

Optimization of Nutritional Constitute for Enhanced *Alpha amylase* Production Using by Solid State Fermentation Technology

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Abstract: Microbial Alpha amylase is highly demanded industrial enzyme in various sectors such as pharmaceuticals, food, textile and detergents, etc. In this study optimized alpha amylase Production by a strain of *Bacillus Subtilis* MAFE 118079 in solid state fermentation (SSF) was initiated and Rice bran was used substrate for alpha amylase production. Linear multiple regression analysis showed that maximum amount of amylase production is 1457.7 U/g in 48 h. process optimized enzyme titre, which was maximum (2311.1 U/g) with pH:7 when SSF was carried out at 37°C for 48 h using a substrate with 75% initial moisture. Supplementation different carbon sources the soluble starch and glucose enhanced enzyme production (Which was maximum 2311.1 U/g) with 1%. And studied on the effect of different nitrogen sources results were showed a positive impact on enzyme synthesis. In different mineral sources calcium which has result in maximum enzyme production shows (1502.2 U/g) with 0.1%. The results of present study suggest that *Bacillus subtilis* MAFE 118079 can be used for the alpha amylase production to cover the growing need of this enzyme in detergent industrial purposes.

Key words: *Bacillus Subtilis* • Alpha amylase • Rice bran • Solid State Fermentation • Optimization

INTRODUCTION

Alpha amylases (endo-1,4-a-D-glucan glucanohydrolase, E.C. 3.2.1.1) are starch degrading endo and extra cellular enzymes that randomly cleave the 1,4-a linkage between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose. Most of the amylases are metalloenzymes, which require calcium ions (Ca²⁺) for their activity [1]. Alpha amylase has been derived from several fungi, yeasts, bacteria and actinomycetes. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors [2, 3]. Amylases are one of the most important industrial enzymes that have a hydrolyzed products are widely applied in the food, paper and textile industries [4]. They are used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups [5]. They are also used as a partial replacement for the expensive malt in the brewing industry, to improve flour in the baking industry and to produce modified starches for the paper industry. In addition to this, they are used to remove starch in the manufacture of textiles. They industrial enzyme traditionally obtained from Solid State and Submerged fermentation technology production

[6]. Several reports of production have shown alpha amylase production from *Bacillus* sp. The most abundantly used bacterial alpha amylases were derived from *Bacillus amyloliquefaciens* [7], *B. licheniformis* and other *Bacillus* sp. And several report explained thermostable a-amylase production was reported from a newly isolated *Bacillus* sp. By the genetic approach the gene encoding the amylase from *Streptomyces albus* was cloned and expressed in *E. coli* and *B. Subtilis* to increase enzyme production. To meet this requirement a study was initiated to search for the optimal combinations of nutritional constituents for enhancing the production of amylase using the response surface methodology [8-10].

MATERIALS AND METHODS

Microorganism: *B. subtilis* MAFE 118079 was isolated from the Japan Culture. The organism was maintained on nutrient agar medium at 37°C.

Preparation of Inoculum: A volume of 50 mL of nutrient broth taken in a 250-mL Erlenmeyer flask was inoculated with a loop full of cells from a 24-hour-old slant and kept at 37°C in a rotary shaker. After 18 h of incubation, 1 mL of this nutrient broth culture was used as the inoculum.

Solid State Fermentation: Rice bran has a potential substrate for SSF alpha amylase productions [11-13]. Rice bran substrate was collected in Rice mill from Dharmapuri District. SSF was carried out by taking 5 g of dry substrate in a 250-mL Erlenmeyer flask to which mineral salt solution containing (in g/L): KH_2PO_4 2, NH_4NO_3 10, NaCl 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 and distilled water was added to adjust the required moisture level. The contents of the flasks were mixed and autoclaved at 121°C for 20 min. The flasks were inoculated using 1 mL of culture broth and incubated at 37°C for pH: 7. the optimization [14, 15], Incubation time (12, 25, 36, 48 and 60 h), incubation temperature (26,30,35,40,45°C), initial moisture content of the substrate 75% (w/w). Studies were also performed to evaluate the influence of different carbon sources (glucose, maltose, starch and sucrose 1% w/v) and nitrogen sources (peptone, urea, ammonium sulphate, ammonium nitrate, ammonium oxide and Sodium nitrate at 1% w/v) and minerals (magnesium sulphate, manganese sulphate, calcium chloride and zinc chloride 0.1% w/v) when added to the fermentation medium.

