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Full Length Research Paper

Production of *β*-mannanase by *Bacillus amylolequifaciens* 10A1 cultured on potato peels

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Different *Bacillus* species were screened for the production of 1,4-*β*- mannanase on solid media. *Bacillus amyloliquefaciens* was selected as the most potent in producing enzyme of high activity. 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. Maximum enzyme activity (61.5 U/mg protien) was obtained with ammonium nitrate as a nitrogen source. The optimum incubation temperature and pH for enzyme production were 35°C and 7, respectively. The influence of inoculum size was so remarkable that, at optimum, a crude filtrate with an enzyme activity of 105.7 U/mg protien was obtained and appeared among the highest levels reported for mannanase titre. Addition of simple carbon source to media containing potato peels cause catabolic repression of mannanase synthesis.

Key words: *Bacillus amyloliquefaciens*, *β*- mannanase, galactomannan, potato peels.

INTRODUCTION

Endo-β-1,4-mannanases (EC.3.2.1.78) randomly hydrolyze the main chain of hetero-mannans, the major softwood hemicellulose (McCleary, 1988). Mannanases have been tested in several industrial processes, such as extraction of vegetable oils from leguminous seeds, viscosity reduction of extracts during the manufacture of instant coffee and manufacture of oligosaccharide (Gubitz et al., 1996; Ademark et al., 1998) as well as applications in the textile industry (Pedersen et al., 1995). In paper industry, mannanases have synergistic action in the biobleaching of the wood pulp, significantly reducing the amount of chemicals used (Khanongnuch et al., 1998). 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 (Ferreira and Filho, 2004; Heck et al., 2005; Juhasz et al., 2005; Jiang et al., 2006; Lin et al., 2007).

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Production of *β*-mannanase by microorganisms is more promising due to its low cost, high production rate and readily controlled conditions.

Lignocellulose is the major structural component of plant cell walls and is mainly composed of lignin, cellulose and hemicellulose, and represents a major source of renewable organic matter. The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value (Malherbe and Cloete, 2003). Large amounts of lignocellulosic “waste” are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries and they pose an environmental pollution problem. However, the huge amounts of residual plant biomass considered as “waste” can potentially be converted into various different value-added products including biofuels, chemicals, and cheap energy sources for fermentation, improved animal feeds and human nutrients. Lignocellulytic enzymes also have an significant applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture (Bhat, 2000; Sun and Cheng, 2002; Beauchemin et al., 2003; Howard et al., 2003). Recently, research for finding suit-

able substrates has been of particular interest.

The aim of the present study was to utilize cheap agro industrial wastes available in large quantities for mannanase production by *Bacillus amyloliquefaciens*.

MATERIAL AND METHODS

Bacterial strains

Bacillus species used in this study (Table 1) were obtained from *Bacillus* Genetic Centre Ohio State University, USA. All stains were maintained as spore stocks, or for short period of time, on Luria-Bertani agar slants.

Chemicals and substrates

Locust bean gum (galactomannan) was purchased from Sigma Chemicals (St. Louis, MO). All other chemicals were of analytical grade. Linen seed meal, sunflower seed meal, soybeans meal, cotton seed meals were obtained from local manufacturers. Wheat straw, wheat bran, rice husk, corn cob, palm seed meal, and peels of orange, mango, apple and potato were collected from farm field or domestic sources. Each of these raw materials was dried and ground to pass through a 30 mm mesh sieve.

Preliminary screening for 1,4-β-mannanase production

Preliminary screening for 1,4-β-mannanase production was performed on mineral salts agar medium (MS) described by Jones and Ballou (1969) and modified to contain (g/l): galactomannan 4; NH_4 NO_3 0.3; Mg SO4.7H_2O 0.2; Fe SO_4.7H_2O 0.01; $CaCl_2$.2H_2O 0.05; K_2HPO_4 7.54; KH_2PO_4 2.32; agar 20; pH was adjusted to 7 and sterilized by autoclaving at 121°C for 15 min. The agar plates were inoculated by first dipping a sterile tooth pick into an actively growing bacterial colony on LB media and incubated for 24 h at 35 ±1°C. Mannanase activity was detected on the cultures by staining the plates with iodine solution for 15 min. Galactomannan hydrolysis was observed by the appearance of clearing zones around the bacterial colony .The activity was calculated as the ratio of the diameter of the clearing zone to the diameter of the colony.

Enzyme production

The mineral salts medium described above without agar was used for enzyme production. The initial pH was adjusted to 7 by HCl or NaOH throughout the work. Erlenmeyer flasks (100 ml) containing 20 ml sterile mineral basal medium (MBM) were inoculated with 0.6 ml seed culture ($O.D_{550}$=1) exponentially growing on LB medium. Submerged batch culture was carried out with agitation (150 rpm) at 35°C for 24 h.

