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Solid State Fermentation of Banana Stalk for Exoglucanase Production

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ABSTRACT

Bacillus subtilis was cultured in solid-state fermentation (SSF) of Banana stalk to produce exoglucanase. The fermented biomass was harvested after 72 h of SSF at pH 7 and temperature 35°C. It was filtered and centrifuged at 10,000 rpm at –10°C and supernatant was collected as crude enzyme extract. Maximum activity of exoglucanase (3.48 IU/mL/min) was obtained from the medium fermented with 70% moisture content, 5 mL inoculum, 0.1% peptone, 0.4% yeast extract and 0.2% Tween-80 at pH 7 and temperature 35°C. SSF was found to be more productive than liquid state fermentation (LSF) in terms of exoglucanase yields. The partial purification of exoglucanase was carried out through $(NH_4)_2SO_4$ precipitation and maximum purification was achieved with 20% $(NH_4)_2SO_4$ saturation.

Key Words: Exoglucanase; *Bacillus subtilis;* Solid State Fermentation; Optimization; Partial purification

INTRODUCTION

Cellulases are among the industrially important hydrolytic enzymes and are of great significance in present day biotechnology. Cellulases are widely used in the food, feed, textile and pulp and paper industries (Nakari & Pentilla, 1996). In textile industry, these are used in biopolishing of cotton fabrics and to produce stone washed look of denim garments. Microbial conversion of cellulosic/lignocellulosic biomass into useful products is a complex process involving combined action of three enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase EC (3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Erikson & Patterson, 1975).

Production of cellulases and their properties have been extensively studied during recent years (Rajoka & Malik, 1997). The development of microbial strains, media composition and process control have all contributed to achievements of high levels of extra cellular accumulation of cellulases for subsequent applications in industrial processes (Gosh *et al*., 1987).

The exoglucanase have the tunnel shape and the tunnel is made up of loops which have strong interaction with the salt linkers, hydrogen bonds (Sinnott, 1997). Exoglucanases are of two types; 1, 4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) which removes cellobiose units and 1, 4-β-D-glucan glucohydolase (EC 3.2.1.74) which removes glucose units both acting from the non-reducing ends of oligosaccharides produced by the action of endoglucanase (Mullings, 1985).

Banana fruit stalk abundantly available in banana production fields and markets appears to be a favorable substrate as it is cheaply available in the tropical and subtropical countries and has a cellulose content of 23.85% (Krishna, 1996). SSF offers advantages over fermentation in liquid broth (submerged fermentation) like higher product yield, better product quality, cheaper product recovery and cheaper technology (Oguntimein *et al.*, 1992). This paper reports the optimization of SSF process for exoglucanase production by *Bacillus subtilis* grown on banana fruit stalk.

MATERIALS AND METHODS

Substrate. Waste banana stalks used as substrate for exoglucanase production were obtained from a fruit shop of University of Agriculture Faisalabad. Substrate was chopped into small pieces of uniform size, spread on trays, dried in oven (70°C) and ground to powder form (2 mm particle size) in an electric grinder.

Fermentation organism and inoculum. Pure culture of *Bacillus subtilis* procured from NIBGE, Faisalabad was maintained on sporulation medium slants at pH 7 and 35°C temperature (Kaukab *et al*., 2002). The defined inoculum medium contained (g/100 ml); glucose 2, yeast extract 0.3, peptone 0.5, NaCl 1.5, $NaH_2PO_4.2H_2O$ 0.61, $Na_2HPO_4.2H_2O$. 1.1, KCl 0.3. $MgSO_4.7H_2O$. The medium was adjusted to pH 7 with M HCl/M NaOH and sterilized (121°C) for 15 minutes. Spores were transferred to this medium and it was incubated for 72 hours at 35°C in incubator/shaker (Gallenkemp) at 150 rpm for 72 hours to get 10^7-10^8 spores/ml.

Solid state fermentation and enzyme extraction. The fermentation flasks (in triplicate) containing 10 g substrate moistured with the mineral salts medium (exculding yeast extract and peptone) were autoclaved and inoculated (5 ml inoculum). The fermentation flasks were incubated at 35°C for 72 hours (unless otherwise indicated) without shaking for solid state fermentation.

Enzyme extraction. The enzyme was extracted by a simple contact method (Krishna *et al*., 1996). The fermented

samples were shaken (150 rpm) with 100 ml of 0.1 M sodium citrate buffer of pH 4.8 for one hour and filtered through Whatman No. 1 filter paper. The filtrates were centrifuged at 1000 rpm (-10°C) to remove spores of the organism and supernatants were used as crude enzyme extracts.

Optimization of conditions. Growth medium of banana stalk moistened with varying volumes of mineral salts medium was fermented with *Bacillus subtilis* for different fermentation periods with varying inoculum size. The effect of varying concentrations of peptone, yeast extract and Tween-80 was also investigated. The level of a parameter optimized in an experiment was maintained in the subsequent studies

Comparison of SSF and LSF. To compare LSF and SSF, the LSF medium was prepared in duplicate by adding 5 g of banana stalk in 100 ml optimum growth medium. After autoclaving and inoculation the LSF flasks were incubated on shaker (120 rpm) for 72 hours under continuous shaking (150 rpm). The SSF flasks containing optimum growth medium were processed as described earlier.

