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Solid Culturing of *Bacillus amyloliquefaciens* for Alpha Amylase Production

*Dhanya Gangadharan, Swetha Sivaramakrishnan, Kesavan Madhavan Nampoothiri and Ashok Pandey**

Biotechnology Division, Regional Research Laboratory, CSIR, Trivandrum 695019, India

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Summary

Fourteen different agroresidues were screened for alpha amylase production using *Bacillus amyloliquefaciens* ATCC 23842. Among them, wheat bran (WB) and groundnut oil cake (GOC) in mass ratio of 1:1 was proved as the best substrate source. Supplementation with 0.01 M KH_2PO_4 and 1 % soluble starch enhanced the enzyme yield considerably. Maximum enzyme recovery from the solid mass was obtained when extracted with 0.1 M acetate buffer, pH=5.0. Maximum enzyme titer expressed as units per mass of dry substrate obtained was 62 470 U/g after 72 hours of fermentation at 37 °C by using the above solid substrate mixture (5 g) with the initial moisture of 85 % and inoculated with *Bacillus amyloliquefaciens* of $2{\cdot}10^9$ CFU/mL.

Key words: alpha amylase, *Bacillus amyloliquefaciens*, solid-state fermentation, agroresidues

Introduction

Alpha amylases (endo-1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) are extracellular endo enzymes that randomly cleave the 1,4-α linkage between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units. Enzymatic hydrolysis of starch has now replaced acid hydrolysis in over 75 % of starch hydrolysing processes due to many advantages, not least its highest yields (*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*). The most abundantly used bacterial α-amylases were derived from *Bacillus amyloliquefaciens*, *B. licheniformis* and *B. stearothermophilus* (*3*). The 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. 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 (desizing) and as additives to detergents for both washing machines and automated dishwashers.

Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. However, solid-state fermentation (SSF) constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly (*2,4–6*). SSF is preferred to SmF because of simple technique, low capital investment, lower levels of catabolite repression and end product inhibition, low waste water output, better product recovery, and high quality production (*7*). On a dry basis, agricultural substrates like corn, wheat, sorghum and other cereal grains contain around 60–75 % (by mass) starch, hydrolysable to glu-

*Corresponding author; Phone: ++91 471 25 15 279; Fax: ++91 471 24 91 712; E-mail: ashokpandey56@yahoo.co.in, pandey@csrrltrd.ren.nic.in

cose, with a significant mass increase, which offers a good resource in many fermentation processes (*8*). With the advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new areas have been opened for their utilization as raw materials for the production of value added fine products (*2*,*9*,*10*). Application of these agroindustrial residues in bioprocesses also solves pollution problems, which their disposal may otherwise cause (*2*,*9*,*11*). Earlier studies by Ramachandran *et al*. (*12*) and Francis *et al*. (*13*) have reported alpha amylase production by *Aspergillus oryzae* on coconut oil cake and spent brewing grains, respectively, as SSF substrates. However, this work was aimed at identifying an effective agroresidue or their combinations as the substrate for the production of alpha amylase from *Bacillus amyloliquefaciens* by solid-state fermentation.

Materials and Methods

Microorganisms and maintenance

Bacillus amyloliquefaciens ATCC 23842 was grown on nutrient agar (Hi-media, Mumbai, India) slants at 37 °C for 24 h. The fully grown slants were stored at 4 °C and were subcultured every two weeks.

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. By serial dilution and plating, the number of viable colonies in the inoculum was found to be $2 \cdot 10^9$ CFU/mL.

Solid-state fermentation

In an attempt to choose a potential substrate for SSF which supports amylase production, various agroresidues like coconut oil cake (COC), groundnut oil cake (GOC), sesame oil cake (SOC), wheat bran (WB), spent brewing grain (SBG), cassava bagasse (CB), jackfruit seed powder (JSP), tamarind seed powder (TSP), rice bran (RB), palm kernel cake (PKC), olive oil cake (OOC), mustard oil cake (MOC), cotton seed oil cake (CSOC) and rice husk (RH) were screened individually as well as in combinations (mass ratio of 1:1). 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, $MgSO_4 \cdot 7H_2O$ 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 24 h.

