OPTIMIZATION OF MANNANASE PRODUCTION FROM *STREPTOMYCES* SP. PG-08-03 IN SUBMERGED FERMENTATION

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Streptomyces sp. PG-08-3 was isolated from the desert of Rajasthan (India). The organism produced mannanase (15 Umg^{-1} protein) in the presence of 0.5% guar gum as a sole carbon source in minimal media by submerged fermentation (SmF). Enzyme production was enhanced by 7.3-fold when 0.5% soyabean meal and 0.25% of leucine were added to the minimal media. Increasing the guar gum concentration in the media by 0.1-1.0% resulted in linearly enhanced the production of mannanase.

Keywords: Mannanase; Optimization; Streptomyces sp. PG-08-03; Production

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INTRODUCTION

Hemicelluloses are complex polysaccharides that are abundant in higher plant cell walls. Galactomannan is the major softwood hemicellulose, containing β-1, 4-linked D-mannopyranose and D-glucopyranose units. The residues in the main chain are partially substituted by α-1-6 linked D-galactosyl side groups. The complete enzymatic degradation of hemicelluloses involves several specific activities. Endo 1,4-β-D-mannanase catalyses the random cleavage of β-D-1, 4-mannopyranosyl linkages within the main chain of galactomannan, glucomannan, galactoglucomannan, and mannan.

β-mannanases are the enzymes useful in several industrial processes, such as extraction of vegetable oils from leguminous seeds and the reduction of viscosity of coffee extracts during the manufacture of instant coffee (Sachslehner et al. 2000). Microbial species known to be actively produced the mannanases include *Vibrio* sp. (Tamaru et al. 1995), *Streptomyces* sp. (Takashi et al. 1984), and fungi (Rose and Zyl 2002; Arisan-Atac et al. 1993). Mannanases are also distributed in higher plants and animals.

In the present study, mannanase production by *Streptomyces* sp. PG-08-3 was carried out by optimization of media components.

EXPERIMENTAL

Isolation and Identification of *Streptomyces* sp. PG-08-03

In order to select the mannanase producing organism, soil specimens were suspended in sterilized water and their supernatants were inoculated into a medium

composed of locust bean gum 0.5%; Na_2HPO_4 0.7%; KH_2PO_4 3%; NH_4Cl 0.1%; and NaCl 0.05%. Final pH was adjusted to 8.0 with 0.1 N NaOH and incubated at 37 ^oC under shaking conditions 150 rpm. After every 24h a loopful of inoculum was streaked onto nutrient agar (pH 8.0) and actinomycetes isolation agar (pH 8.0) composed of asparagine 0.01%; sodium propionate 0.4%; K_2HPO_4 0.05%; $MgSO_4.7H_2O$ 0.01%; $FeSO_4.7H_2O$ 0.0001%; and agar 2.0%, which was subsequently incubated at 37 ^oC. The isolate thus obtained was transferred on locust bean gum nutrient agar plate (pH 8.0) by point inoculation. Plates were incubated at 37 ^oC for 3 to 5 days. The mannanase producing isolates gave a yellow zone against a red background upon staining with 0.5% Congo red and destaining with 1M NaCl.

Mannanase Production under Submerged Fermentation Condition (SmF)

Each Erlenmeyer flask (250ml) contained 50ml of minimal media, (gL^{-1}): guar gum, 5.0; Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NH_4Cl_2, 1.0; and NaCl, 0.5. Final pH was adjusted to 8.0±0.2 with sterilized 10% sodium carbonate. Media broth was inoculated with 2%, 48h old seed culture of *Streptomyces* sp. PG-08-3 and incubated at 37 ^oC for 72h at 200 rpm shaking conditions. Biomass of 72h old culture was measured by the centrifuging 20 ml culture of *Streptomyces* sp. PG-08-03 at 10000 *g* and drying the pellet at 50ºC until constant weight.

Effect of Nitrogen Sources on Mannanase Production

50 ml minimal media with 0.5% guar gum was supplemented with various organic (1.0%, w/v) and inorganic (1.0%, w/v) nitrogen sources. The media was inoculated with 2% of Streptomyces sp. PG-08-3 inoculum and incubated at 37 ^oC under shaking (200rpm) conditions for 72h. Thereafter dry biomass and mannanase activity were determined.

