Optimization of physicochemical parameters for maximum amylase production by indigenously isolated *Bacillus cereus* AS2 strain

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Abstract: Amylases are enzymes that catalyze the hydrolysis of starch into highly valuable products of economic significance. Amylases are used extensively in various industrial sectors. Microbial sources particularly *Bacillus* species are well known for the cost effective commercial production of amylase enzyme. Present study focuses on the enhancement of amylase enzyme production from an indigenously isolated *Bacillus cereus* AS2 strain via one variable at a time (OVAT) optimization of different physical and chemical factors. Purposely, eight parameters possibly affecting the amylase production including temperature, pH, incubation time, inoculum size, substrate concentration, metal ions, carbon and nitrogen sources were investigated. According to the results, amylase production was significantly boosted at maximum when the *Bacillus cereus* AS2 was grown at 45°C on pH 7.0 for 72 hours in the medium supplemented with 4% starch and 0.5% glycine. Among the different metal ions tested, $CaCl_2$ (0.05%) was found significant to accelerate extracellular amylase production.

Keywords: *Bacillus cereus*, amylase, extracellular, physicochemical parameters, optimization.

INTRODUCTION

Amylases in general, catalyze the hydrolysis of glucosidic linkages of starch subunits to produce varied sized oligosaccharides. Amylases are extracted from different origins like microorganisms, plants and animals, but microbial source are often favored due to vast availability, plasticity and cost effectivity in the production (Serin *et al.*, 2012). Among the microbial sources, members of genus *Bacillus* are used significantly for the production of amylase commercially. Extracellular protein secretion, enzymes with desired properties and high activity at extreme conditions (temperature, pH, pressure, osmolarity etc.), environment-friendly, short fermentation period, cost effectiveness and safe-handling has made members of this genus most important for commercial production of enzymes and a large number of fine bio chemicals. About 60% of commercially available enzymes are being produced by *B. cereus*, *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquifaciens*. Amylases offer a vast range of applications like fermentation, baking, brewing, food, beverages, paper, detergent and textile industry and bio fuel production (Ahlawat *et al.*, 2009). Nowadays, amylases are also of great significance in the sludge treatment processes for reduction of solid content and pre-treatment of animal feed to enhance its digestibility (Regulapati *et al.*, 2007; Saxena *et al.*, 2007).

Optimization of the conditions for fermentation is central to the enhancement in the enzyme production. Physio-chemical parameters such as temperature, pH, fermentation period, inoculum size, carbon and nitrogen concentrations, metal requirements etc. are of key importance in enzyme production processes. Although different *Bacillus* species share a common growth pattern and enzyme profile but at strain level their optimized fermenting conditions may vary, therefore it is very challenging to obtain a strain producing enzyme that can meet industrial demands. Thus, present study was carried out to maximize the yield of amylase enzyme by optimizing the physical and chemical parameters during fermentation from indigenously isolated *Bacillus cereus* AS2.

MATERIALS AND METHODS

Bacterial source for amylase production

Eighty nine (89) different bacterial strains were isolated from garden soil samples. On the basis of morphological, biochemical and cultural characteristics, 39 were identified as the member of the genus *Bacillus*. These strains were screened qualitatively for extracellular amylase production and five strains with enzyme index greater than 1.8 were selected for quantitative analysis of amylolytic potential. Finally, *Bacillus* AS2 was selected because of its maximum amylase producing potential (3179.6 IU/ml/min) (Rehman and Saeed, 2015). The selected *Bacillus* AS2 strain was also subjected to molecular identification by the amplification of 16s rDNA through real time PCR and gene sequencing.

Fermentative production of amylase

Bacillus AS2 was inoculated in L.B broth (Luria Basal) and incubated at 45°C for 24 hrs. Overnight seed culture (8%) was introduced into flasks containing 20 ml fresh L.B broth and incubated with continuous agitation (150

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rpm) for 24 hrs at 45°C. After 24 hrs, broth was centrifuged at 4000 rpm for 15 min (at 4°C). The cell free culture supernatant (CFCS) was further used in enzyme assay as a crude enzyme solution.