Enzyme Extraction: Crude enzyme was extracted by mixing a known quantity of fermented matter with distilled water containing 0.1% Tween-80 to a total extract volume amounts of 50 ml. Contents were mixed thoroughly by shaking for 1 h at room temperature in a rotary shaker (Certomat, B. Braun Biotech) at 180 rpm. The suspension was then centrifuged at 8000rpm at 4°C for 10 min and the supernatant was used for enzyme assay [16].

Enzyme Assay: Alpha amylase activity was determined as Okolo *et al.* [17]. The reaction mixture consisted of 1.25 ml 1% (w/v) soluble starch (Merck) solution, 0.25 ml, 0.1M sodium acetate buffer (pH 5.0), 0.25 ml of distilled water and 0.25 ml of properly diluted crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) method [18]. The color developed was read at 540 nm using a Shimadzu UV-160A spectrophotometer. Glucose was used as standard. Appropriate blanks were used. One unit (U) of Alpha amylase is defined as the amount of enzyme releasing 1 μ mol glucose equivalent per minute under the assay conditions.

Effect of Washing Performance with Crude Enzyme: Clean Cloth pieces (6 cm x 6cm) were soiled with chocolate, bread jam and food waste with out pre treatment were applied to cloth piece and then dried.

Chocolate was heated under liquefaction and then 100 μ l was added and then dried. The strained cloth pieces were subjected to wash treatments with commercial solid detergent diluted in distilled water at 5mg/ml, supplemented with and without crude enzyme. The stained cloth pieces were taken in separate containers, with 100ml of final volume, A (flask with distilled water only), B (flask with distilled water and commercial detergent) and C (commercial detergent with crude enzyme). The alpha amylase activity is 50U/100ml. All flasks were incubated at 37°C for 60 min under agitation (220rpm). After incubation cloth pieces were taken out, rinsed with water and dried [19].

RESULT AND DISCUSSION

Medium and Optimization of Enzyme Production: The improving fermentation media and optimization conditions were successfully applied for the production of alpha amylase by *B.subtilis* MAFE 118079 and the enzyme yield was increased considerable amount. The previous reports were described rice bran as a good source for amylase production [20, 21]. The bacillus sp result also confirms an earlier report well amylase producer with rice bran as a substrate.

Effect of Incubation Time: The enzyme assays carried out with the extract collected from the fermented samples revealed a growth related production of alpha amylase [22, 23]. SSF was carried out with 1ml of suspension on rice bran with the initial moisture content adjusted to 75% at 37°C. The diluted crude enzymes extracted from fermented samples were used for enzyme sources. After 12 h incubation, 1031 U/g of the enzyme was produced, which is increased maximum amount 1457.7 U/g after 48 h incubation. After 48 h incubated decreased enzyme yield. The reason for this might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium [24]. It could have been also be due to the fact that the microorganism was on its decline phase during the third day of fermentation and resulted in the decreased the enzyme production (Fig.1). And during long time the proteins components were denaturated in the SSF medium.

Effect of Moister Content in Enzyme Production: Moister is one of the most important parameters in SSF that influence the organism and thereby enzyme production. Low and high moister levels of the substrate affect the growth of the microorganism resulting in lower

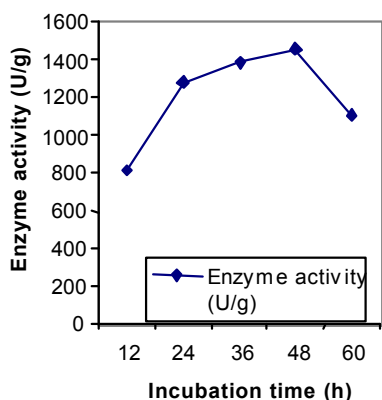


Fig. 1: Effect of incubation period (h).

