Journal of Industrial Microbiology & Biotechnology (1999) 22, 622–626

http://www.stockton-press.co.uk/jim

Production of raw-starch digesting amyloglucosidase by *Aspergillus* sp GP-21 in solid state fermentation

G Mamo and A Gessesse

Department of Biology, Addis Ababa University, PO Box 1176, Addis Ababa, Ethiopia

***Aspergillus* sp GP-21 produced a raw-starch digesting amyloglucosidase which showed optimum activity at 65°C and pH 5.0–5.5. At 50°C the enzyme converted about 40% of raw corn starch to glucose within 48 h. Enzyme production was studied in solid state fermentation using wheat bran. Productivity was affected by the level of moisture, incubation temperature and the presence or absence of supplements. Maximum enzyme production was observed at a moisture level of 75% and at 30°C. Enzyme production was stimulated by supplementing wheat bran with 0.25% proteose peptone, 1% trace mineral solution, 0.01% $CaCl_2$ and 0.01% $MgSO_4$.**

Keywords: amyloglucosidase; glucoamylase; raw starch hydrolysis; *Aspergillus*; solid state fermentation

Introduction

In conventional starch processing, starch slurry is heated at temperatures up to 105°C, a process known as gelatinization. This increases the viscosity of the slurry and poses problems with mixing and pumping. To overcome such viscosity-associated problems, thermostable α-amylase is added which can liquefy starch at this elevated temperature. Liquefied starch is then saccharified using fungal amyloglucosidase (AMG) at 60°C. This process of starch saccharification, although currently used by starch processing industries, is energy intensive thus increasing the production cost of starch-based products. Hence, with the view of reducing the energy cost of starch processing, currently there is an ongoing search for efficient raw starch-digesting amylases. Such enzymes have been reported from different fungal [11,19,20] and bacterial [2,4,12,13,21] strains.

Raw starch-digesting amyloglucosidases reported so far have been produced using submerged fermentation (SmF) [19,21,24]. On the other hand, the use of solid state fermentation (SSF) is more advantageous than SmF and allows cheaper production of enzymes [23,25]. In this study, we report the properties of a raw-starch digesting amyloglucosidase (AMG) and its production under SSF.

Materials and methods

Organism

Raw-starch hydrolyzing fungal strains were isolated from garden soil. Based on the properties of the AMG produced and the level of production, one isolate, designated GP-21, was selected for further studies. The isolate was identified as a strain of the genus *Aspergillus*.

Solid state fermentation

Appropriately moistened wheat bran in the presence or absence of different supplements was autoclaved at 121°C for 15 min, and inoculated with 1 ml spore suspension (6×10^7 ml^{-1}). After 72 h of incubation at 30°C the culture was extracted with distilled water and filtered. The filtrate was used as the source of enzymes.

Submerged fermentation

The medium used for SmF was composed of (g L^{-1}): starch, 10; proteose peptone 2.5; KH_2PO_4, 1.5; NaCl, 1; $CaCl_2$, 0.1; $MgSO_4$, 0.1; trace mineral solution, 1 ml. One hundred milliliters of medium in a 500-ml baffled flask were inoculated with 1 ml spore suspension (6×10^7 ml^{-1}) and incubated at 30°C with rotary shaking. After 72 h the culture was harvested and the cell-free supernatant phase was used as the enzyme source.

Enzyme assay

The activity of AMG was determined at 60°C by mixing 0.9 ml of 1% (w/v) soluble starch dissolved in 50 mM acetate buffer, pH 5 with 0.1 ml of appropriately diluted enzyme source. The reducing sugar released was measured following the dinitrosalicylic acid (DNS) method [17]. One unit of AMG activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to glucose per minute under the assay condition. Enzyme productivity using solid state fermentation was given as U g^{-1} dry mouldy bran (DMB). Xylanase and cellulase activity were determined in 50 mM acetate buffer, pH 5.0 at 60°C as described by Gessesse and Mamo [9] and Khyami-Horani [14], respectively. Protease activity was measured as described earlier [8]. All values given are averages of three determinations.