Enzyme assay

Enzyme activity was determined according to the method of Bernfeld (1955). Enzyme production was quantified by the amount of maltose (reducing sugar) which is produced by the activity of enzyme on the substrate i.e., starch. Reaction mixture for determining the enzyme activity was prepared by adding 100µl of soluble starch (in 20mM sodium phosphate buffer) as a substrate to 100µl of CFCS (as a crude enzyme solution). This reaction mixture was kept for 15 min at 50°C. In order to stop the reaction 100 µl of 3, 5-di nitro salicylic (DNS) reagent (96mM) was added, followed by boiling for 15 min. It was cooled to room temperature and distilled water (900µl) was added. By using the spectrophotometer, absorbance was recorded at 540 nm. Enzyme units (IU/ml/min) were calculated using Standard Maltose Curve (Rehman, 2015).

Process of optimization

One variant at a time approach (OVAT) was applied for the optimized production of amylase. Factors such as incubation period, inoculums size, pH, temperature, metal ions, different carbon and nitrogen supplementations are optimized for the maximum recovery of enzyme (Asad *et al.*, 2014).

Incubation period

Bacillus AS-2 was grown in LB broth at 45ºC for 24 hrs. Five flasks, each containing 100 ml of selected medium (pH 7.0) were inoculated with the 24 hrs old culture and then kept at 45ºC for different time periods i.e., 24, 48, 72, 96 and 120 hrs (Gangadharan *et al.*, 2006). Enzyme assay of cell free culture supernatant (CFCS) for each flask was performed.

Temperature

Bacillus AS-2 was grown in LB broth at 45ºC for 24 hrs. Five flasks, each containing 100 ml of selected medium having pH 7.0, were inoculated with the 24 hrs old culture (8%) and then incubated at 30°C, 35°C, 40°C, 45°C and 50°C (Choubane *et al.*, 2015). Enzyme activity of cell free culture supernatant (CFCS) of each flask was monitored.

pH

A series of media were prepared with different pH values (i.e., 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0). HCl (1M) and NaOH (1M) were used to adjust the pH. *Bacillus* AS-2 was grown in LB broth at 45ºC for 24 hrs. Overnight culture (8%) was transferred into flasks (having different pH) and kept at 45°C for 72 hrs. Enzyme activity was then calculated by performing the enzyme assay (Sivakumar *et al.*, 2012).

Inoculum size

Bacillus AS-2 was grown in LB broth at 45ºC for 24 hrs. Five flasks, each containing 100 ml of selected medium having pH 7.0 were inoculated with 4%, 8%, 12%, 16% and 20% of 24 hrs old culture and then incubated at 45ºC for 72 hrs Amylase production was quantified after running the standard enzyme assay (Mazutti *et al.*, 2007).

Nitrogen source

For optimizing the nitrogen source, fermentation media (pH 7.0) were added with various nitrogen supplements such as casein, tryptone, yeast extract, gelatin, glycine, malt extract, urea and peptone at a final concentration of 0.5% (w/v). Each of the medium was added with 8% over night grown culture of *Bacillus* AS2 and incubated at 45ºC for 72 hrs. Enzyme production was then assayed using standard protocol.

Carbon source

Growth media (pH 7.0) were prepared by using 1% (w/v) of different carbon supplements including sorbitol, sucrose, lactose, xylose, galactose, arabinose, fructose, maltose, mannose and starch. Overnight culture (8%) was inoculated in the media supplemented with different sources of carbon and kept at 45ºC for 72 hrs . Enzyme activity was measured in each of the media (Akcan, 2011).

Substrate concentration

Basal medium was supplemented with various starch concentrations ranging from 0.5% to 5.0% (0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5%). Each of the medium was inoculated (8%) with 24 hrs old culture of *Bacillus* AS-2 and incubated at 45ºC for 72 hrs. Amylase production was noticed in each media by standard enzyme assay protocols (Dutta *et al.*, 2016).

Metal salts

Different media (pH 7.0) supplemented with 0.05 % (w/v) of various salts including $MgSO_4$, NaCl, $FeCl_3$, $HgCl_2$, $ZnSO_4$, $CUSO_4$, and $CaCl_2$ were inoculated with 24 hrs old culture (8%) and kept at 45°C for 72 hrs (Kanthi Kiran and Chandra, 2008). Enzyme activity was then measured by standard assay protocols.

STATISTICAL ANALYSIS

Optimization experiments were performed in triplicate. ANOVA and Tukey's test were applied to all the tested parameters using Minitab 17. At 95% confidence limit, all values with P values <0.05 were found statistically significant (Rehman, 2018).

