PRODUCTION OF SINGLE CELL PROTEIN, ESSENTIAL AMINO ACIDS, AND XYLANASE BY *PENICILLIUM JANTHINELLUM*

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Microbial biomass having 46% crude protein content and enriched with essential amino acids as well as extracellular xylanase activity (100-150 IU/ml) was produced by an efficient fungal strain, *Penicillium janthinellum* (NCIM St-F-3b). Optimization studies for maximum xylanase and biomass production showed that the fungus required a simple medium containing bagasse hemicellulose as carbon source and ammonium sulphate as the nitrogen source. Therefore bagasse, which is a waste product of the sugar industry, can be efficiently used in microbioal biomass protein preparation for animal feed.

Keywords: Penicillium janthinellum; Microbial biomass production; Xylanase; Bagasse hydrolysate; Crude protein

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INTRODUCTION

In recent years there has been an increasing research focus on efficient utilization of agro-industrial residues.These residues will not only produce food, feed and fuels, but also reduce environmental pollution. Microbial biomass protein also referred to as Single Cell Protein(SCP) can be a very attractive alternative as it can be produced from a variety of materials including hydrocarbons, lignocellulosic materials, waste from wood industry, whey, molasses etc. Single cell protein thus produced will be an alternative to conventional proteins like casein, soyabean meal, egg protein or meat protein in animal feed.However, high nucleic acid content of microbial biomass protein should be reduced by enzymatic or other methods when used as human food (Kunhi and Rao 1995).

In India, bagasse is a byproduct of sugarcane industry produced annually in millions of tons. Bagasse is used as a fuel directly by sugar industry. Another significant applications of bagasse has been for the production of protein –enriched cattle feed and enzymes. Bagasse is rich in fermentable carbohydrates (≈40% hexosan and ≈30% pentosan and ≈20% lignin) with low protein and ash content.(Pandey et al 2000). Pretreatment is necessary to remove a major part of lignin so that microorganisms can utilize the hemicellulose and cellulose portion for hydrolysis or use it as a carbon source . Moo-Young et al (1993) cultivated the food grade fungus, *Neurospora sitophila* on bagasse for food and fodder grade mycoprotein production. Rodriguez and Gallardo(1992) used mixed bacterial culture (*Cellulomonas* and *Pseudomonas*) for SCP production using bagasse pith as substrate.

The use of fungi to convert agricultural waste offers advantages such as easy filtration and drying of the product without the necessity of using sophisticated

equipment, unlike bacterial fermentations. It is also potentially adaptable to relatively simple technology applicable to rural situations in developing countries(Pandey et al 2000).

In the present study, steam treatment is used as a pretreatment method to obtain bagasse hydrolysate. At NCL, a process has been developed based on steam explosion to seperate lignin, cellulose and hemicellulose along with relevant downstream processing(Patent application 1893DEL2007dated 27August, 2007).Downstream processing led to pure cellulose(α-cellulose>92%) and lignin, both of which have commercial applications.

EXPERIMENTAL

Materials

Malt extract, Yeast extract, Ammonium sulphate were purchased from Himedia , India. Glucose agar and potassium hydrogen phosphate were purchased from Qualigens, India. Xylose and Tween 80 were purchased from Loba Chemicals, India. Dinitrosalicylic acid and Xylan were purchased from Sigma Chem. Co. USA. Peptone was purchased from Sarabhai Chemicals, India. All other chemicals used were of analytical grade. Native bagasse and Wheat bran were purchased from local market.

Methods

Pretreatment of bagasse

Steam treatment was given to bagasse as per Patent No 1893/DEL/2007. Bagasse was exposed to high steam pressure (15kg) and extracted with water. The hydrolysate was filtered off and concentrated in a rotary evaporator to the desired concentration levels. Dry powder so obtained was used in the medium. It was characterized using Dionex HPIC consisting of GS 50, quaternary gradient pump, ED-50 elctrochemical detector Rheodyne injector and chromeleon software. Solutions were made in aqueous alkali (NaOH). The chromatogram showed 75-80% xylose content in hydrolysate. Bagasse hydrolysate thus obtained retained the hemicellulose portion which served as carbon source for fungus *Penicillium janthinellum* during SCP in addition to xylanase production.

