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Optimization of mannanase production by *Bacillus* sp. MG-33

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ABSTRACT

Various *Bacillus* species were screened for the production of mannanase in submerged fermentation. *Bacillus* sp. MG-33 was selected as the most potent strain for production of enzyme with high activity. Organisim produced mannanase (13Uml^{-1}) in presence of 0.5% guar gum as a sole carbon source in minimal media. Enzyme production was increased by 3.3, 2.5 and 1.0 fold when 0.25 and 1% DL-Leucine, wheat bran and tryptone were added to the minimal media respectively. When increasing (0.1-1.0%) the guar gum concentration in media then as a result linearly enhanced the production of mannanase by *Bacillus* sp. MG-33.

KEYWORDS

Mannanase;
Production;
Bacillus sp. MG-33;
Submerged fermentation.

INTRODUCTION

Hemicelluloses are complex polysaccharides that are abundant in higher plant cell walls. Galactomannan is major softwood hemicellulose contains β-1, 4-linked D-mannopyranose and D-glucopyranose units. 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. In paper industry, mannanases have synergistic action in the biobleaching of the wood pulp, significantly reducing the amount of chemicals used[1]. The growing interest in mannanase production for Industrial applications is due to its importance in the bioconversion of agro-industrial residues. Various mannanases from fungi, yeasts and bacteria as well as from germinating seeds of terrestrial plants have been produced[2-5]. Production of mannanase by microorganisms is more promising due to its low cost, high production rate and readily controlled conditions. In the present study mannanase production by *Bacillus* sp. MG-33 was carried out by optimization of media components.

METHODOLOGY

Isolation and identification of *Bacillus* sp. MG-33

For the selection of mannanase producing organism soil samples were suspended in sterilized water and their supernatants were inoculated into a medium, as described previously[6]. After every 24h a loopful of inoculums was streaked onto nutrient agar (pH 7.0).

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The isolate thus obtained was transferred on locust bean gum nutrient agar plate (pH 7.0-7.5) by point inoculation. Plates were incubated at 30°C 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 destining with 1M NaCl.

Mannanase production under submerged fermentation condition (SmF)

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; NaCl, 0.5. Final pH was adjusted to 7.0±0.2 with sterilized 10% sodium carbonate. Media broth was inoculated with 1%, 48h old seed culture of *Bacillus* sp. MG-33 and incubated at 30°C for 72h at 200 rpm shaking conditions. Cell count of culture was measured by the CFU of the isolate or by measuring the optical density of culture at 600 nm.

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 1% of *Bacillus* sp. MG-33 inoculum and incubated at 30°C under shaking (150 rpm) conditions for 72h. Thereafter cell growth and mannanase activity were determined.

Effect of amino acids on mannanase production

The effect of amino acids (0.25%) 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) and inoculated with 2% of inoculum and incubated at 37°C under shaking (150 rpm) conditions for 72h. Thereafter cell count, 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 3,5-dinitrosalicylic acid (DNSA) method of Miller[7]. The amount 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 6.5 and temp. 65ºC) assay conditions. Mannanase activity was expressed in terms of Uml^{-1}. Protein was measured by the Lowry's method[8].

RESULTS

Isolation and Identification of the organism

Organism was isolated from the soil 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[9]. Organism was Gram positive, appears rod shaped under the compound microscope at 100X resolution. On nutrient agar plate this strain produced transparent colonies with regular margins, bulged surface. The isolate was catalase and oxidase positive and was not able to reduce citrate, MR and VP test were negative and positive respectively. Organism was capable of hydrolyzing carboxylmethyl cellulose, tributyirin, xylan, locust bean gum, guar gum, casein but could not hydrolyzes tannic acid, pectin and chitin.

The isolate exhibited metabolism of D-glucose, D-galactose, D-sucrose, D-xylose, arabinose and mannose accompanied by acid production. The detailed morphological, biochemical and physiological characteristics of the isolate are given in TABLE 1. Organism gave the $13Uml^{-1}$ of mannanse when grown in minimal media contained only salts and 0.5% guar gum as a sole carbon source after 72h at 30ºC at 150 rpm shaking conditions.