Effect of Amino Acids on Mannanase Production

The effect of amino acids on mannanase production was studied by supplementing different amino acids in minimal media with 0.5% guar gum. Mannanase activity and growth of the organism were observed for 72h.

Effect of Guar Gum Concentration on Mannanase Production

50 ml of minimal media was supplemented with different concentrations of guar gum, ranging from (0.1-1.0% w/v), inoculated with 2% of inoculum, and incubated at 37^oC under shaking (200 rpm) conditions for 72h. Thereafter biomass, protein concentration, and mannanase yield were determined.

Enzyme Assay

Mannanase activity was determined by using locust bean gum (0.5% w/v) as substrate. The mannanase activity was measured in the terms of the amount of reducing sugars released from locust bean gum by action of enzyme. Reducing sugars were measured by using the 3,5-dinitrosalicylic acid (DNSA) method of Miller (1959). The amounts of sugars released (mg ml^{-1}) were determined by using mannose as a standard. The one international unit of mannanase activity was defined as the amount of enzyme

that is required to release the 1µmole of mannose under standard (pH 8.0 and temp. 75ºC) assay conditions. Mannanase activity was expressed in terms of Umg^{-1} protein. Protein was measured by Lowry's method (1951).

RESULTS

Isolation and Identification of *Streptomyces* sp. PG-08-3

The organism was isolated from the desert of Rajasthan (India). Identification and taxonomical studies of the isolate were carried out according to the standard techniques and protocols mentioned in Bergey's Manual of Systematic Bacteriology (Sneath 1994). Identification was on the basis of morphological characteristics, such as aerial mycelia that were extensively branched top of aerial mycelium having a spiral form and each spore chain having about 5 to 10 spores. The surface of spore was smooth. The color of aerial mycelium was white to grey. Malanoid pigment was observed when grown on peptone yeast extract, iron agar or tyrosine agar.

The isolate exhibited metabolism of D-glucose, D-galactose, D-sucrose, D-xylose, arabinose, and mannose accompanied by acid production, but no gas production. The detailed morphological, biochemical, and physiological characteristics of the isolate are given in Table 1. The organism gave the 15 Uml^{-1} of mannanse when grown in minimal media containing only salts and 0.5% guar gum as a sole carbon source after 72h at 37 ºC and 200 rpm shaking conditions.

Effect of Nitrogen Sources on Mannanase Production

The effect of various nitrogen sources on mannanase production by *Streptomyces* sp. PG-08-3 was examined in minimal media containing 0.5% guar gum (Fig.1). Soyabean meal gave the maximum (33 Uml-1) enzyme production among the other organic and inorganic nitrogen sources.

Effect of amino acids on mannanase production

Amino acids and their analogues were tested for the enhanced production of mannanase by *Streptomyces* sp. PG-08-3. Amino acids at 0.25% (w/v) concentration in minimal media with 0.5% guar gum exhibited a significant level of stimulation on mannanase production. Amino acid such as DL-leucine stimulated the mannanase production by 2.8-fold (Table 2).

Optimization of Guar Gum Concentration for Mannanase Production

The effect of guar gum concentration on the mannanase production by *Streptomyces* sp. PG-08-3 in minimal medium was studied. Guar gum was added in various concentrations between 0.1 and 1.0% to the medium. There was linear increase in mannanase production with an increase in concentration of guar gum up to 0.5%. With further increase the concentration of guar gum in medium there was no additional enhancement in enzyme production (Fig. 2).