Partial purification. In order to achieve maximal precipitation of exoglucanase, several (0, 20, 40, 60 and 80%) $(NH_4)_2SO_4$ solution concentrations were tested. Crude enzyme was saturated by adding different amount of $(NH_4)_2SO_4$ in crude extract under constant stirring and it was left undisturbed overnight. Precipitation was then allowed for 1 hr at 4°C. The supernatant obtained after centrifugation was separated. Both the supernatant and filtrate were subjected to enzyme assay and protein estimation (Barda & David, 1949).

Enzyme assay. An appropriately diluted culture filtrate was used to determine exoglucanse activity in 0.1 M sodium citrate buffer (pH 4.8) by a spectrophotometric method using filter paper strips 1.0x6.0 cm as substrate and dinitrosalicylic acid (DNS) as coupling reagent (Gosh, 1987). One unit of enzyme activity in each case was defined as the amount of enzyme which released one βmole of glucose per minute.

RESULTS AND DISCUSSION

Fermentation parameters were optimized for production of exoglucanase by *Bacillus subtilis* in SSF medium of banana stalk and results have been discussed as under:

Fermentation period. SSF medium of banana stalks was inoculated and incubated at pH 7 and 35°C and triplicate flasks were processed for different time periods. The exoglucanse activity increased upto 72 h (1.83 IU/mL/min) of incubation and decreased, thereafter (Fig. 1) Romero *et al*. (1999) found the maximum activity of cellulases at 100 h but after that a steep decrease was observed by increasing the fermentation time.

Moisture level. The experiment was carried out to study the effect of varying moisture content. Maximum exoglucanase

Fig. 1. Effect of varying Fermentation period on Exoglucanase production by B. subtilis

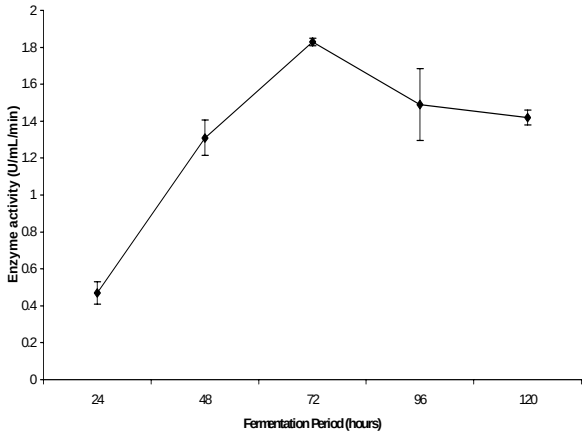

Fig. 2. Effect of varying concentrations of peptone on exoglucanse production by Bacillus subtilis

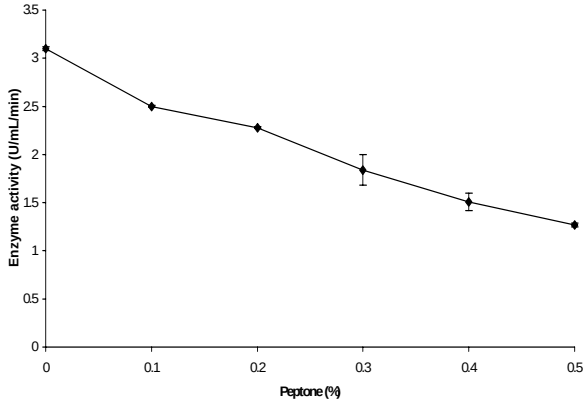

Fig. 3. Effect of varying concentration of tween-80 on exoglucanase production by B. subtilis

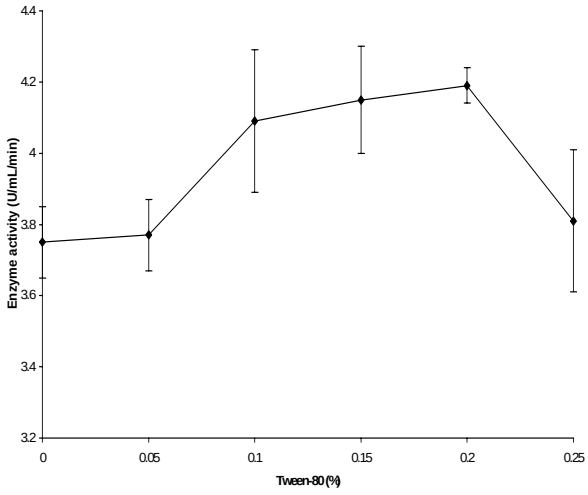

Table I. Activity of exoglucanase produced by *Bacillis subtilis* with varying Moisture levels

Moisture level (%)	Exoglucanse activity (IU/mL/min)
40	1.14
50	1.98
60	2.40
70	3.48
80	1.75

* pH 7; temperature 35°C (Krishna, 1996)

Table II. Activity of exoglucanase produced by *B. subtilis* with varying Inoculum size

