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ORIGINAL PAPER

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Banana waste as substrate for α-amylase production by *Bacillus subtilis* (CBTK 106) under solid-state fermentation

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Abstract *Bacillus subtilis* CBTK 106, isolated from banana wastes, produced high titres of α-amylase when banana fruit stalk was used as substrate in a solid-state fermentation system. The effects of initial moisture content, particle size, cooking time and temperature, pH, incubation temperature, additional nutrients, inoculum size and incubation period on the production of α-amylase were characterised. A maximum yield of 5 345 000 U mg^{-1} min^{-1} was recorded when pretreated banana fruit stalk (autoclaved at 121 °C for 60 min) was used as substrate with 70% initial moisture content, 400 μm particle size, an initial pH of 7.0, a temperature of 35 °C, and additional nutrients (ammonium sulphate/sodium nitrate at 1.0%, beef extract/peptone at 0.5%, glucose/sucrose/starch/maltose at 0.1% and potassium chloride/sodium chloride at 1.0%) in the medium, with an inoculum-to-substrate ratio of 10% (v/w) for 24 h. The enzyme yield was 2.65-fold higher with banana fruit stalk medium compared to wheat bran.

Introduction

Banana (*Musa sapientum*) is grown extensively in tropical and subtropical countries, and 14.37% of the world's production is shared by India. From the banana produce, in addition to the fruit wastes, the stem, leaves and pseudostem are also accumulated as wastes in the environment, posing serious environmental problems. Several attempts have been made to utilise these wastes through ensilaging, and to eliminate or reduce the negative nutritional effects (Le Dividich et al. 1976). Protein enrichment of banana waste, skin and pulp using the yeast *Pichia spartinae* (Chung and Meyer 1979), enrichment of dry green banana pulp with protein by solid-state fermentation employing *Aspergillus niger* (Baldensperger et al. 1985), the production of biogas (Del Rosario and Pamatong 1985), ethanol (Iizuka et al. 1985) and starch from the pseudostem (Sharma et al. 1988), biomass production utilising banana skin with *Saccharomyces uvarum* (Enwefa 1991) and fermentation of whole banana waste liquor for the production of lactic acid (Lopez-Baca and Gomez 1992) have been reported. However, there is no information available on the use of these banana wastes as solid substrates for the production of industrial enzymes.

During the course of the study we found that banana fruit stalk contained 56.8% total sugar, 27.0% starch, 4.65% reducing sugar and 4.3% protein on a dry-weight basis, and *Bacillus subtilis*, isolated from banana wastes, could produce α-amylase at significant levels compared to other strains recovered from the same source. Hence in the present investigation an attempt was made to develop a suitable process for the production of α-amylase, using banana fruit stalk as solid substrate and employing *B. subtilis*.

Materials and methods

Microorganism

Bacillus subtilis CBTK 106, isolated from banana wastes collected from the market and maintained on nutrient agar slants, were used in the present investigation.

Preparation of substrate

Banana fruit stalk (peduncle) was sliced, spread on trays and oven-dried at 70 °C for 24 h. The dried slices were ground and sieved through standard-mesh sieves to obtain particles of various sizes

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ranging from 0.4 mm to 3.2 mm that were stored in polyethylene bags at room temperature (30 ± 2 °C) until use.

Medium

The mineral-salt medium recommended by Ramesh and Lonsane (1989) for α-amylase production under solid-state fermentation (SSF) using wheat bran was used in the present study. The composition of the medium used included 1.1 g $Na_2HPO_4 \cdot 2H_2O$, 0.61 g $NaH_2PO_4 \cdot 2H_2O$, 0.3 g KCl, 0.01 g $MgSO_4 \cdot 7H_2O$ and 100 ml distilled water, pH 7.0. Banana fruit stalk substrate, added to the medium, served as the source of carbon. This medium was used for optimising the various process parameters and additional nutrients. Necessary changes were made in the composition of the medium to provide optimal requirements for maximal enzyme production after standardisation.