Microorganism

Fungal culture: *Penicillium janthinellum* (NCIM-St-F-3b) was obtained from National Collection of Industrial Microorganisms and routinely maintained on PDA slant.

Growth and enzyme production

The inoculum was prepared by inoculating 5.0 ml of MGYP medium (0.3% malt extract, 1.0% glucose, 0.3% yeast extract, and 0.5% peptone) with four days old slant followed by incubation at 28°C for 48 hrs with shaking at 200 rpm. Optimization studies were carried out by transferring 10% inoculum in 50 ml fresh MGYP medium and replacing glucose with xylose, xylan, wheat bran, bagasse and bagasse hydrolysate and

nitrogen sources yeast extract and peptone by ammonium sulphate and diammonium hydrogen phosphate followed by incubation at 28°C for 48 h with shaking at 200 rpm. The extracellular broth was collected by centrifugation at 10,000 rpm for 20 min and used for estimation of reducing sugars as well as xylanase and cellulase activities. Biomass was washed twice with water, dried to constant weight and used to determine protein content. All experiments were performed in duplicate.

Determination of xylanase activity

Xylanase activity was determined according to Mishra et al (1985) by incubating 1.0 ml of reaction mixture containing suitably diluted enzyme with 0.5 ml of 1.0% of D-xylan in acetate buffer (final concentration 0.05M (pH 5.0) for 30 min at 50°C. The reducing sugar formed was determined by DNS method (Miller 1959) using D-xylose as standard. One unit (IU) of enzyme activity was defined as the amount of enzyme which produces one µmol of reducing sugar/min under the assay conditions.

Determination of Cellulase activity

Cellulase activity was determined according to Mishra et al(1985) by incubating suitably diluted enzyme and 25 mg Whatman No.1 filter paper in 0.05M acetate buffer (pH5.0) for 1h at 50°C. One unit (U) of filter paper activity was determined as the amount of enzyme reducing 1.0 µmol of glucose/min under the assay conditions.

Protein estimation

Biomass obtained while harvesting extracellular broth was washed free of nutrient media and dried till constant weight. The dried biomass was powdered and analysed for its nitrogen content by CHN-S autoanalyser (FLASH EA 1112 series) and crude protein value was expressed as N×6.25.

Estimation of Amino Acids:

1 mg of biomass was hydrolysed with 6N HCl (110ºC, 24 h) finally suspended in 1mM HCl and derivatized with AccQ-Taq fluor reagent (6-aminoquinolyl-N-hydroxysucinimidyl carbamate or AQC).Amino acid analysis was done by HPLC (Water AccQ-Tag column NovaPak TM C 18, 4µM column. The mobile phase comprising a linear gradient acetate –phosphate buffer(solution A) and 60% acetonitrile (solution B) for 35 min. step gradient as given in AccQ-Tag.Chemistry package at 37°C at a flow rate of 1ml/min.

RESULTS AND DISCUSSION

Effect of Carbon Sources on SCP and Xylanase Production

The fungal culture showed growth in MGYP and MXYP media (Table -1). Malt extract was omitted in subsequent experiments as it was not essential for growth of the organism. Milagres et al (1993) reported 9.8 IU/ml and 3.8 IU/ml of xylanase activity from a local isolate of *Penicillium janthinellum* culture using xylan and bagasse as carbon sources respectively. *Penicillium janthinellum* (ST-F-3B) culture produced 4.0 IU/ml

with xylan and 4.4 IU/ml with bagasse (Table-1). Xylose and xylan are costly carbon sources therefore replaced by wheat bran and native bagasse. In case of native bagasse and wheat bran there was apparent increase in biomass weight probably due to unutilized bagasse and wheat bran present in the biomass. Maximum xylanase was detected when bagasse hydrolysate was used as carbon source (38.3IU/ml). Surfactants have been reported to increase the yield of extracellular enzymes (Reese and Maguire, 1969). When cellulose powder was added in production medium with T.80, an increase in xylanase (55.3IU/ml) was detected (Table-2) but again unutilized cellulose powder added to the biomass weight. Cellulase activity was not detected in any culture broth (data not shown).