Raw starch adsorption

To 1 ml of AMG preparation (12 U), 0.5 g of raw potato or corn starch was added and gently stirred for 15 min at room temperature. After centrifugation at $2500 \times g$ for 10 min, the level of activity in the supernatant phase was determined. The adsorption rate (AR) was calculated according to the equation:

$$AR\ (\%) = (0 - R)/O$$

Correspondence: Dr G Mamo, Department of Biology, Addis Ababa University, PO Box 1176, Addis Ababa, Ethiopia
Received 27 October 1998; accepted 15 March 1999

where R and O stand for residual and original enzyme activity, respectively.

Raw starch hydrolysis

The reaction mixture in a final volume of 10 ml (50 mM acetate buffer, pH 5.0) was composed of 1% starch and 6.5 U ml^{-1} enzyme. The mixture was incubated at 50°C with rotary shaking and samples were withdrawn at intervals of time. The reducing sugar released was determined as described above.

Results

Properties of the enzyme

Effect of temperature on activity was determined between 30–75°C at intervals of 5°C. The enzyme was optimally active at 65°C and displayed 90% of its peak activity at 70°C (Figure 1). Thermal stability was determined in the presence and absence of Ca^{2+} at 60°C and pH 5.0. Addition of Ca^{2+} did not improve its thermal stability (Figure 2). In the presence and absence of Ca^{2+} the half-life of the enzyme was 90 min. The optimum pH for activity was in the range of 5.0–5.5 (Figure 3). Maximum stability was also observed in this pH range.

The enzyme was absorbed to raw corn and potato starch with an adsorption rate of over 70%. However, the degree of hydrolysis of corn and potato starch granules was different. About 40% of corn and 4% of potato raw starch was hydrolysed after 48 h of incubation (Figure 4).

Enzyme production

Aspergillus sp GP-21 was grown both in solid and liquid medium. Compared to SmF the level of enzyme production under SSF was very high. As indicated in Table 1, under SSF, in addition to AMG the organism produced cellulase, xylanase and protease. The level of AMG and xylanase activity was high, while productivity of the other enzymes was low.

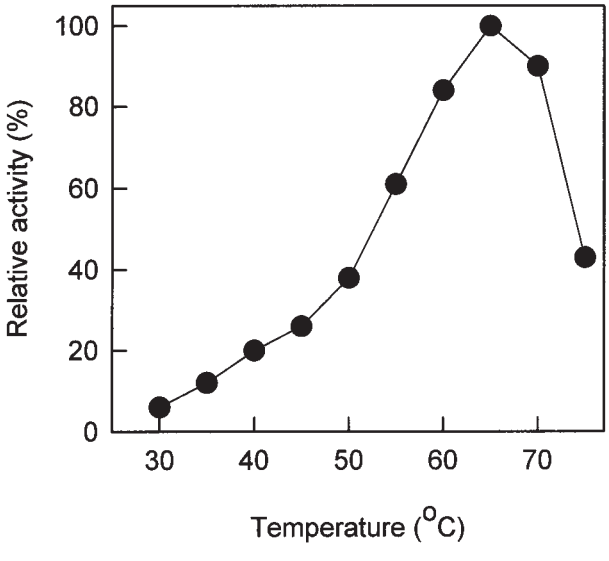

Figure 1 Effect of temperature on the activity of *Aspergillus* sp GP-21 amyloglucosidase. Enzyme activity was determined at different temperatures using 50 mM acetate buffer, pH 5.0.

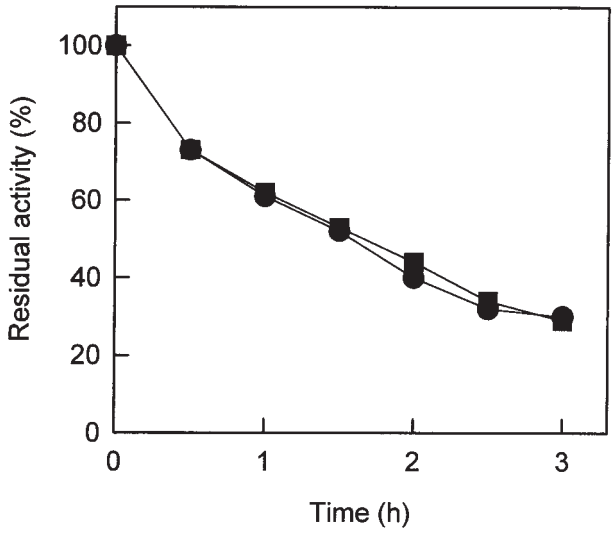

Figure 2 Thermal stability of *Aspergillus* sp GP-21 amyloglucosidase at 60°C in the presence (■) and absence (●) of 5 mM Ca^{2+}.