α-Amylase production using banana fruit stalk under SSF

Procedures used for α-amylase production by *Bacillus* sp. using wheat bran during SSF, reported by Ramesh and Lonsane (1989), were adopted for SSF studies using banana fruit stalk. The following procedure was used unless otherwise stated. Banana fruit stalk particles (size 600–1200 μm), weighing 10 g, were moistened in 250-ml conical flasks with the prepared mineral medium to an initial moisture content of 65%, autoclaved at 121 °C for 60 min, cooled to about 30 °C and inoculated with a 10% (v/w) cell suspension. The contents of the flasks were mixed thoroughly to ensure uniform distribution of the inoculum and left for incubation in a slanting position at 35 °C for 48 h; samples were removed after 12 h for analysis. The contents were extracted (Ramesh and Lonsane 1989) and assayed for α-amylase.

Optimisation of process parameters

The various process parameters that influence the enzyme yield during SSF were optimised over a wide range. The strategy followed was to optimise each parameter independently of the others, and subsequently optimal conditions were employed in all experiments.

In a sequential order the various process parameters were optimized for maximal enzyme production: initial moisture content (30%–90%), suitable particle size (400–3200 μm), cooking temperature and time (100–130 °C and 0–120 min), initial pH of the medium (4–10 using HCl/NaOH), incubation temperature (20–55 °C), additional nutrients [$(NH_4)_2SO_4$, $NaNO_3$, peptone, beef extract, glucose, sucrose, starch, maltose, NaCl and KCl, each at concentrations of 0.1%–3.0% w/w on the basis of the weight of dry banana fruit stalk], inoculum size (5%–40%) and incubation period (0–120 h).

α-Amylase production under optimised conditions using banana fruit stalk

After optimisation of all the process parameters, α-amylase production under SSF using banana fruit stalk, was carried out under optimal conditions as described above. After extraction of the enzyme, the residual solid particles of banana fruit stalk were dried at 60 °C overnight and assayed for total sugars, reducing sugars, starch and protein in order to determine whether bacteria could utilize banana fruit stalk as a carbon source during solid-state fermentation. Appropriate controls were run using fresh, and unfermented banana fruit stalk particles for comparison. Whether banana fruit stalk could support α-amylase production was also ascertained by growing *B. subtilis* in the mineral-salts medium under optimised conditions without addition of banana fruit stalk particles and other carbon sources, under submerged fermentation.

Production of α-amylase under SSF using wheat bran

The methods adopted for substrate and media preparation, inoculation, incubation and enzyme extraction were similar to those reported by Ramesh and Lonsane (1987, 1989). The comparative production of the α-amylase was evaluated with media containing wheat bran with and without added soluble starch in the range 0%–3.0% (w/w).

Enzyme assays

α-Amylase activity was assayed by the method of Medda and Chandra (1980). One unit of α-amylase activity, expressed as dextrinizing activity, is defined as the amount of enzyme required to bring about the hydrolysis of 0.1 mg starch at optimal pH and temperature after 10 min of incubation.

Biochemical analyses

Samples were analysed for starch (Clegg 1956), total sugars (Dubois et al. 1956), reducing sugars (Miller 1959) and protein (Lowry et al. 1951).

Results

Suitability of banana fruit stalk as substrate for α-amylase production

Fresh unfermented banana fruit stalk contained 56.8% total sugar, 27.0% starch, 4.65% reducing sugar and 4.3% protein on a dry-weight basis. After fermentation it was observed that there was a drastic reduction in starch (75%) total sugars (85%) and reducing sugars (95%) and an increase in protein content (50%). There was no production of α-amylase in the mineral salts medium in the absence of any carbon source or banana fruit stalk.

Optimisation of process parameters for α-amylase production, using banana fruit stalk, under SSF

Optimal conditions that favoured maximal production of α-amylase by *B. subtilis* on banana fruit stalk as solid substrate were determined as 70% initial moisture content (Fig. 1), 400 μm particle size (Fig. 2), mixed particles of reduced size (Table 1), 60 min cooking time at 121 °C (Fig. 3), pH 7.0 (Fig. 4) and incubation at 35 °C (Fig. 5). In all these cases, either increase or decrease in the level of the variables led to significant reductions in the enzyme yield. Addition of either ammonium sulphate or sodium nitrate at 1.0% (w/w) (Fig. 6), and beef extract or peptone at 0.5% (w/w) as an additional