Effect of nitrogen sources on mannanase production

The effect of various nitrogen sources on mannanase production by *BacillusI* SP. MG-33 was examined in minimal media contained 0.5% guar gum (Figure 1). Tryptone gave the maximum (1.0 fold increased) en-

TABLE 1 : Morphological, Physiological, and Biochemical Characteristics of *Bacillus* sp. MG-33

Characteristics	Observation
Gram's stain	+ve
Morphology	Rods (2.0-2.5 µm)
Spore	+ve
Metabolism	Aerobic
Motility	+ve
Catalase	+ve
Oxidase	+ve
Nitrate reduction	+ve
Methyl red (MR)	-ve
Voges prosker (VP)	+ve
Citrate	+ve
Glycerol	-ve
Phenylalanine deaminase	-ve
Arginine dihydrolase	-ve
Lysine decarboxylase	-ve
H_2S production	-ve
Indole	-ve
Growth pH range	5-8 (Optimum 7.0)
Growth temperature range	25-35°C(Optimum 30°C)
Growth in presence of 4–13% NaCl	+ve
Hydrolysis profile	
Xylan, Urea, Tributyrin, Pectin	+ve
Mannan and Casein	+ve
Carboxymethyl cellulose	+ve
Tannic acid	-ve
Starch	+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% NaCl	+ve
Acid from sugar (1%, w/v)	
D glucose, D galactose, sucrose, D xylose, arabinose, mannose	+ve
maltose, lactose, mannitol, sorbitol	-ve

zyme production among the other organic and inorganic nitrogen sources.

Effect of carbon sources on mannanase production

Different carbon sources were tried to enhance the mannanase production by *Bacillus* SP. MG-33 in minimal media contained 0.5% guar gum (Figure 2). Wheatbran stimulated the maximum (2.5 fold increase)

TABLE 2 : Effect of amino acids on mannanase production from *Bacillus* SP. MG-33 at pH 8.0 and 37°C under shaking conditions

Amino acids (0.25%, w/v)	Growth A_{600}nm	Enzyme activity (U ml^{-1})
Control	0.79	15
Casaminoacids	0.81	30.2
DL-Alanine	0.61	15
DL-2 Amino-N butyric acid	0.71	21
DL-Norleucine	0.9	46
DL-Leucine	0.81	50
DL-Isoleucine	0.85	31
L-Lysine Monohydrochloride	0.85	35
L-Ornithine monohydrochloride	0.72	26
L-Arginine monohydrochloride	0.81	26
L-Histidine monohydrochloride	0.79	20
DL-β-Phenylalanine	0.72	30.00
DL-Norvaline	0.79	46
DL-Methionine	0.795	19
DL-Aspartic acid	0.79	16
DL-Serine	0.71	18
DL-Tryptophan	0.79	15
L-Glutamic acid	0.85	15
L-Proline	8.2	18
L-Hydroxyproline	7.90	15
DL-Threonine	8.2	15
L-Tyrosine		

enzyme production among the other organic and inorganic nitrogen sources.

Effect of amino acids on mannanase production

From the tried amino acids, leucine maximally increased (3.3 fold) the mannanase production by *Bacillus* SP. MG-33 among the other tried amino acids TABLE 2.

Effect of guar gum concentration on the mannanase production

When guar gum was added in various concentrations between 0.1 to 1.0% in the medium. There was linearly increased in mannanase production with an increasing the concentration of guar gum up to 0.4%. With further increase the concentration of guar gum in medium there was no additional stimulation in enzyme production by *Bacillus* SP. MG-33 (Figure 3).

Production of the mannanase by *Bacillus* sp. Mg-33 in the optimized media

It was observed that 0.5% guar gum, 1.0% tryptone, 1% wheat bran and 0.25% DL-leucine when added to the minimal media then higher (50 Uml^{-1}) production of mannanase was acheived as compared to the production of enzyme in sole minimal media (13U

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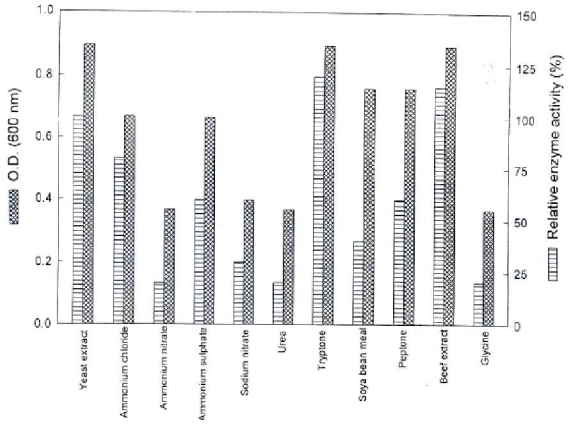

Figure 1 : Effect of various nitrogen sources (1%) on the production of mannanase by *Bacillus* sp. MG-33 in minimal media at 30 °C, pH 7.0 at 150 rpm for 72H