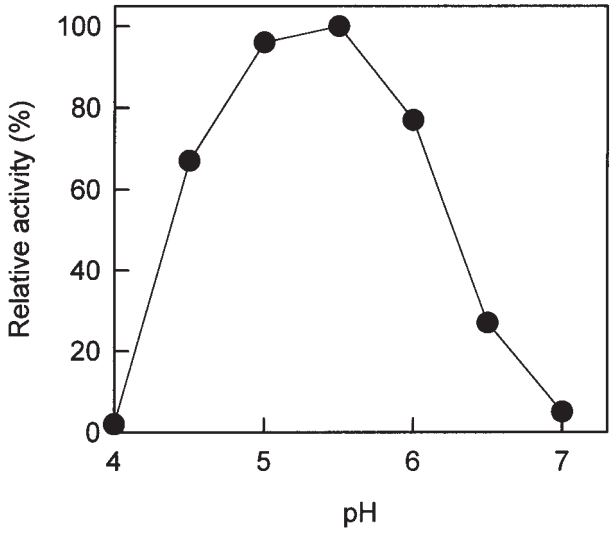

Figure 3 Effect of pH on the activity of *Aspergillus* sp GP-21 amyloglucosidase at 60°C. Buffers used were 50 mM acetate (pH 4.0–6.0) and phosphate (pH 6.5 and 7.0).

Enzyme production was affected by the moisture content of the substrate and the incubation temperature. Maximum activity was detected at an initial moisture level of 75% (Figure 5) and an incubation temperature of 30°C (Table 2). The amount of enzyme produced at room temperature (23 ± 2°C) was 95% of that produced at 30°C. On the other hand, only 40% of the maximum production was measured at 37°C (Table 2).

Wheat bran at a moisture level of 75% was supplemented with 0.25 g of yeast extract, peptone, tryptone and ammonium chloride. AMG production was slightly lower in the presence of yeast extract while addition of tryptone and peptone resulted in slight improvement on the level of enzyme production (Table 3).

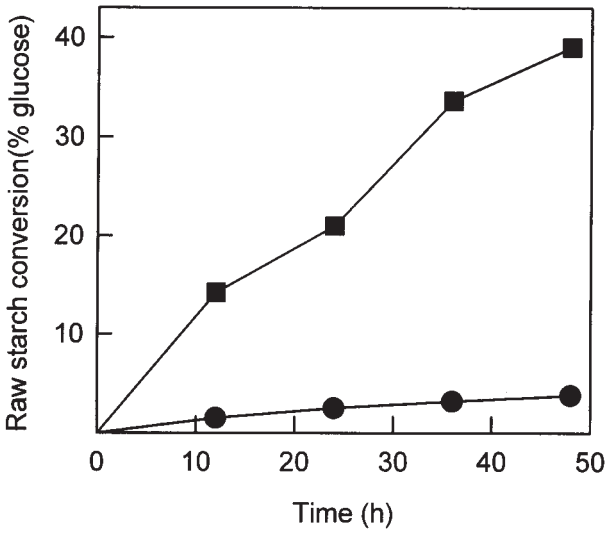

Figure 4 Raw corn (■) and potato (●) starch hydrolysis by *Aspergillus* sp GP-21 amylase. The mixture was composed of 0.1 g raw starch and 65 U of AMG in 10 ml acetate buffer and incubated at 50°C.