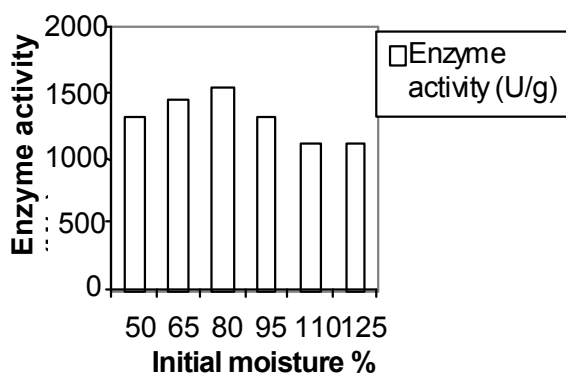


Fig. 2: Effect of initial moisture content (%).

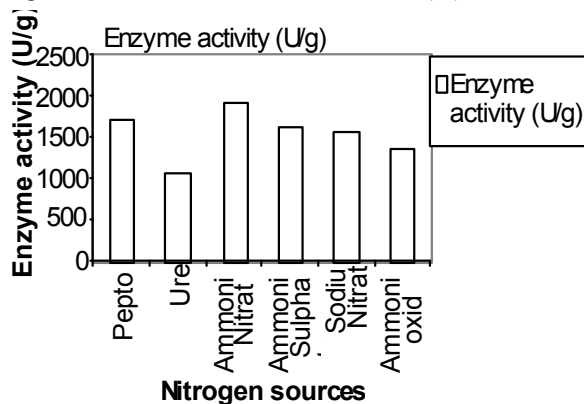


Fig. 3: Effect of Nitrogen sources (1%).

enzyme production. High moisture content leads to reduction in substrate porosity, changes in the structure of the substrate particles and reduction of gas volume. Bacteria are generally known to require initial moisture of 70-80%. With the initial moisture content 50%, alpha amylase yield was 1315 U/g, which considerably increased moisture content maximum yield 80% (1530U/g) results shows Fig.2. Higher moisture content was sufficient level decreased enzyme yield. And higher moisture level

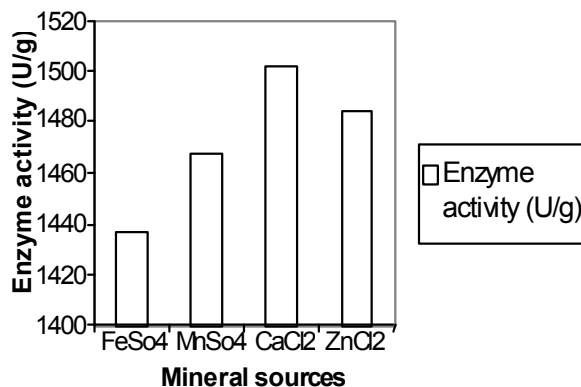


Fig. 4: Effect of Carbon sources (1%).

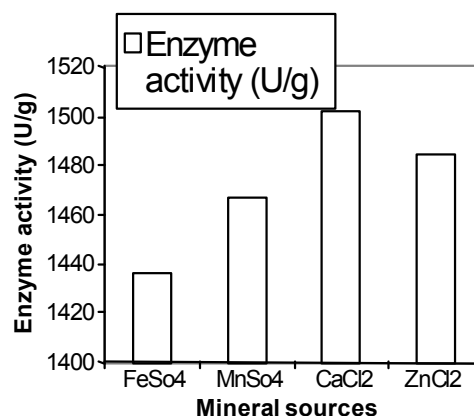


Fig. 5: Effect of Different Mineral sources (0.1%)

decreases porosity, promotes development of stickiness, increase the change of contamination [25].

Effect of Supplementation of Nitrogen Sources on SSF Medium: Previous research shows the nitrogen sources was increased enzyme production. Soya bean meal was best nitrogen sources for alpha amylase production by Bacillus sp. [26, 27]. However, in our studies, as results shown in among the 1% of nitrogen sources peptone is given maximum enzyme yield 1724.4 U/g. and in organic nitrogen sources ammonium salts also enhanced the enzyme activity results shows in (Fig.3).