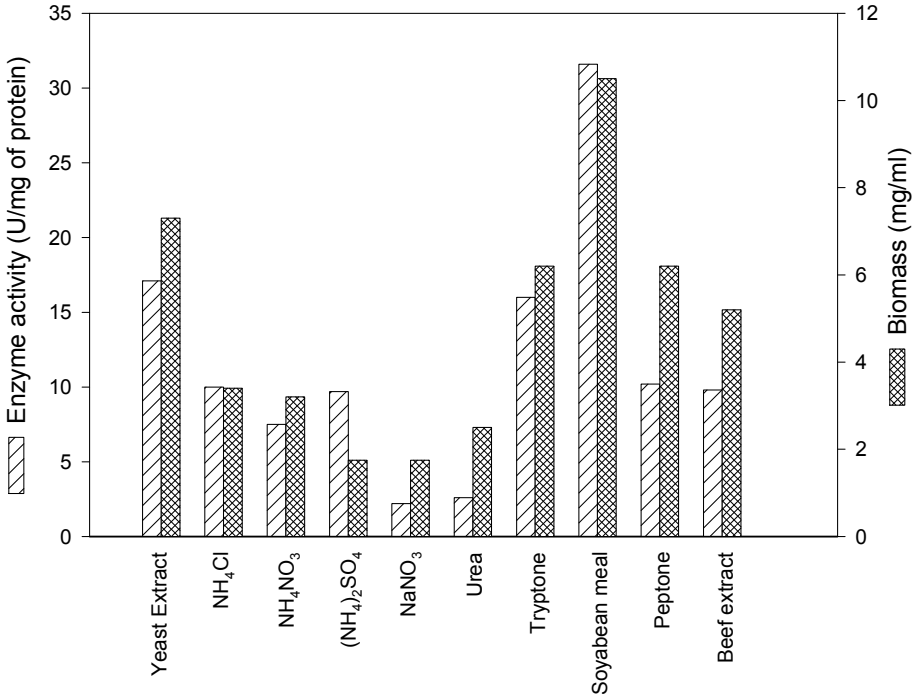

Fig. 1. Effect of various nitrogen sources (1%) on production of mannanase in the presence of 0.5% guar gum, at 37ºC and pH 8.0 under shaking (200 rpm) conditions for 72h

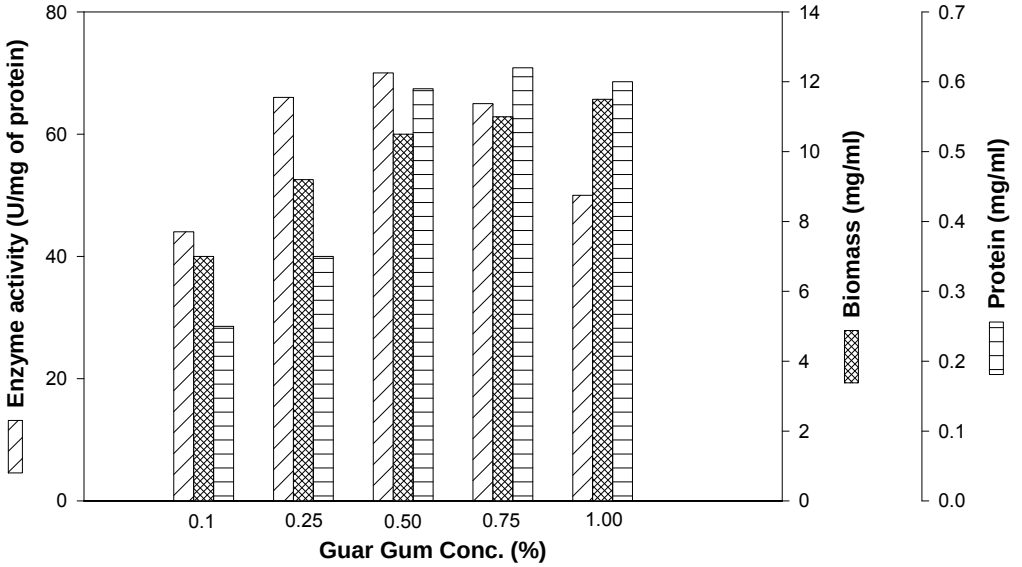

Fig. 2. Effect of guar gum concentration on the production of mannanse by *Streptomyces sp.* PG-08-03

Table 1: Morphological, Physiological, and Biochemical Characteristics of *Streptomyces* sp. PG-08-3