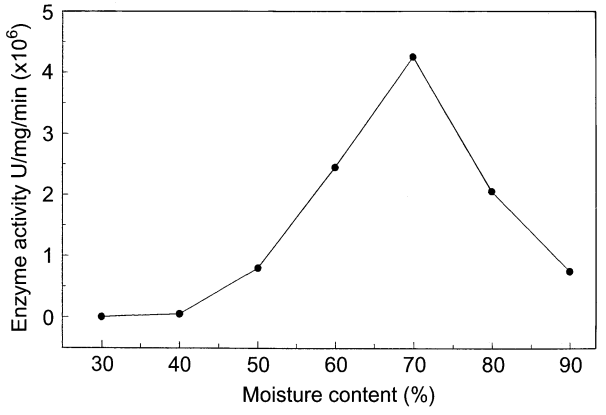

Fig. 1 Effect of initial moisture content (%) of the medium on α-amylase production by *Bacillus subtilis* CBTK 106 in a solid-state fermentation (SSF) system. Particle size 600 μm, cooking time and temperature 121 °C for 60 min, pH 7.0, incubation temperature 35 °C, inoculum size 10% (v/w)

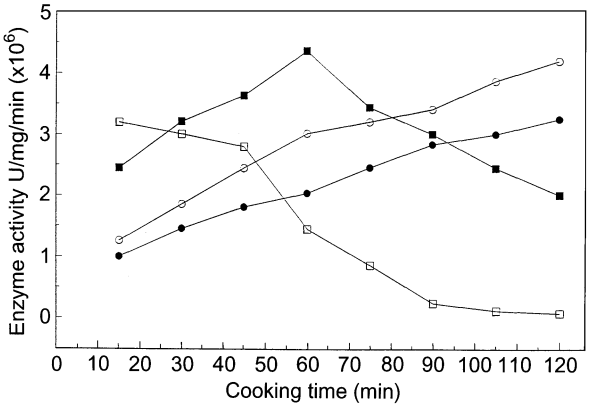

Fig. 3 Effect of cooking time (min) and temperature (°C) of the substrate on α-amylase production by *B. subtilis.* CBTK 106 in a SSF system. ● 100 °C, ○ 110 °C, ■ 121 °C, □ 130 °C. Moisture content 70%, particle size 400 μm, pH 7.0, incubation temperature 35 °C, inoculum size 10% (v/w)

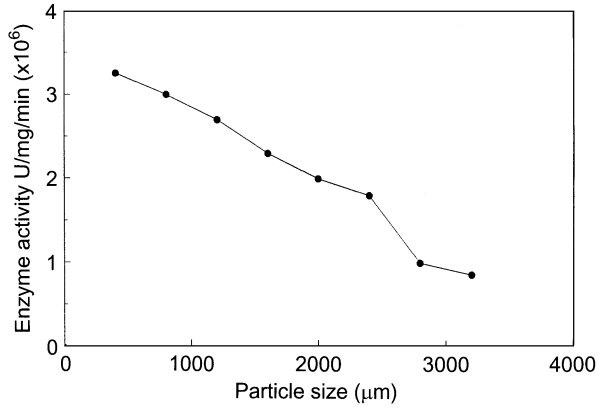

Fig. 2 Effect of substrate particle size(μm) on α-amylase production by *B. subtilis* CBTK 106 in a SSF system. Moisture content 70%, cooking time and temperature 121 °C for 60 min, pH 7.0, incubation temperature 35 °C, inoculum size 10% (v/w)

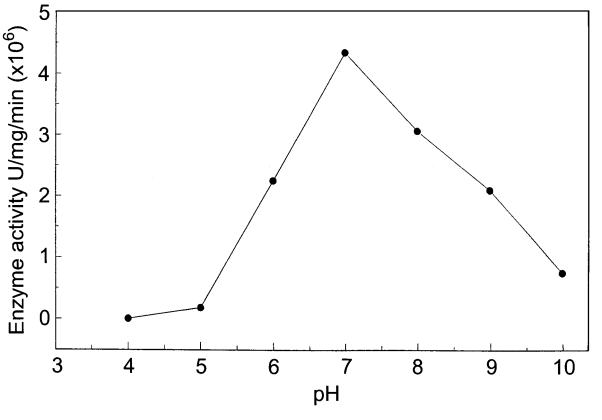

Fig. 4 Effect of pH variation of the medium on α-amylase production by *B. subtilis* CBTK 106 in a SSF system. Moisture content 70%, particle size 400 μm, cooking time and temperature 121 °C for 60 min, incubation temperature 35 °C, inoculum size 10% (v/w)