Table 1. Effect of Carbon Sources on Xylanase and Microbial Biomass Production, and Sugar Utilization of *Penicillium janthinellum* (NCIM-St-F-3b)

Medium	% Sugar (Xylose) utilized	Dry wt of biomass (gm) in 50 ml	Xylanase activity IU/ml
MGYP	-	0.35	-
MXYP	84	0.38	3.3
YPXylan	97	0.3	4
YPWB	99	0.77	9.7
YPNB*(0.5)	100	0.54	4.2
YPNB(1.0)	100	0.74	4.4
YPNB(2.0)	99	1.41	2.6
YPH*(0.5)	98	0.03	27.5
YPH(1.0)	98	0.17	31.3
YPH(2.0)	93	0.63	38.3

MGYP represents malt extract, glucose, yeast extract and peptone medium.
MXYP represents malt extract, xylose, yeast extract and peptone medium.
YPNB* represents yeast extract, peptone and native bagasse (The figure in the bracket is % of bagasse in the medium).
YPH* represents yeast extract, peptone and bagasse hydrolysate.(The figure in the bracket is % of sugar) in the medium.
YPWB represents yeast extract, peptone and wheat bran medium

Effect of Nitrogen Sources on Xylanase and SCP Production

When different nitrogen sources like peptone, yeast extract, ammonium sulphate and diammonium hydrogen phosphate were used in the production medium, ammonium sulphate gave highest (151 IU/ml) xylanase activity (Table-2). Use of ammonium sulphate will reduce the cost of the medium as organic nitrogen sources are costlier than inorganic nitrogen sources. In order to reduce cost further, fertilizer and commercial grade diammonium hydrogen phosphate were used in the production medium but resulted in poor biomass and xylanase activity. Increasing bagasse hydrolysate to 3% in the production medium showed less biomass and xylanase activity probably due to inhibitory material present in the hydrolysate (Table-2). Adsul M. et al (2004) reported 130 IU/ml

of xylanase activity from *Penicillium janthinellum* NCIM1171 strain in 6 days using NaClO2 treated bagasse sample. *Penicillium janthinellum* culture used in the present studies requires only 2 days suggesting that productivity is better than 1171 culture.Addition of wheat bran,cellulose powder and surfactant (T.80) to NHK* medium yielded less xylanase activity as compared to only NHK medium .

Table 2. Effect of Nitrogen Sources on Microbial Biomass Production and Sugar Utilization of *Penicillium janthinellum* (NCIM-St-F-3b)

Medium with 2% hydrolysate as carbon source	%sugar(xylose) utilized	Dry wt biomass(gm) in 50 ml	Xylanase activity IU/ml
PHK*	95	0.43	76.70
NHK*(0.5%)	98	0.03	42.70
NHK(1.0%)	98	0.17	104.20
NHK(2.0%)	93	0.68	151.70
NHK(3.0%)	82	0.62	104.20
YHK*	74	0.37	75.30
$(NH_4)_2HPO_4$HK (commercialgrade)	35	0.14	9.50
$(NH_4)_2HPO_4$HK (fertilizer grade)	30	0.13	2.80
NHK(2%)+WB(2%)+ T.80+CP(1%)	85.5	1.72	55.50
NHK(2%)+WB(2%)+T.80	85.5	1.03	97.30

PHK* represents peptone, bagasse hydrolysate and potassium dihydrogen phosphate medium.
YHK* represents yeast extract, bagasse hydrolysate and potassium dihydrogen phosphate medium
NHK* represents ammonium sulphate, bagasse hydrolysate and potassium dihydrogen phosphate medium.
$(NH_4)HPO_4$HK represents diammonium hydrogen phosphate,bagasse hydrolysate and potassium hydrogen phosphate medium.