Characteristics	
Gram's stain	+ve
Morphology	Long branching filament
Spore	Grey/white
Metabolism	Aerobic
Catalase	+ve
Oxidase	-ve
Nitrate reduction	-ve
Citrate	+ve
Glycerol	-ve
Phenylalanine deaminase	-ve
Arginine dihydrolase	-ve
Lysine decarboxylase	-ve
H_2S production	-ve
Indole	-ve
Growth pH range	4-9
Growth temperature range	25-42°C
Growth in presence of 4–13% NaCl	+ve
Hydrolysis profile	
Xylan, Urea, Tributyrin, Pectin,	+ve
Mannan and Casein	+ve
Carboxymethyl cellulose	-ve
Starch, Tannic acid	-ve
Growth in presence of inhibitory compounds	
Crystal Violet 0.01 %	-ve
Phenol 0.01 %	-ve
Growth of organism in presence of 4, 7, 10 & 13% NaCl	+ve
Acid from sugar (1%, w/v)	
{D-glucose, D-galactose, sucrose, D-xylose, arabinose, mannose}	+ve
{maltose, lactose, mannitol, sorbitol}	-ve
Antibiotic resistance of organism	
{Penicillin, Cephalexin, Norfloxacin Cotrimoxazole, Cefotaxime, Ampicillin, Ciprofloxacin, Cefadroxil, Amoxycillin, Cloxacillin, Ceftazidime, Ofloxacin, Pefloxacin, Cefazolin}	Resistant
{Cefuroxime, Amikacin, Kanamycin Ceftriaxone, Netilnicin, Tobramycin Gentamycin, Piperacillin, Tetracycline Erythomycin, Lincomycin}	Sensitive

Table 2: Effect of Amino Acids on Mannanase Production from *Streptomyces* sp. PG-08-03 at pH 8.0 and 37°C under Shaking Conditions

Amino acids (0.25%, w/v)	Biomass $mgml^{-1}$	Enzyme activity (U mg^{-1} protein)	Yield Index (fold)
Control	10	30.23	1.00
Casaminoacids	10.50	62.87	2.08
DL-Alanine	9.75	30.23	1.00
DL-2 Amino-N butyric acid	10.00	45.64	1.51
DL-Norleucine	11.00	60.2	2.02
DL-Leucine	10.50	84.64	2.80
DL-Isoleucine	8.25	30.00	1.00
L-Lysine Monohydrochloride	8.50	60.46	2.00
L-Ornithine monohydrochloride	8.75	37.78	1.25
L-Arginine monohydrochloride	9.75	51.39	1.70
L-Histidine monohydrochloride	9.25	37.78	1.25
DL-β-Phenylalanine	10.50	60.46	2.00
DL-Norvaline	10.00	30.00	1.00
DL-Methionine	10.25	52.90	1.75
DL-Aspartic acid	9.00	37.78	1.25
DL-Serine	8.25	30.15	1.00
DL-Tryptophan	8.50	30.23	1.00
L-Glutamic acid	8.50	30.23	1.00
L-Proline	8.75	30.25	1.00
L-Hydroxyproline	9.25	36.02	1.20
DL-Threonine	10.00	30.23	1.00
L-Tyrosine	10.25	30.23	1.00

Production of the Mannanase by *Streptomyces* sp. PG-08-3 in the Optimized Media

Optimization was carried out for various nutritional parameters (nitrogen source, amino acid, and guar gum) of *Streptomyces* sp. PG-08-3 for the optimized production of mannanase. After standardization we found that when 0.5% guar gum, 0.5% soyabean meal, and 0.25% DL-leucine were added to the minimal media, higher (110 Umg^{-1} protein) production of mannanase was acheived as compared to the production of enzyme in sole minimal media (15 U mg^{-1} protein) under similar environmental conditions (Fig. 3).