Table 1 Effect of mixed particle size on α-amylase production by *B. subtilis* CBTK 106 in a solid-state fermentation system

Particle size (μm)	$10^{-6} \times$ Enzyme activity (U mg^{-1} min^{-1})
> 400	2.65
400–1000	3.1
1000–1600	2.3
1600–2200	1.9
2200–2800	1.0
2800–3200	0.6
< 3200	0.1

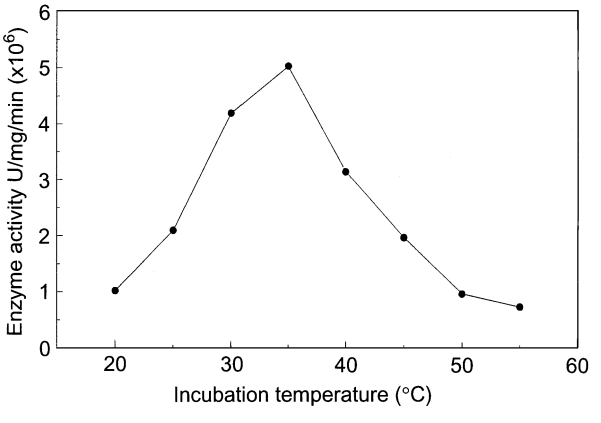

Fig. 5 Effect of incubation temperature (°C) on α-amylase production by *B. subtilis* CBTK 106 in a SSF system. Moisture content 70%, particle size 400 μm, cooking time and temperature 121 °C for 60 min, inoculum size 10% (v/w)

nitrogen source (Fig. 7), starch, maltose, glucose or sucrose at 0.1% (w/w), as an additional carbon source (Fig. 8) and NaCl or KCl at 1% (w/w) (Fig. 9) resulted in enhanced amylase synthesis. Further increase in the

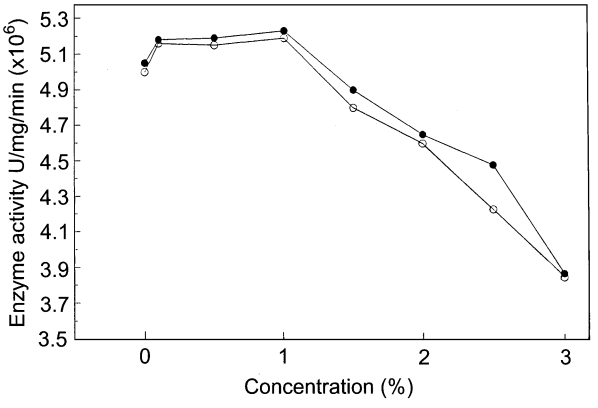

Fig. 6 Effect of additional inorganic nitrogen sources in the medium for α-amylase production by *B. subtilis* CBTK 106 in a SSF system. ● Ammonium sulphate, ○ sodium nitrate. Moisture content 70%, particle size 400 μm, cooking time and temperature 121 °C for 60 min, incubation temperature 35 °C, inoculum size 10% (v/w)

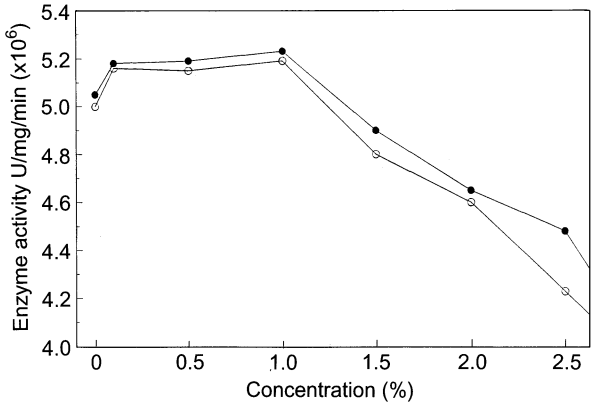

Fig. 7 Effect of additional organic nitrogen sources in the medium for α-amylase production by *B. subtilis* CBTK 106 in a SSF system. ● Beef extract, ○ peptone. Moisture content 70%, particle size 400 μm, cooking time and temperature 121 °C for 60 min, incubation temperature 35 °C, inoculum size 10% (v/w)