Unless it is specified otherwise, crude enzyme was extracted by mixing a known quantity of fermented matter with distilled water on a rotary shaker (180 rpm) for 1 h. The suspension was then centrifuged at 8000 × *g* at 4 °C for 10 min and the supernatant was used for enzyme assay. Dry mass of the SSF samples was determined by drying them in a hot air oven at 80 °C for 16 h.

Effect of process parameters on amylase production in SSF

The optimization of medium components and fermentation process is of primary importance in any fermentation process. Combinations of the best substrates were employed for further optimization of process parameters, namely initial moisture content (55, 60, 65, 70, 75, 80, 85 and 90 %), incubation time (24, 48, 72, 96 and 120 h), incubation temperature (30, 35, 40, 45 and 50 °C), initial pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) of the medium, inoculum size (0.5, 1, 2, 4, 6 and $8 \cdot 10^9$ CFU/mL), while nutrient supplementation such as inorganic nitrogen sources (0.125 M) (ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulphate), 1 % (by mass) organic nitrogen sources (peptone, tryptone, yeast extract, soybean meal and corn steep solid) and added phosphate (KH_2PO_4) concentration (0.005, 0.01, 0.02 and 0.03 M) were optimised. To study the efficacy of various inducers, the medium was supplemented independently with 1 % soluble starch, hydrolysed starch, maltose, lactose and glucose. Varying concentrations (0.5, 1.0, 1.5 and 2.0 %) of the best inducer were also studied.

Distilled water, 0.1 M acetate buffer (pH=5.0), 0.1 % solution of various detergents like Tween 20, Tween 40, Tween 80 and Triton X-100 were used independently to find the best extraction medium for the enzyme.

Analytical methods

Alpha amylase activity was determined as it is described by Okolo *et al*. (*14*). The reaction mixture consisted of 1.25 mL of 1 % soluble starch, 0.5 mL of 0.1 M acetate buffer (pH=5.0), and 0.25 mL of 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 (*15*). The colour developed was read at 510 nm using a Shimadzu UV-160A spectrophotometer. Glucose was used as standard. The blank contained 0.75 mL of 0.1 M acetate buffer (pH=5.0) and 1.25 mL of 1 % starch solution. One unit (IU) of α-amylase is defined as the amount of enzyme releasing one μmol of glucose equivalent per minute under the assay conditions. The enzyme activities used for representations are the average values of three independent experiments.

Results and Discussion

Screening of agroresidues as substrates for SSF

Among the various substrates screened for SSF, WB gave the highest enzyme activity (1 566 U/g) (Fig. 1). As it is shown in Table 1, all mixtures of substrates containing WB gave significant increase in the enzyme activity, but the highest was WB+GOC (1 671 U/g). The production on different ratios of WB+GOC showed that the substrate in the ratio 1:1 yielded the highest enzyme activity (Table 2). WB has been widely reported to be the best substrate for enzyme production in SSF (*16*,*17*).

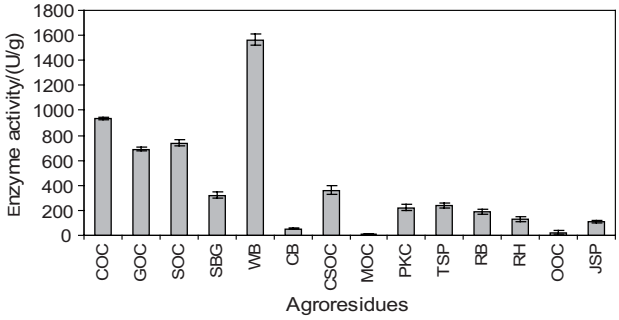

Fig. 1. Screening of agroresidues for the production of α-amylase using *B. amyloliquefaciens*