Inoculum size (mL)	Exoglucanse activity (U/mL/min)
3	2.01
4	2.71
5	3.52
6	3.48
7	2.85

Table III. Activity of exoglucanse produced by *B. subtilis* with varying concentrations of yeast extract

Yeast Extract (%)	Exoglucanse activity (IU/mL/min)
Control	3.46
0.1	3.68
0.2	3.96
0.3	4.28
0.4	4.75
0.5	3.92

Table IV. Activities of exoglucanse produced by *B. subtilis* in SSF and LSF under optimum conditions*

Type of Fermentation*	Exoglucanase activity (IU/mL/min)		
	A	B	Mean
SSF	2.75	2.45	2.6
LSF	0.97	0.71	0.84

* Optimum conditions
pH = 7
Temperature = 35°C
Fermentation period = 72 hours
Inoculum = 5 mL
Yeast extract = 0.4%
Tween-80 = 0.2%

(3.48 IU/mL/min) production by *Bacillus subitilis* was observed with 70% moisture (Table I). The results indicated that when moisture level increased beyond a certain limit the enzyme activity started decreasing. This decline may be attributed to poor aeration in SSF and partial adsorption of enzyme to the substrate. Xia *et al.* (1999) studied the cellulase production by solid state fermentation on lignocellulosic waste and reported that water content of solid substrate is one of the key factors in cellulase production experiments. SSF at a water content of 70% was found to be the most suitable for cellulase production.

Inoculum size. There was a gradual increase in enzyme production by *Bacillus substilis* by increasing inoculum size and optimum exoglucanase (3.52 U/mL/min) was recorded in the medium receiving 5 ml inoculum under pre-optimized conditions. A further increase of inoculum upto (6-7 ml) showed decreasing trend (Table II). Results of our study are in line with those of Zhang *et al*. (2001) who investigated the effect of inoculum size on cellulase synthesis by *Trichoderma viride* and described that the impact of the amount of inoculant on cellulase production was small and 5% inoculum was the most suitable.

Peptone. Production of hydrolytic enzymes is enhanced by the additional nitrogen sources like $(NH_4)_2SO_4$, peptone and yeast extract. In our case all concentrations of peptone inhibited the microbial growth and enzyme production (Fig. 2). Control (with no peptone) gave the maximum exoglucanase yield (0.1 mL/min at 0.1 max). Our results are in contrast with the work of Enari *et al*. (1977) who reported that good cellulase production can be obtained with the organic nitrogen sources such as yeast extract and peptone but these sources were not effective replacement for inorganic nitrogen sources.

Yeast extract. The medium with 0.4% yeast extract gave maximum (4.75 U/mL/min) exoglucanase activity under preoptimized process conditions. Further addition of yeast extract (0.5%) caused a decrease in enzyme production (Table III). The results are comparable to those of Oguntimein *et al*. (1992) who reported that CMCase and Fpase (exoglucanase) activities were affected by all nitrogen sources but non significant effects on β-glucosidase was observed. With 0.5 g/litre yeast extract as sole nitrogen source when added to the medium gave highest exoglucanase activity.

Effect of tween-80. Exoglucanse production by *Bacillus subtilis* was studied with varying concentrations of Tween-80 in the preoptimized banana stalk medium. An enhanced enzyme production by the bacteria was observed with the addition of Tween-80. Maximum activity of exoglucanase was observed at concentration of 0.2% (4.19 U/mL/min) as shown in Fig. 3 and with further increase of this surfactant, the yield of enzyme was decreased. The results are well in line with the work of Oguntimein *et al*. (1992) who studied the effect of Tween-80 on cellulase production and observed increased enzyme activity when it was added to enzyme production medium. When Tween-80 was added (0.05% v/v) to medium used for fungal species such as *T. reesei*, the FPase production was increased (Mandals & Weber, 1969).

Comparison of SSF with LSF. Triplicate flasks containing the optimum growth medium for exoglucanse production were subjected to SSF and LSF for 72 hours. Results given in Table IV indicated that SSF showed more production of exoglucanse (2.6 IU/mL/min) as compared to LSF (0.84 IU/mL/min). Murthy *et al*. (1993) described that SSF involves the growth of microorganisms on solid substrate and through SSF process cellulase can be produced on a large scale with high productivity and uniform quality (Xia

et al., 1999).

Partial Purification through ammonium Sulfate $(NH_4)_2SO_4$. Exoglucanse produced by *Bacillus subtilis* was partially purified by the addition of $(NH_4)_2SO_4$ into the crude enzyme extract. Maximum exoglucanase purification was achieved with 20% $(NH_4)_2SO_4$ precipitation. The analysis of residual enzyme protein yielded 0.094 and 0.12 mg/mL protein in supernatants and residues, respectively (Table V). Specific activity of exoglucanase was 230.93 and 229.95 for residue and the supernatant, with 1.51 and 1.50 fold purification, respectively. Andrade *et al*. (1994) purified complete celluloytic system produced which included an exoglucanse, endoglucanse and β-glucosidase to about 36-fold with a recovery of about 12%

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