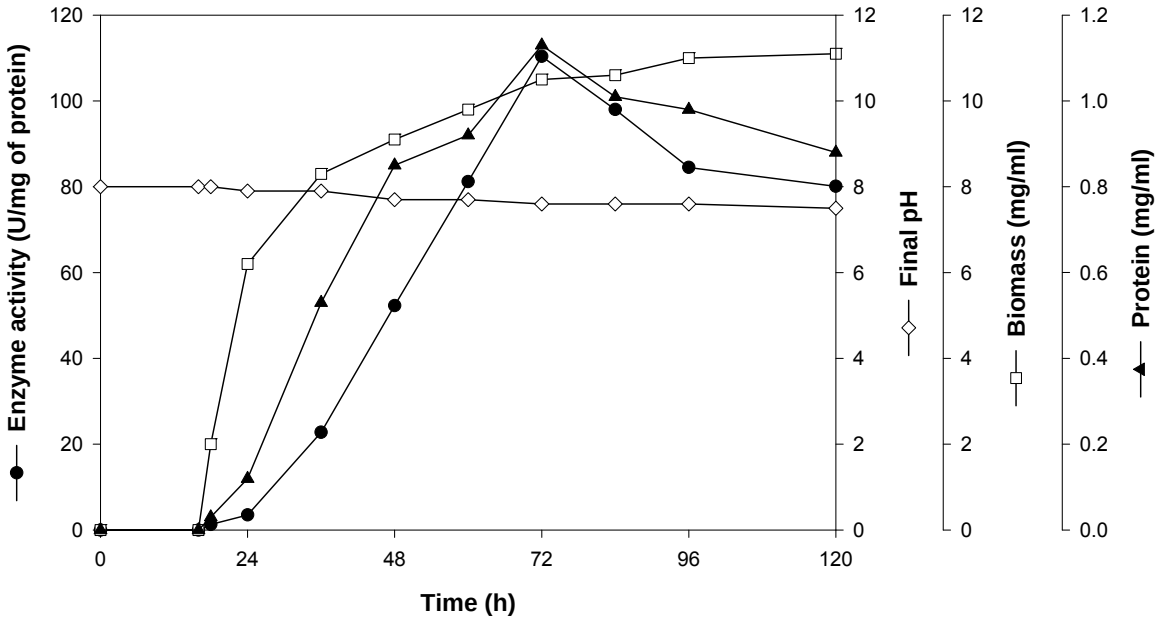

Fig. 3. Time course for growth, mannanase production, pH shift, and protein concentration by *Streptomyces* sp. PG-08-3 in the optimized media (0.5% guar gum, 0.5% soyabean meal, and 0.25% DL-leucine) at 37°C, initial pH 8.0, and 200 rpm

DISCUSSION

Streptomyces sp. PG-08-3 isolated from sand was selected over the other isolates, because it produced a high yield of mannanase without the use of any expensive nutrient component. The organism was gram positive with branching filaments and grown well in the temperature and pH range of 25-42 °C and 5.0-9.0 respectively. This organism is a successful environmental biodegrader saprophyte, because it hydrolyzes the locust bean gum, guar gum, pectin, urea, casein and tributyrin.

Mudau and Setati (2008) reported that *Scopulariopsis candida* strains LMK004 and LMK008 were isolated from the solar saltern and cultivated in Vogel's medium supplemented with NaCl and locust bean gum as a carbon source produced 180 nkat ml^{-1} and 116 nkat ml^{-1} of mannanase, respectively. Petrus et al. (2009) reported a 13-fold increase in enzyme production by the successful expression of β-mannanase gene of *Aspergillus aculeatus* MRC11624 in *Aspergillus niger* under control of the *A. niger* glyceraldehyde-3-phosphate dehydrogenase promoter (gpdP) and the *A. awamori* glucoamylase terminator (glaAT). The highest reported mannanase production is 18,403 nkat ml^{-1} and was achieved by the heterologous expression and characterization of man gene from *Bacillus subtilis* in *Pichia pastoris* (Qiao et al. 2008).

The remarkable differences in the enzyme production by the use of different nitrogen sources was not understood, it may be due to different requirements by the isolate. Similar results have been reported from *Rhodothermes marinus* with yeast extract

(Gomes and Steiner 1998), *Bacillus* sp. with polypeptone (Akino et al. 1987) as a suitable nitrogen source.

It was observed that mannanase production was enhanced by different amino acids and their analogues to a variable extent, but there was not much difference in growth of the organism. Several studies have already established that amino acids are responsible for enhancing the production of several enzymes such as β-galactosidase and xylanase. But in particular, published information about the effect of aminoacids on mannanase production is rare.

The present work indicated that mannanase production was not only induced by mannan present in the medium, but was also enhanced by soyabean meal in production medium. Moreover, this fact has already been established that amino acids are responsible for enhancing the production of several enzymes such as β-galactosidase and xylanase.

CONCLUSION

Optimization of nutritional components for the production of alkalophilic mannanase from newly isolated *Streptomyces* sp. was achieved successfully. Attempts to clone this mannanase gene in order to understand the regulatory control of production in *Streptomyces* sp. PG-08-03 are being carried out.

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