Table 1 Enzymes produced by *Aspergillus* sp GP-21 under SSF using wheat bran as a substrate

Enzyme	Activity (U g^{-1} DMB)
AMG	207
Xylanase	151
Cellulase	7
Protease	4

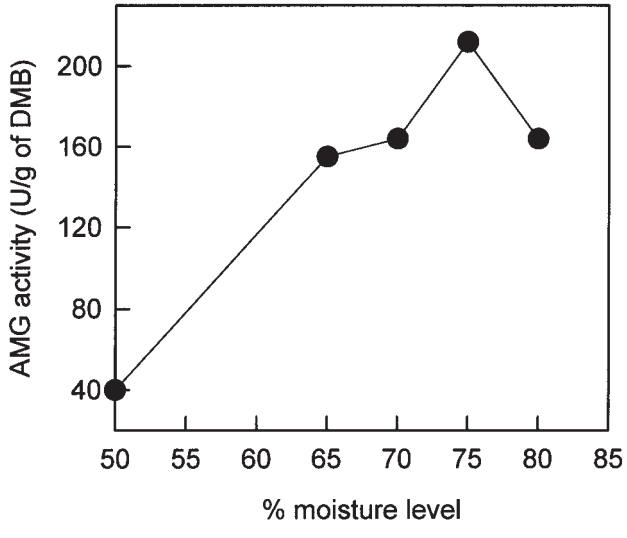

Figure 5 Effect of moisture level on enzyme production by *Aspergillus* sp GP-21 under solid state fermentation. Wheat bran supplemented with proteose peptone and minerals was moistened to a different level. The culture was incubated at 30°C and enzyme activity was determined after 72 h.

Table 2 Effect of incubation temperature on the level of enzyme production by *Aspergillus* sp GP-21 under solid state fermentation

Incubation temperature (°C)	U g^{-1} of DMB
Room temperature (23 ± 2)	198
30	209
37	86

DMB: dry mouldy bran.

Table 3 Effect of different supplements on amyloglucosidase production by *Aspergillus* sp GP-21 under solid state fermentation

Supplement		U g^{-1} DMB
Nitrogen sources	Control	127
	Yeast extract	109
	Proteose peptone	155
	Tryptone	151
	NH_4Cl	126
Minerals	Trace	146
	$CaCl_2$ + $MgSO_4$	151
	Trace + $CaCl_2$ + $MgSO_4$	179

Trace mineral solution (1 ml), $CaCl_2$ (0.01 g) and $MgSO_4$ (0.01 g), and a combination of these were used to supplement the wheat bran. As shown in Table 3, addition of these minerals slightly stimulated enzyme production.

The time course of enzyme production was studied using wheat bran supplemented with proteose peptone, $CaCl_2$, $MgSO_4$ and trace mineral solution. Maximum enzyme production (221 U g^{-1} of DMB) was achieved after 72 h, followed by a gradual decline in productivity (Figure 6).

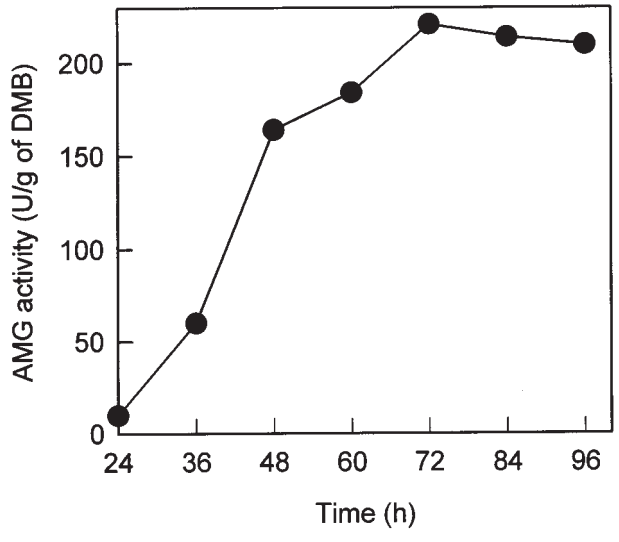

Figure 6 Time course of amyloglucosidase production by *Aspergillus* sp GP-21 under solid state fermentation. Wheat bran at a moisture level of 75% and supplemented with proteose peptone and minerals was inoculated with spores and incubated at 30°C.