RESULTS

Molecular identification

Real time PCR was performed to amplify 16s rDNA. On agarose gel, the amplicon size was found to be of 1.5 kb. Purification and sequencing of re-amplified 16s rDNA

resulted in a sequence with 638-nucleotides. The amplified sequence when subjected to BLAST (Basic Local Alignment Search Tool) analysis, displayed about 96.16% similarity with the strains of *Bacillus cereus* (Rehman, 2018). The retrieved sequence was also submitted in Gene Bank with accession number MK640654.

Optimization of incubation period

Gradual increase in the activity of amylase was noticed by the gradual increase in incubation period started with 24 hrs up to 72 hrs. Subsequently, decrease in enzyme production was recorded with increase in the incubation period (fig. 1). Maximum enzyme production occurred at 72 hrs.

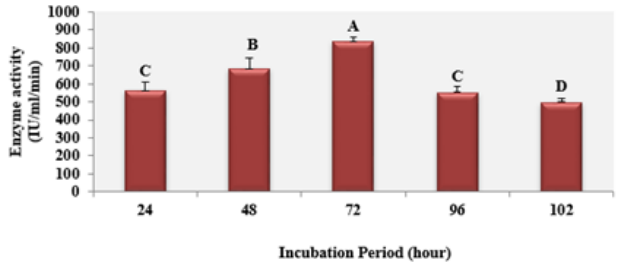

Fig. 1: Optimization in extracellular amylase production at different incubation periods. Mean with different letters differ statistically as reported by Tukey's test ($p<0.05$).

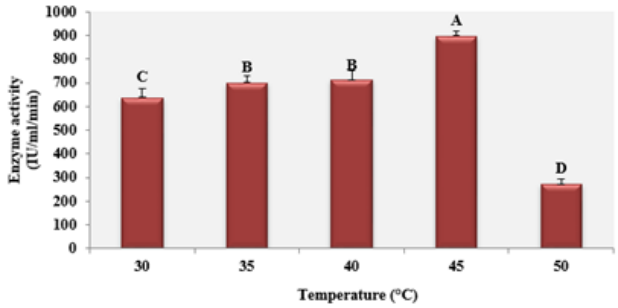

Fig. 2: Optimization in extracellular amylase production at different fermentation temperatures. Mean with different letters differ statistically as reported by Tukey's test ($p<0.05$).

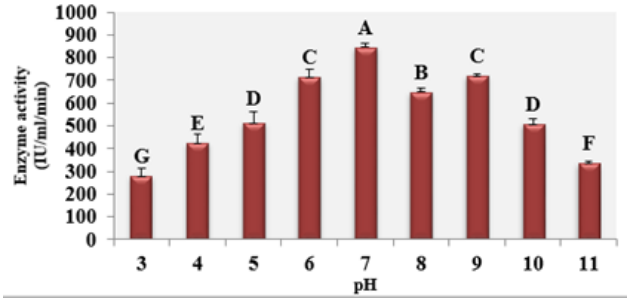

Fig. 3: Optimization in extracellular amylase production at different fermenta pH values. Mean with different letters differ statistically as reported by Tukey's test ($p<0.05$).

Optimization of temperature

Optimum amylase yield was recorded at a temperature of 45°C (fig. 2). Beyond 45°C enzyme production was drastically decreased.

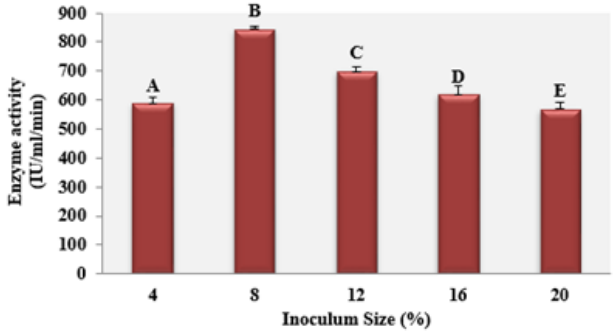

Fig. 4: Optimization in extracellular amylase production at different inoculum size. Mean with different letters differ statistically as reported by Tukey's test ($p<0.05$).

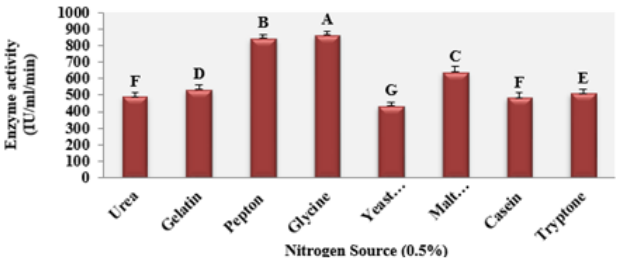

Fig. 5: Optimization in extracellular amylase production at different nitrogen sources. Mean with different letters differ statistically as reported by Tukey's test ($p<0.05$).

