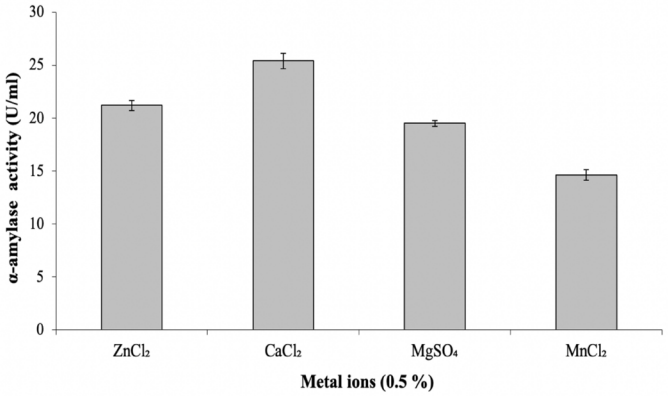

Figure 6: Effect of metal ions on the bioproduction of α-amylase.
The standard deviation (±SD) among replicates is represented as Y-error.

Conclusions and Recommendations

The cultural conditions and nutritional necessities of *Bacillus subtilis* for the optimal biosynthesis of alpha-amylase via SSF are optimized successfully. The optimal amylase bioproduction was attained when the fermentation was carried out for 48 h at 37°C using 10 g wheat bran. Alpha amylase production was improved by using the diluent having pH 7. Maximum alpha-amylase production (25.4±0.73 U/ml) was achieved when diluent was supplemented (%, w/v) with soluble starch 0.25, NH_4NO_3, peptone 0.25 and $CaCl_2$ 0.5. However, the production of alpha-amylase can be further improved by mutation prior to scale-up studies.

Acknowledgments

We acknowledge PCSIR, Laboratories, Lahore for providing the bacterial strain and facilities.

Novelty Statement

we have reported the optimized conditions for the alpha-amylase biosynthesis by *B. subtilis* that resulted in a 12.1-fold (1109.5% increase) in enzyme productivity.

Author's Contribution

UH, AL, MAK: Design of design, experimental work, data analysis and manuscript writing.
MA: Manuscript preparation.
IH: Approval of the experiment design.

Conflict of interest

The authors have declared no conflict of interest.

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