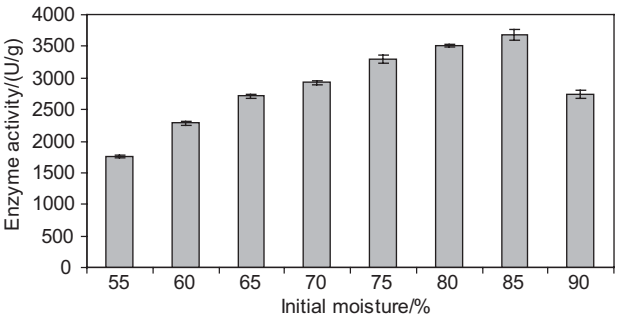

Fig. 2. Effect of initial moisture content of the SSF medium on α-amylase production by *B. amyloliquefaciens*

Table 1. Screening of substrate mixtures (1:1) for the production of α-amylase by *B. amyloliquefaciens*

Substrate mixtures (1:1)	Enzyme activity/(U/g)
COC+GOC	822
GOC+SOC	556
SOC+WB	923
WB+SBG	141
COC+SOC	137
COC+WB	152
WB+GOC	1 671
COC+SBG	88
GOC+SBG	108
SOC+SBG	84

Table 2. Production of α-amylase by *B. amyloliquefaciens* on different ratios of WB and GOC

WB:GOC	Enzyme activity/(U/g)
1:1	3 672
2:1	2 946
3:1	2 637
1:2	2 885
1:3	2 778

Effect of initial moisture content of the medium on the production of α-amylase

Fig. 2 indicates that moisture is a critical factor for the production of enzymes under SSF. Generally, bacteria require higher water activity for their growth. The necessary moisture in SSF exists in absorbed or complex form within the solid matrix, which is likely to be more advantageous for growth because of the possible efficient oxygen transfer process (*18*). Enzyme production was found to gradually increase with moisture content. If the quantity of water becomes insufficient and does not allow a good diffusion of solutes and gas, the cell metabolism slows down, or it can stop completely, because of the lack of substrates or due to too high concentration of inhibitive metabolites in or near the cell (*19*). Similar findings were reported by Ramesh and Lonsane (*20*). Similarly, higher moisture level decreases porosity, changes particle structure, promotes development of stickiness, reduces gas volume and decreases diffusion, which results in lowered oxygen transfer (*20*). For this bacterial system, the maximum enzyme production (3 677 U/g) was observed when the substrate moisture was set at 85 %. It is worth mentioning that it is the special nature of the substrate combination which helped to retain 85 % moisture without producing any free water so that the entire system remained in solid state. In the case of *B. licheniformis*, optimal moisture level was found to be 60–75 % for α-amylase production (*21*).

Influence of various extraction media on the enzyme yield under SSF

The medium used for the extraction of crude enzyme from the fermented matter was found to have a profound effect on the enzyme yield. Results from Table 3 show that maximum enzyme yield (15 114 U/g) was observed when 0.1 M acetate buffer (pH=5.0) was used. This profound influence is due to the inactivation of alkaline and neutral proteases at acidic pH provided by the buffer. The enzyme profile of the bacterial strain had shown significant activity of neutral and alkaline proteases (data not shown). The degradation of alpha amylases by proteases is one of the reasons for lower activity when distilled water was used as extraction medium.

Table 3. Effect of extraction solvents on the production of α-amylase by *B. amyloliquefaciens*

Extraction solvents	Enzyme activity/(U/g)
Distilled water	4 738
0.1 % Tween 20	12 768
0.1 % Tween 40	8 730
0.1 % Tween 80	11 104
0.1 M acetate buffer (pH=5.0)	15 114

Influence of incubation period on enzyme production

The incubation time is governed by characteristics of the culture and also based on growth rate and enzyme production (22). Fig. 3 shows a gradual increase in enzyme production through 24, 48 and maximum at 72 h (52 641 U/g). The enzyme yield showed a gradual decrease on further extension of fermentation period. The decrease in enzyme yield after the optimum level may