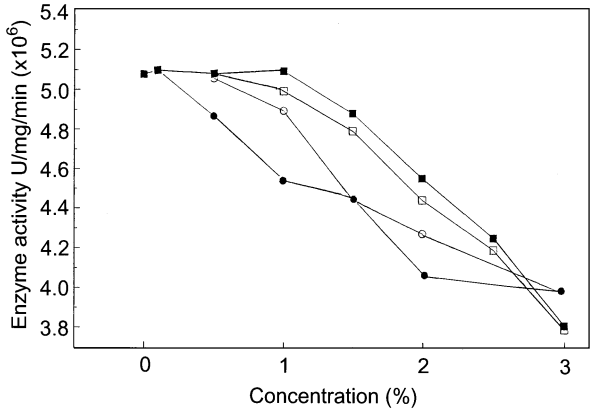

Fig. 8 Effect of additional carbon sources in the medium for α-amylase production by *B. subtilis* CBTK 106 in a SSF system. ○Glucose, ● sucrose, ■ starch, □ maltose at various concentrations (%, w/w). Moisture content 70%, particle size 400 μm, cooking time and temperature 121 °C for 60 min, incubation temperature 35 °C, inoculum size 10% (v/w)

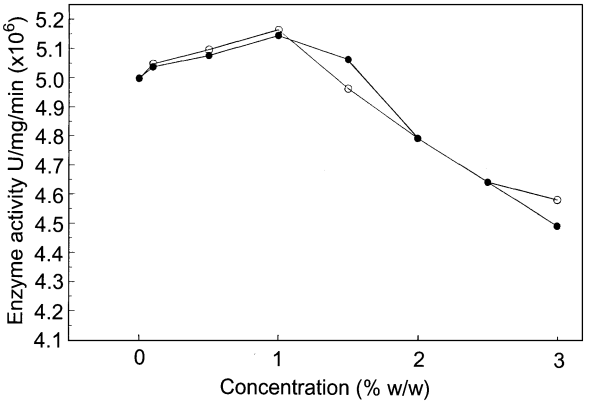

Fig. 9 Effect of additional salts in the medium for α-amylase production by *B. subtilis* CBTK 106 in a SSF system. ● Potassium chloride, ○ sodium chloride at various concentrations (%, w/w). Moisture content 70%, particle size 400 μm, cooking time and temperature 121 °C for 60 min, incubation temperature 35 °C, inoculum size 10% (v/w)

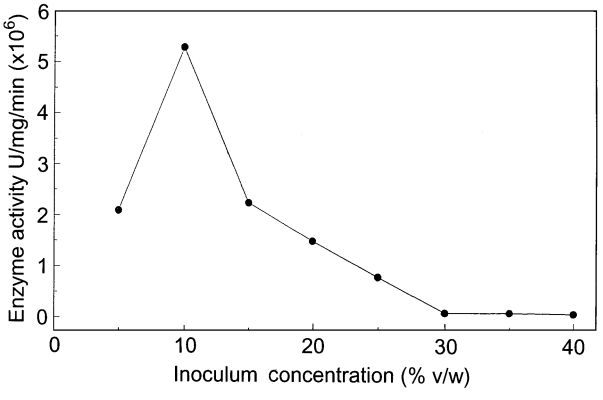

Fig. 10 Effect of inoculum size (%, v/w) on α-amylase production by *B. subtilis* CBTK 106 in a SSF system. Moisture content 70%, particle size 400 μm, cooking time and temperature 121 °C for 60 min, incubation temperature 35 °C)

concentrations of additional nitrogen, carbon or salts above the optimal level led to a sharp decline in enzyme yield. The inoculum-to-substrate ratio (v/w) had a significant impact on enzyme production. Data presented in Fig. 10 indicate that a considerable amount of inoculum, to the 10% (inoculum-to-substrate ratio, v/w) level, was required to promote maximal α-amylase activity. Inoculum ratios less or higher than 10% drastically affected the enzyme yield.