Effect of Supplementation of Carbon Sources on SSF Medium: Influence of supplementary Carbon sources, soluble starch increased the amylase production (2311.1 U/g) followed by glucose Glucose and Sucrose (Fig). Earlier results shows soluble starch as the best carbon source supplement for amylase production by B.licheniformis and Bacillus sp I-3 [28] and B.stearotherophilus. Among this carbon sources glucose was helped to microbes for fast growth in

fermentation media, high concentration of microbial growth was give high enzyme production. Previous study shows soluble starch is one of the best carbon source in fermented media, the next one glucose is given maximum enzyme yield [29]. Among this ammonium source ammonium nitrate is given maximum enzyme production 1920 U/g (Fig.4).

Effect of Supplementation of Different Minerals:

Most of alpha amylases are known to be metalloenzymes, Supplementation of different metal ions provided good growth of microorganisms and thereby better enzyme production. Previous result reported shows addition of CaCl_2 to the fermentation media increased enzyme production, however in our study, 0.1% of different mineral sources (FeSO_4 , MnSO_4 , CaCl_2 and ZnCl_2), among this various mineral source CaCl_2 is the increased best production. Followed results shown in (Fig.5). S.Mishra studied Increase in enzyme productivity by induced oxidative stress in *Bacillus Subtilis* cultures and analysis of its mechanism [30].

Effect of Washing Performance: The detergent amylases work best by hydrolyzing large insoluble starch materials in the bulk wash liquids. Starch soils are initially removed from the fabric surface of components of the detergent. In this crude enzyme and it is laundry detergents were investigated. As report shows in fig, *Bacillus subtilis* [MAFE 118079] strain crude enzyme was relatively stable towards all solid and liquid detergents tested at 37°C. Additionally the earlier report shows the alpha amylase showed excellent stability towards all commercial liquid and solid detergents tested and retained more than 96% of its activity after 1 h incubation at 40 and 50°C. And this stability results shows amylase have more stability compare to protease.

Including these findings are different previous works reported shows the alkaline protease from *Bacillus* sp. SSR1 retained 37% of its initial activity after 1 h incubation at 40°C in the presence of Ariel at a concentration 5mg/ml[31]. in this results shows that *Bacillus subtilis* [MAFE 118079] Crude enzyme can remove variety of stains, such as chocolate, bread jam and grave (Fig.6) This agreement has in several reports shows usefulness of alkaline protease and amylase from *Bacillus licheniformis* NH1. However according to the reported demonstrate, the enzymatic preparation in detergent industry in more effective. In our result suggest strongly The *Bacillus subtilis* [MAFE 118079] producing alpha amylase activities is more effective.

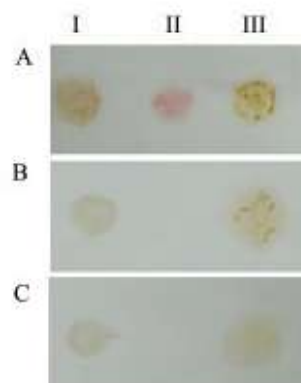


Fig. 6: Washing performance of the *Bacillus subtilis* [MAFE 118079] enzyme in the presence of the commercial detergent. I. chocolate, II. Bread jam and III. Gravy. (A) Stained cloth washed only tap water, (B) Stained cloth washed with detergent only and (C) Stained cloth washed with detergent with the crude enzyme of *Bacillus subtilis* [MAFE 118079].

Therefore *Bacillus subtilis* [MAFE 118079] sp enzyme preparation containing amylases activities could be considered as a potential for use a cleaning detergent to facilitate the release starch based stains.

CONCLUSION

This study showed that *Bacillus subtilis* [MAFE 118079] could be good microbial species for alpha amylase production by rice bran as a substrate. Our results shows proved necessary need for minerals, carbon and nitrogen sources in fermentation media with best moisture level is 80% were given maximum enzyme production. Supplementation of 1% starch, 1% peptone, 0.1% calcium chloride and ammonium nitrate to the 2311.1 U/g, the crude enzyme exhibited a high stability in the presence of commercial detergents and could effectively remove the chocolate, bread jam and food waste. Considering its enzymatic preparation containing alpha amylase activities, simultaneously *Bacillus subtilis* [MAFE 118079] strain may find potential application in detergent industries.

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