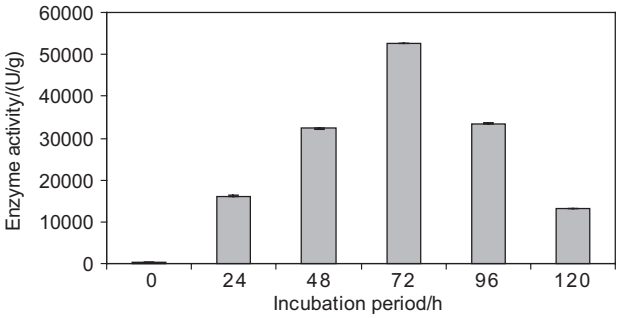

Fig. 3. Effect of incubation period on α-amylase production by *B. amyloliquefaciens*

be because of denaturation or decomposition of α-amylase due to interaction with other components in the medium as it is reported elsewhere (*23*).

Influence of inoculum size on the production of enzyme under SSF

There was no significant increase in the enzyme yield when size of the inoculum was increased from 2 to $6{\cdot}10^9$ CFU/mL. Lower inoculum such as 0.5 mL ($1{\cdot}10^9$ CFU) resulted in lower biomass production and hence lower enzyme yield (40 569 U/g). The maximum enzyme yield was 52 587 U/g when 1 mL of inoculum containing $2{\cdot}10^9$ CFU was used. After that, there was a gradual decrease in enzyme production and it was 48 363 U/g when inoculum size was increased to $8{\cdot}10^9$ CFU/mL. This may be due to the limiting nutrients at higher inoculum size. Thus 1 mL was used as inoculum for further studies.

Effect of initial pH of the medium for higher enzyme yield

Among the physicochemical parameters, the pH of the growth medium plays an important role by inducing morphological changes in the organism and in enzyme secretion. The production of alpha amylase is very sensitive to initial pH of the fermentation medium (*24*). Haq *et al*. (*25*) reported pH=7.5–8.0 to be the best for the production of alpha amylase by *Bacillus subtilis*. The enzyme production was maximum when initial medium pH was 4.0, which yielded 52 820 U/g enzyme units (Table 4). Variations in pH result from the substrate consumption (*e.g.* protein hydrolysis) and/or metabolite production (*e.g.* organic acids). They are indicators of changes in metabolic activity (*26*). Results show that enzyme production was generally stable from pH=4–7.0, which indicates excellent buffering property of the agroresidues used for solid-state fermentation.

Table 4. Effect of initial medium pH and incubation temperature on α-amylase production by *B. amyloliquefaciens*

pH	Enzyme activity/(U/g)	Incubation temperature/°C	Enzyme activity/(U/g)
4.0 (control)	52 791	37 (control)	52 877
3.0	32 238	30	11 098
4.0	52 820	35	50 369
5.0	49 078	40	13 365
6.0	48 431	45	4 588
7.0	47 217	50	1 865
8.0	32 484		
9.0	21 032		

Influence of incubation temperature on α-amylase production

The influence of temperature on amylase production is related to the growth of the organism. The results from Table 4 show that the enzyme yield was maximum (52 887 U/g) in the control at 37 °C. It was interesting to note that at 30 °C, the yield was 11 098 and at 45 °C it was 4 588 U/g. The range from 35–37 °C is necessary for maximum enzyme titre. It has also been reported that the metabolic heat generated during microbial cultivation in SSF exerts harmful effects on the microbial activity (*27*) and thus the initial set temperature is vital.

Effect of supplementation of nitrogen sources on enzyme production

Added nitrogen sources have been reported to have an inducing effect on the production of various enzymes including alpha amylase in an SSF system (*24,28*). Earlier reports show that among various inorganic nitrogen sources tested, ammonium sulphate, ammonium chloride and ammonium hydrogen phosphate favoured growth and enzyme secretion (*29*). Similar observations were recorded by Chandra *et al*. (*30*) and Babu and Satyanarayana (*31*). However, in our studies, as shown in Table 5 in comparison with the control (52 279 U/g), there was no significant increase in enzyme yield in the case of the supplementation of either inorganic or organic nitrogen sources. A marginal increase was noted with the addition of peptone (53 624 U/g) or tryptone (53 691 U/g), indicating that any of the sources can be alternatively used. Apart from a good carbon source, wheat bran also serves as a nitrogen source, thus an increase in the complex nitrogen source adversely influenced the production of alpha amylase (*17*). This finding is also in agreement with the work reported earlier by Pedersen and Nielsen (*24*). Since mixed substrate was used, nitrogen requirement could be met not only from WB but from GOC too.