After standardisation of all process parameters, the time course of maximal enzyme production was standardized. Data presented in Fig. 11 demonstrate the fact that the incubation period influences the overall enzyme yield. Thus the enzyme yield increased significantly along with the incubation time, reaching a maximum of 5 345 000 U mg^{-1} min^{-1} at 24 h. Further increase in incubation period led to a significant reduction in enzyme activity.

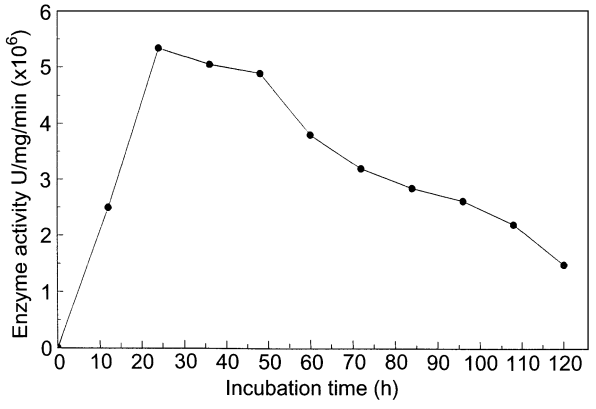

Fig. 11 Effect of incubation period on α-amylase production by *B. subtilis*, CBTK 106 in a SSF system. Moisture content 70%, particle size 400 μm, cooking time and temperature 121 °C for 60 min, incubation temperature 35 °C, inoculum size 10% (v/w)

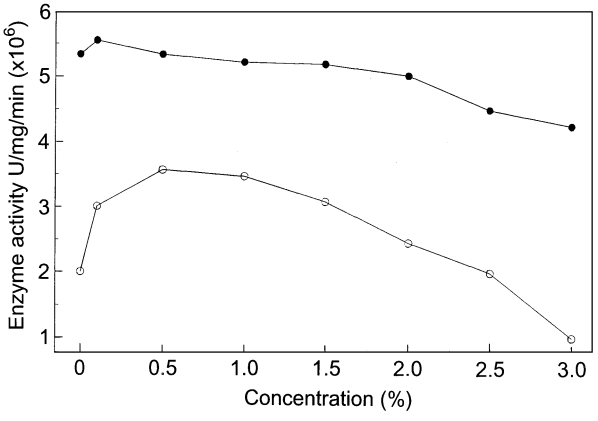

Fig. 12 A comparative study using wheat bran as substrate for α-amylase production by *B. subtilis* CBTK 106 in a SSF system. ○ Wheat bran, ● banana fruit stalk. Moisture content 70%, particle size 400 μm, cooking time and temperature 121 °C for 60 min, incubation temperature 35 °C, inoculum size 10% (v/w)

Comparison of α-amylase production under SSF using banana fruit stalk with that using wheat bran as the solid substrate

A comparative analysis of data obtained for α-amylase production with both wheat bran and banana fruit stalk suggested that banana substrates favoured higher levels of α-amylase production than wheat bran (Fig. 12). Addition of soluble starch to wheat bran medium, up to a concentration of 0.5%, led to a significant increase in amylase yield when compared to the insignificant increase recorded with 0.1% banana fruit stalk. Further increase in the starch concentration led to inhibition of α-amylase synthesis. On the whole, an approximately 2.65-fold increase in overall α-amylase yield could be recorded with banana fruit stalk as substrate compared to wheat bran without addition of soluble starch to the medium.

Discussion

Results obtained for the optimization of process parameters for SSF production of α-amylase with banana fruit stalk as substrate demonstrated clearly the impact of the process parameters on the gross yield of enzymes as well as their independent nature in influencing the organism's ability to synthesize the enzyme.