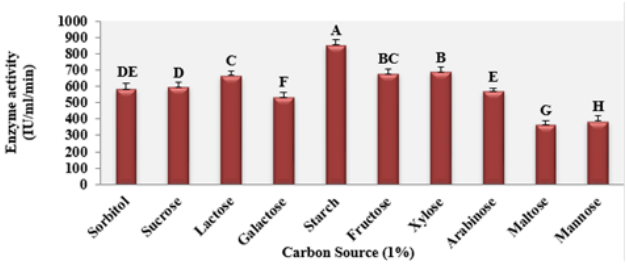

Fig. 6: Optimization in extracellular amylase production at different carbon sources. Mean with different letters differ statistically as reported by Tukey's test ($p<0.05$).

Optimization of pH

Bacillus AS2 was grown in different growth medium with pH ranges from 3.0-11. Maximum growth and amylase production was found at pH 7.0 (fig. 3). At pH below and above the optimum limits, the amylase production was found significantly low.

Optimization of inoculum size

Production of amylase by *Bacillus* AS2 was found to be increased up to 8% of the inoculum. Further increase in the inoculum size exerted a negative effect on amylase enzyme production (fig. 4).

Optimization of a nitrogenous source

Among the different nitrogenous sources tested, glycine was recorded as the best nitrogen source for maximum growth as well as enzyme production (fig. 5). Other nitrogen sources that were found stimulator for extra cellular amylase production by *Bacillus* AS2 were tryptone, peptone, gelatin and malt extract. It was also found that when urea, casein and yeast extract were supplemented in the fermentation medium, there was no such increase in amylase yield. However, addition of urea showed little increase in enzyme production as compare to yeast extract and casein.

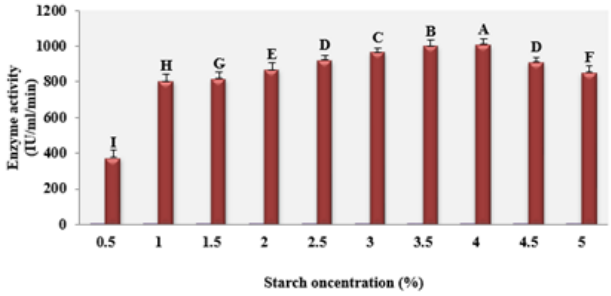

Fig. 7: Optimization in extracellular amylase production at different starch concentrations. Mean with different letters differ statistically as reported by Tukey's test ($p<0.05$).

Optimization of a carbon source

Different carbon supplements when used in the medium, effect of starch was found to be more pronounced on amylase production (fig. 6). Galactose, sucrose, arabinose, fructose and lactose also showed some stimulatory effect towards amylase production.

Optimization of concentration of starch (substrate)

For the maximum yield of amylase, the most frequently used substrate is starch. Among the various starch concentrations investigated, maximum enzyme activity was noticed at 4% of starch (fig. 7).

Optimization of metal ions

Amylase production by the test organism (*Bacillus* sp. AS2) was increased when fermentation medium was supplemented with $CaCl_2$ (fig. 8). NaCl was also reported as to increase the amylase production whereas $MgSO_4$, $ZnSO_4$, $HgCl_2$, $CUSO_4$ and $FeCl_3$ showed inhibitory effect on amylase production.

DISCUSSION

Nowadays enzymes are used as eco-friendly alternatives to the chemical hydrolysis of substrates at commercial level. Recent study is concerned with the optimization of different physical as well as chemical parameters for obtaining maximum amount of amylase enzyme from *Bacillus* sp. AS2. Maximum enzyme production was noticed when fermentation broth containing starch (4%), glycine (0.5%) and $CaCl_2$ (0.05%) was incubated for 72 hrs at 45°C, while the pH was adjusted to 7.0.