Table 5. Effect of supplementation of nitrogen source on α-amylase production by *B. amyloliquefaciens*

Nitrogen source		Enzyme activity/(U/g)
Control (without any nitrogen supplementation)		52 279
Inorganic (0.125 M)	Ammonium nitrate	52 981
	Sodium nitrate	50 794
	Ammonium chloride	52 923
	Ammonium sulphate	52 248
Organic (1 %)	Peptone	53 624
	Tryptone	53 691
	Yeast extract	51 512
	Soya bean meal	48 259
	Corn steep solid	47 660

Effect of various phosphate concentrations on enzyme production

Phosphate serves as the construction material of cellular components such as cyclic AMP, nucleic acids, phospholipids, nucleotides and coenzymes. α-Amylase synthesis was found to be stimulated by phosphate (*32*). Our results indicated that a supplementation of 0.01 M KH_2PO_4 gave relatively higher enzyme titre (55 070 U/g) in comparison with the control (52 772 U/g).

Influence of effector molecules on higher enzyme yield

Alpha amylase is an inducible enzyme, which is generally induced in the presence of starch or its hydrolytic product maltose (*33*). Results (Fig. 4) obtained in enzyme production indicate that addition of soluble starch (1 % by mass) gave the highest enzyme yield (62 470 U/g), followed by maltose (58 499 U/g). *Bacillus thermooleovorans* preferred starch, glucose, lactose, maltose and maltodextrin as favourable carbon sources for amylase secretion (*29*). Hydrolyzed starch and glucose were found to repress the enzyme yield, which may be due to feedback inhibition caused by the presence of reducing sugars. Easily metabolizable carbohydrates may result in the better growth of the bacteria along with reduction in the enzyme formation (*32,33*). Since soluble starch was found to induce enzyme production, experiment was conducted to find out the appropriate concentrations for enhanced enzyme production. Using 1.5 %, the yield rose to 65 275 U/g and it was found to be the optimum since further increase resulted in gradual decrease in enzyme titre.

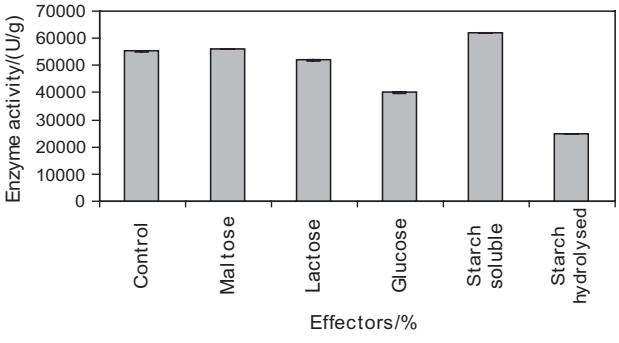

Fig. 4. Influence of various carbon sources as effectors on α-amylase production by *B. amyloliquefaciens*

Conclusion

Study on evaluation of different agroresidues as a substrate and effect of various fermentation parameters such as fermentation period, fermentation temperature, inoculum size, extraction solvents, pH of the production medium, inorganic and organic nitrogen sources, phosphate concentration, effector molecules for the production of alpha amyalse by *Bacillus amyloliquefaciens* under solid-state fermentation was carried out. Even though SSF is widely applied for enzyme production using filamentous fungi, the results of the present study proved that a bacterial culture such as *Bacillus amyloliquefaciens* can be successfully used for the production of alpha amylase employing WB and GOC in a mass ratio of 1:1 with relatively higher moisture level (85 %) within a relatively shorter time interval of three days.

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