As in the present study, a 70% moisture content was reported earlier to enhance maximal fungal pectinase production on cassava fibrous waste residue (Budiatman and Lonsane 1987). A reduction in enzyme yield at high initial moisture content might be due to the steric hindrance of the growth of the organism through reduction in interparticle spaces and impaired oxygen transfer (Nigam 1990; Sandhya and Lonsane 1994). A lower moisture content also resulted in a decline in enzyme yield. Perhaps this results from suboptimal growth, less substrate swelling and high water tension during low moisture (Lonsane et al. 1985).

The particle size (specific surface area) is a critical factor in SSF. Banana fruit stalk particles of 400 μm favoured maximal α-amylase production compared to larger particles. A similar trend was reported for glucoamylases with wheat bran (Pandey 1991) and cellulase production with coir pith of small particle size (Muniswaran and Charyulu 1994). With smaller particles the surface area for growth is greater but the interparticle porosity is less. With larger sizes, the porosity is greater but the saturated surface area is less. These two opposing factors, decrease in surface area and increase in porosity, probably interact to determine the values corresponding to optimum growth and enzyme production (Muniswaran and Charyulu 1994).

Banana fruit stalk, which contains about 27% starch, required pretreatment before it could be used as a solid substrate for α-amylase production. From the results it is inferred that prolonged cooking above 60 min at 120 °C resulted in a reduction in enzyme yield. Perhaps the starch granules, which might have lost their semi-crystallinity when subjected to a longer cooking period, then contributed to the increased viscosity consequently causing decreased growth and enzyme yield.

The initial pH of the medium influenced the rate of enzyme production on banana fruit stalk, as was reported for wheat bran (Ramesh and Lonsane 1987). The present results clearly indicate that variations in pH influence the efficiency of the organism whatever the type of solid substrate used.

The usual temperature maintained in SSF systems is in the range 25–32 °C, depending on the growth kinetics of the microorganism employed for the fermentation purposes (Lonsane et al. 1985). However, the optimal temperature recorded for maximal growth and α-amylase production by *B. subtilis* in the present study (35 °C) is almost identical to that reported for *B. licheniformis* growing on wheat bran (Ramesh and Lonsane 1989). These results indicate the independent

nature of the temperature effect irrespective of the type of solid substrate used.

Addition of either ammonium sulphate or sodium nitrate enhanced α-amylase production significantly. These results advocate nitrogen enrichment of the medium for maximal α-amylase production using banana fruit stalk. A similar increase in the carbohydrate utilisation and reduction in fermentation time was reported for sugarcane-press mud medium enriched with 1.8% ammonium sulphate (Sandhya and Lonsane 1994).

Additional carbon sources, both glucose and sucrose, tended to inhibit enzyme synthesis, although there was a marginal increase when the sugars were added at the 0.1% level. Banana fruit stalk, which has a high starch content, when attacked by α-amylase during fermentation could have undergone degradation resulting in the accumulation of reducing sugar, which would have led to enhancement of the sugar concentration of the substrate and catabolite repression of α-amylase synthesis.

Inoculum size controls the initial lag phase (Nystorm and Kormuta 1975). A smaller inoculum extends the lag phase, whereas larger inoculum size increased the moisture content to a significant extent. The free excess liquid present in an unabsorbed form will give rise to an additional diffusional barrier together with that imposed by the solid nature of the substrate and lead to a decrease in growth and enzyme production (Muniswaran et al. 1994). Observations of the effects of inoculum size on maximal enzyme production in the present study agree well with the facts stated above.

The decline in enzyme activity observed after 24 h of incubation, where a maximum was noted, might be due to denaturation and/or decomposition of α-amylase as a result of interactions with other compounds in the fermented medium (Ramesh and Lonsane 1987) or due to inactivation by proteases secreted into the system (data not shown). A similar trend was also reported by Zhu et al. (1994).

Substrates traditionally used in solid-state fermentation include rice, wheat, millet, barley, corn and soya bean (Hesseltine 1972; Yang 1988). On the basis of the results of the present study it is concluded that the utilisation of banana fruit stalk as solid substrate could lead to large-scale production of industrial enzymes and also contribute to safe and economic waste management in the environment, where these wastes are continuously accumulated and cause serious pollution problems.

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