Nitrogen and Protein Content of Biomass of Penicillium janthinellum

Maximum crude protein (46%) and protein productivity (19.3) is obtained when 2% bagasse hydrolysate is used as carbon source (Table-3). Srinivasan et al (1983) and Rao et al (1983) used alkali treated and washed rice straw as substrate but hemicellulose portion was lost during washing the straw resulting in low protein (20 %) and (30 %) content respectively .Samir et al (1994) used white rot fungus *Pleurotus ostreatus* NRRL-2336 and chemically treated bagasse as substrate to obtain 22.6% crude protein after 14 days fermentation. As compared to other fungal cultures like *Aspergillus terreus* (Garg S.K and Neelakantan S., 2004) and Polyporus BH1and BW1(Nigam, 1990)which take 72h and 192h.to produce 20.6%,18-22% and 21-23 % crude protein, *Penicillium janthinellum* produced 40-50% protein in less time (48 h).

Table 3. Protein Content and Productivity of *Penicillium janthinellum*

Sample	% Nitrogen	% Protein In biomass	Protein Productivity (G/l/h)
PHK(2.0%)	4.77	29.81	12.4
NHK(0.5%)	6.83	42.68	17.4
NHK(1.0%)	6.74	42.12	17.5
NHK(2.0%)	6.93	46.31	19.3
NHK(3.0%)	7.05	44.06	18.4
YHK(2.0%)	5.12	32.00	13.3

% Nitrogen is determined by CHN-S autoanalyser (FLASH EA 1112 series) and protein value was expressed as N×6.25.
The fig in the bracket represents % sugar in the medium

Estimation of Essential Amino Acids

Table 4 showed the presence of all essential amino acids. When compared with FAO standard proteins, fungal biomass contained high content of lysine, valine, and tyrosine but low content of methionine. However content of cysteine and methionine was adequate.

Table 4. Comparison of Essential Amino Acids (g amino acid / 100g protein)

Amino Acid	*P. Janthinellum*	FAO standard
Threonine	3.3	2.8
Lysine	14.0	4.2
Valine	9.0	4.2
Leucine	2.4	4.8
Tyrosine	4.6	2.8
Phenylalanine	2.7	2.8
Methionine	0.3	2.2
Cystine*+ methionine	2.6	2.2

Biomass is hydrolysed with 6 N HCl (110^{o}C,24h.) and then amino acids were analysed as described in materials and methods.* Cystine content was determined by the method of Habeeb A. (1972)

CONCLUSIONS

The fungal culture *Penicillium janthinellum* (St-F-3b) was grown on simple medium containing bagasse hydrolysate, ammonium sulphate, and potassium dihydrogen phosphate for the production of microbial biomass protein (46 %). Steam treatment used in this work was developed in NCL and retained hemicellulose portion of bagasse which is otherwise wasted in other pretreatments using acid or alkali. Harvesting before onset of sporulation and high growth rate are the key factors of this work. This will avoid the production of mycotoxins which are secondary metabolites produced during idiophase (Srinivasan *et al,* 1983).*Penicillium janthinellum* also produced xylanse activity (150 IU/ml) simultaneously which is obtained as a value added by product. The hemicellulose fraction of bagasse has no utility for steam and power generation (Pandey *et al,* 2000). For the cellulose bioconversion process to become economically viable, cellulose as well as hemicellulose both should be used from agricultural residues. The culture produced cellulase free xylanase activity even when cellulose powder was used as an inducer indicating that *Penicillium janthinellum* culture has the potential to produce xylanse activity in an inexpensive medium using waste bagasse hemicellulose fraction in a brief fermentation cycle. Cellulase free xylanase has extensive application in paper and pulp industry as pre-bleach agents (Balakrishnan *et al.* 1992).

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