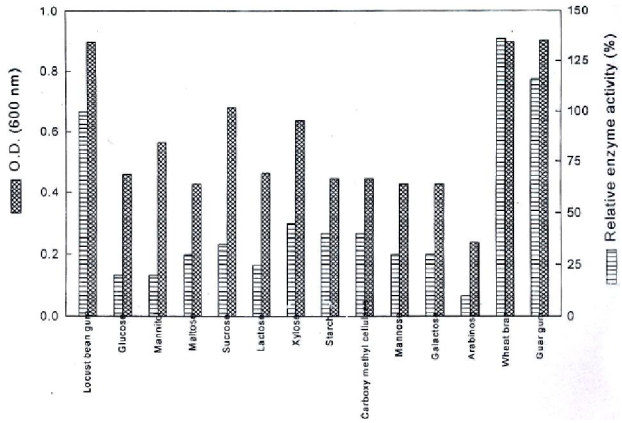

Figure 2 : Effect of different carbon sources (1%) on the production of mannanase by *Bacillus* sp. MG-33 in minimal media at 30°C, pH 7.0 at 150 rpm for 72H

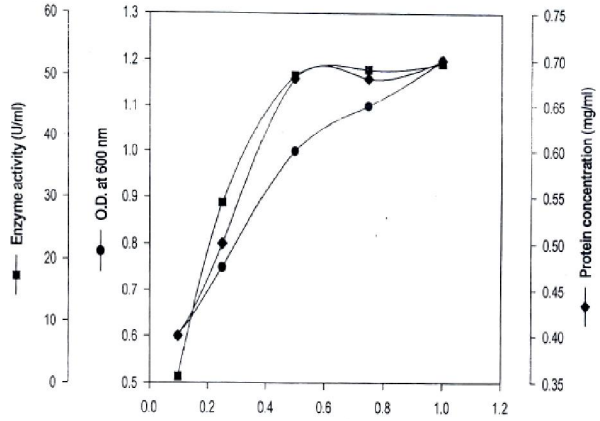

Figure 3 : Effect of guar gum concentration on the production of mannanse by *Bacillus* sp. MG-33

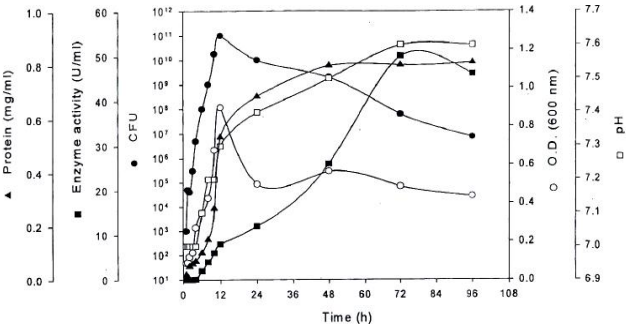

Figure 4 : Time course for growth (O.D, 600nm & CFU), mannanase production, pH shift and protein concentration by *Bacillus SP.* MG-33 in the optimized media (0.5% guar gum, 1.0% tryptone, 1% wheat bran and 0.25% DL-leucine) at 30°C , initial pH 7.0 and 150 rpm for 72h

ml^{-1}) under similar environmental conditions (Figure 4).

DISCUSSION

Bacillus SP. MG-33 was isolated from the soil, it produced a high yield of mannanase without the use of any expensive nutrient component. Organism was gram positive with rods and grown well in the temperature and pH range of 25-35°C and 5.0-9.0 respectively. This organism is a successful environmental saprophyte, because it hydrolyzes the carboxymethyl cellulose, starch, locust bean gum, guar gum, pectin, urea, casein and tributyrin. Mabrouk et al.[10] reported that different *Bacillus* species were screened for the production of mannanase. *Bacillus amyloliquefaciens* was selected as the most potent in producing enzyme of high production. Utilization of various agro-industrial residues on mannanase production was evaluated. Potato peels at a concentration of 14 g/l was found to be the most effective carbon source. Addition of simple carbon source to media containing potato peels cause catabolic repression of mannanase synthesis.

Santiago et al.[11] reported that mutant strains of *Aspergillus niger,* GS1-S059 and GS1-S067 significantly increased their level of mannanase, xylanase and cellulase production, compared to the parental strain, improving their potential industrial applications. Maria et al.[12] reported *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.[13] reported that 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.[14].

The demarkable differences in the enzyme production by the use of different nutritional sources was not understood, it may be due to different requirements by the isolate. 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 particularly the effect of aminoacids on mannanase production is not common. Bhoria et al.[6] reported that mannanase production was enhanced by 7.3 fold when 0.5% soyabean meal and 0.25% of leucine were added to the minimal media.

The present work indicated that mannanase production was not only induced by guar gum present in the medium, but was also enhanced enzyme production by wheatbran and tryptone in production medium. Moreover, this fact has already been established that amino acids are responsible for increasing the production of mannanase by prokaryotes[6].

CONCLUSION

Optimization of different nutrient components for the production of mannanase by newly isolated *Bacillus* SP. MG-33 was achieved successfully. Further work will be done to clone this mannanase gene in order to elucidate the regulatory control of production of enzyme in *Bacillus* SP. MG-33 being carried out.

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