Discussion

Compared to conventional starch processing, direct raw-starch saccharification is considered to be cost effective. However, up to now the efficiency of enzymatic raw-starch digestion is very low and remains economically unattractive [20]. There are reports showing improvement on the efficiency of raw starch digestion around 60°C compared to lower temperatures [1,4]. In this regard the raw-starch digesting AMG of *Aspergillus* sp GP-21 which was optimally active at 65°C and showed high thermal stability could be a good candidate for efficient hydrolysis of raw starch. Even for conventional starch saccharification these properties are highly desirable. In fact the search for thermostable AMGs is one future goal of conventional starch saccharification [6]. *Aspergillus* sp GP-21 AMG displayed 90% of the peak activity at 70°C and it did not require Ca^{2+} as a stabilizer. This is an interesting property, especially in the production of starch-based sweeteners. Calcium is an inhibitor of glucose isomerase, the enzyme used for the isomerization of glucose to fructose [5,16,27].

In addition to AMG, α-amylases from different sources digest raw starch [5,11,12]. Previous workers demonstrated a synergistic effect of α-amylases and AMGs on raw starch degradation [10,19]. This is expected to improve the efficiency of enzymatic raw-starch digestion. To exploit this opportunity of synergism, the temperature and pH optima (for activity and stability) of α-amylases and AMGs need to be compatible. However, while the pH optima of most AMGs is at or near 4.0 [3,7], α-amylases which are active and stable at this low pH are extremely rare [6]. The AMG of *Aspergillus* sp GP-21 was optimally active at pH 5.5 and 65°C, where many raw-starch degrading α-amylases are also active [12,18].

Given the potential usefulness of the enzyme for direct saccharification of raw starch, development of methods for cheaper production of the enzyme was important. One alternative was the use of SSF. In this study the use of SSF was found a cheap way of producing a high level of AMG by *Aspergillus* sp GP-21. Enzyme production was decreasing with increasing moisture level of the substrate, which could be attributed to a reduction in the degree of aeration with increasing moisture level. Enzyme production under SSF by different microbial strains is affected by the level of moisture [15,22,25]. The incubation temperature also affects the level of enzyme production under SSF [26,28]. *Aspergillus* sp GP-21 showed optimum productivity at 30°C and room temperature (23 ± 2°C). Maximum production at lower temperatures may be advantageous for it can reduce the rate of evaporation during incubation.

Supplementation of the wheat bran with trace minerals and nitrogen sources (with the exception of yeast extract) stimulated AMG production. When the nitrogen source and the mineral sources were used together the level of production was improved.

Production of protease together with AMG under SSF may affect the stability of the enzyme. To avoid this problem development of protease-negative mutants through mutagenesis and/or the use of appropriate protease inhibitors during enzyme extraction could be beneficial. Previously, Hayashida and Teramoto [10] reported on the development of a protease-negative α-amylase-producing *Aspergillus ficum* mutant from a protease-positive strain.

Acknowledgements

The financial support of the Swedish International Development Cooperation Agency (SIDA/SAREC) is gratefully acknowledged. The authors thank Fekadesilassie Wubie and Aster Mekasha for technical assistance.