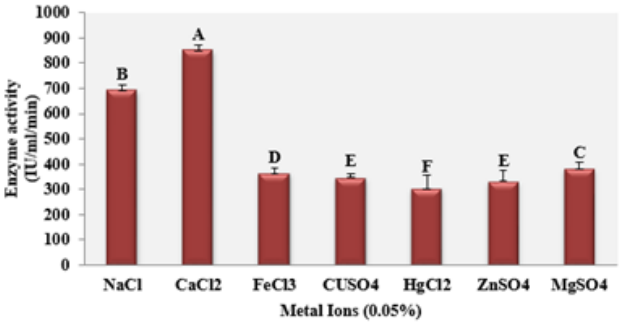

Fig. 8: Optimization in extracellular amylase production at different metal ions. Mean with different letters differ statistically as reported by Tukey's test ($p<0.05$).

Incubation period affects cultural characteristics, growth rate and enzyme production (Chowdary *et al.*, 2018). *Bacillus* strain used in the study showed increase in amylase production with the increase in incubation period from 24 hrs to 72 hrs. When the incubation period was increased further, it resulted in a decrease in enzyme production. Reduction in amylase production after the optimum value might be associated with amylase denaturation as a result of interaction with components of the media or related with the decline phase of growth which results in depletion of the nutrients as well as accumulation of toxic substances in the media that results in reduced growth and ultimately lower levels of enzyme (Baysal *et al.*, 2003). The Temperature as well as the pH are the most important factors that regulate a bioprocess. Optimum amylase yield was recorded at a temperature of 45°C (fig. 2). This is because 45°C is the optimum temperature for both of the responses i.e., bacterial growth as well as enzyme production. Beyond 45°C enzyme production was drastically decreased that may be associated with the thermal denaturation of enzyme as well as that of bacterial culture.

On the contrary, low temperature also affects amylase production by decreasing metabolic rate which lowers enzyme synthesis (Mazutti *et al.*, 2007). Another important factor is the pH especially when the activity of enzyme is concerned as most of the enzymes have a selected range of pH for the action of enzyme and the activity ceases at below or beyond that range. Optimum amylase production at pH value between 4.0 to 8.0 from *Bacillus subtilis and Bacillus amyloliquifaciens* was documented by Ikram-ul-Haq *et al.* (2003) and Gangadharan *et al.* (2006). Inoculum size is a significant factor for enzyme yield. High inoculum level may result in increased moisture content and biomass production which consequently reduce product yield. In contrast, low inoculum level results in insufficient biomass which

requires a prolong incubation period for utilization of substrate and formation of a desired product (Balkan and Ertan, 2007).

Nitrogen stimulates and down regulates the enzyme production by microorganisms. In the recent study glycine was recorded as the most suitable nitrogen source for growth as well as the enzyme production by AS2 strain (fig. 5). Glycine was used as a nitrogen supplement in the fermentation medium (Vijayabaskar *et al.*, 2012). Peptone, gelatin and malt extract were also found to stimulate the extracellular amylase production by the test specie. Gangadharan *et al.* (2006) found high yield of amylase in presence of peptone as a nitrogen source by *Bacillus amyloliquifaciens.* Carbon sources either monosaccharide or polysaccharide in fermentation medium has significant effect on amylase production. Our results showed starch as the most significant source of carbon for maximum enzyme production (fig. 6). Amylases utilize starch as a substrate but starch is also required for the accelerated production of amylase. Same results were exhibited by Ashwini *et al.* (2011) and Vishnu *et al.* (2014). Supplementation of selected medium with the metal salts provides better growth that ultimately enhances enzyme production. Halide ions especially chloride ions are necessary for amylase activity (Swetha *et al.*, 2006). Ca^{2+} had significant effect on physiology and metabolism of an organism; in addition it plays a significant role in enzyme activity and stability against proteases (Bekler *et al.*, 2018). NaCl also increased amylase production in our study whereas $MgSO_4$, $ZnSO_4$, $HgCl_2$, $CUSO_4$ and $FeCl_3$ showed inhibitory effect on amylase production. Other investigators also reported that $Fecl_3$ and $MgSO_4$ have inhibitory effect on enzyme production (Viswanathan and Surlikar, 2001). Inhibitory effects of metal salts may be related with the change in the pH required for maximum enzyme production.

CONCLUSIONS

Bulk Production of enzyme is a prerequisite for multipurpose applications of industrial interest as well as for its purification and characterization. The studied *Bacillus cereus* AS2 strain can efficiently secrete extracellular amylase enzyme in large quantities under optimized set of parameters. Although, amylases from *B. cereus* resemble with other known enzyme counterparts but have an advantage of immediate industrial applications owing to their unique properties.

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