References

1 Abe J, FW Bergmann, K Obata and S Hizukuri. 1988. Production of the raw starch digesting amylase of *Aspergillus* sp K-27. Appl Microbiol Biotechnol 27: 447–450.
2 Achi O and A Njokuobi. 1992. Production of raw starch saccharifying amylase by *Bacillus alvei* grown on different agricultural substrates. World J Microbiol Biotechnol 8: 206–207.
3 Ali S and Z Hossain. 1991. Characteristic of glucoamylase from *Aspergillus terreus*. J Appl Bacteriol 71: 144–146.
4 Campus BG, FG Priest and JR Stark. 1992. Hydrolysis of starch granules by the amylase from *Bacillus stearothermophilus* NCA 26. Proc Biochem 27: 17–21.
5 Chen WP. 1980. Glucose isomerase (a review). Proc Biochem 15: 36–41.
6 Crabb WD and C Mitchinson. 1997. Enzymes involved in the processing of starch to sugars. Trends Biotechnol 15: 349–352.
7 Fogarty WM and CP Benson. 1983. Purification and properties of a thermophilic amyloglucosidase from *Aspergillus niger*. Eur J Appl Microbiol Biotechnol 18: 271–278.
8 Gessesse A and BA Gashe. 1997. Production of alkaline protease by an alkaliphilic bacteria isoalted from alkaline soda lake. Biotechol Lett 19: 479–481.
9 Gessesse A and G Mamo. 1998. Purification and characterization of an alkaline xylanase from alkaliphilic *Micrococcus* sp AR-135. J Ind Microbiol Biotechnol 20: 210–214.
10 Hayashida S and Y Teramoto. 1986. Production and characteristics of raw-starch-digesting α-amylase from a protease-negative *Aspergillus ficum* mutant. Appl Environ Microbiol 52: 1068–1073.
11 Iefugi M, M Chino, M Kato and Y Iimura. 1996. Raw-starch-digesting and thermostable alpha-amylase from the yeast *Cryptococcus* sp S-2: purification, characterization, cloning and sequencing. Biochem J 318: 989–996.
12 Itkor P, N Tsukagoshi and S Udaka. 1989. Purification and properties of divalent cation-depending raw-starch-digesting α-amylase from *Bacillus* sp B1018. J Ferment Bioeng 68: 247–251.
13 Kelly CT, MA McTigue, EM Doyle and WM Fogarty. 1995. The raw starch-degrading alkaline amylases of *Bacillus* sp IMD 370. J Ind Microbiol 15: 446–448.
14 Khyami-Horani H. 1996. Partial purification and some properties of an alkaline cellulase from an alkaliphilic *Bacillus* sp. World J Microbiol Biotechnol 12: 525–529.
15 Krishna C and M Chandrasekaran. 1996. Banana waste as substrate for α-amylase production by *Bacillus subtilis* (CBTK 106) under solid state fermentation. Appl Microbiol Biotechnol 46: 106–111.
16 Lehmacher A and H Bisswanger. 1990. Comparative kinetics of d-xylose and d-glucose isomerase activities of the d-xylose isomerase from *Thermus aquacticus* HB8. Biol Chem Hoppe-Seyler 371: 527–536.
17 Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31: 426–428.
18 Monma M, N Kagi and K Kainuma. 1989. Raw starch digestion by *Chalara paradoxica* immobilized in calcium alginate cells. Starch 41: 355–357.
19 Morita H and Y Fujio. 1997. High specific activity of raw starch digesting-glucoamylase producing *Rhizopus* sp A-11 in liquid culture. Starch 49: 293–296.
20 Oates CG. 1997. Towards an understanding of starch granule structure and hydrolysis. Trends Food Sci Technol 8: 375–379.
21 Okolo BN, LI Ezeogu and CO Ebisike. 1996. Raw starch digesting amylase from *Thermoactinomyces thalpophilus* F13. World J Microbiol Biotechnol 12: 637–638.

22 Pandey A. 1992. Production of starch saccharifying enzyme (glucoamylase) in solid cultures. Starch 44: 75–77.
23 Pandey A. 1992. Recent process development in solid state fermentation. Proc Biochem 27: 109–117.
24 Pandey A. 1995. Glucoamylase research—an overview. Starch 47: 305–309.
25 Ramadas M, O Holst and B Mattiasson. 1996. Production of amyloglucosidase by *Aspergillus niger* under different cultivation regimens. World J Microbiol Biotechnol 12: 267–271.
26 Sani A, FA Awe and JA Akinyanju. 1992. Amylase synthesis in *Aspergillus flavus* and *Aspergillus niger* grown on cassava peel. J Ind Microbiol 10: 55–59.
27 Smith A, M Rangarajan and S Hartley. 1991. d-Xylose (d-glucose) isomerase from *Arthrobacter* strain NRRL B3728. Biochem J 277: 255–261.
28 Yu RC and YD Hang. 1990. Amylolytic enzyme production by *Rhizopus oryzae* grown on agricultural commodities. World J Microbiol Biotechnol 6: 15–18.