Production of β-mannanase using different carbon sources

The fermented broth medium was modified by replacing mannan with several types of agro industrial by products. Palm seed, orange peel, apple peel, corn cob, wheat straw, potato peel, mango peel, and rice husk were used as substrates for β-mannanase production by *B. amyloliquefaciens* in comparison to galactomannan (control).

Table 1. Mannanase activity of the tested strains. Activity ratio was calculated from clear zones formed on agar plate containing mannan as the sole carbon source and incubated at 35°C for 24 h.

Strain	Activity ratio
B. thuringiensis 4HJ1	1.4
B. thuringinesis 4AH1	1.4
B. subtilis 168	1.3
B. lichenformis Mo1	1.3
B. polymyxa 25A1	1.0
*B. amyloliquefaciens*10A1	1.6
B. sphaericus	-
B. thuringinesis 4A1	-
B. thuringinesis	1.2

Production of β mannanase using different nitrogen sources

Various inorganic nitrogen sources ($(NH_4)_2$ SO_4, NH_4Cl, $NaNO_3$, $(NH_4)_2HPO_4$, NH_4NO_3) were examined in MS broth medium. The nitrogen source which will give the highest mannanase activity shall be replaced one at a time with different agro industrial by products serving mainly as nitrogen source such as wheat bran, soybean meal, sun flower meal, cotton seed meal, and linen seed meal.

The fermented broth was clarified by centrifugation at 10,000 rpm for 15 min and the clear supernatant was used for assay of enzyme and soluble protein. All experiments were performed in duplicate.

Determination of enzyme activity

Mannanase was assayed by measuring the reducing sugars using dinitrosalicylic acid (DNS) method (Miller, 1959). The mannanase assay mixture contained 0.5 ml of 0.5% (w/v) locust bean gum (substrate), prepared in 50 mM phosphate buffer, pH 7 and 0.5ml of appropriately diluted culture broth. The reaction mixture was maintained at 50°C for 30 min. After incubation, 1 ml of DNS reagent was added and boiling took place from 5 – 15 min. The developed red brown color was measured at 575 nm. One unit of enzyme activity (U) was defined as the amount of enzyme liberating 1 µmol of mannose per minute under the assay conditions. Specific activity was expressed as U/mg of protein. Controls were routinely included in which enzyme preparation or substrate was omitted.

Protein determination

Soluble protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Chemical analysis of potato peels

Oven dry weight of potato peels were used to estimate its content of ash (Browne and Zerban, 1948), protein (Daughton et al., 1984), carbohydrate (Dubois et al., 1956), and easily utilizable sugars (Miller, 1959).

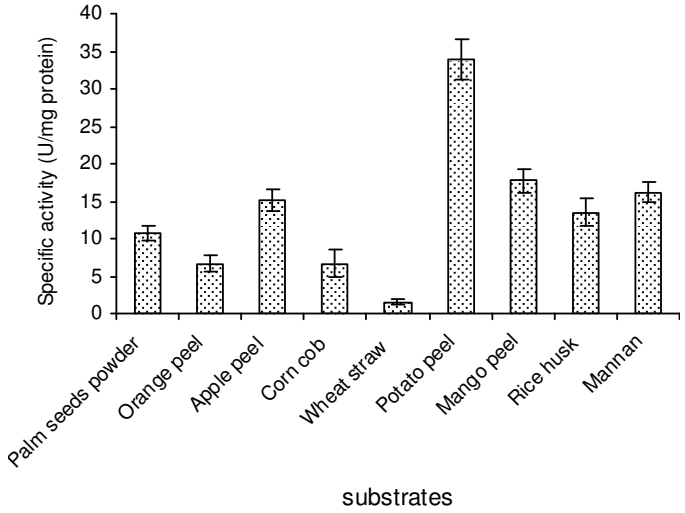

Figure 1. Specific activity of mannanase produced by *B. amyloliquefaciens* grown on different lignocellulosic materials.Cells were grown on mineral salt medium with each tested substrate (10 g/l) and incubated at 35°C for 24 h

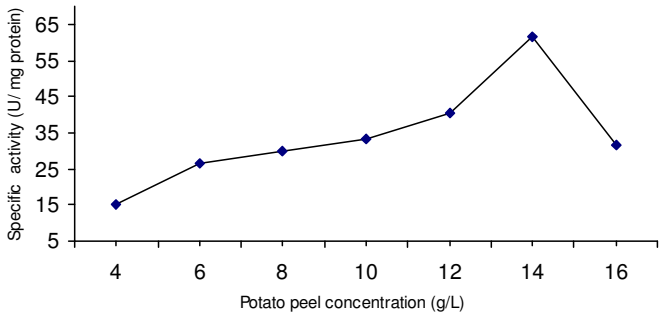

Figure 2. Effect of potato peels concentration on mannanase production by *B. amyloliquefaciens* incubated at 35°C for 24 h.

Table 2. Proximate chemical composition of potato peel.

Component	% of dry weight
Moisture	11.2
Ash	7.56
Sugars	3.45
Carbohydrates	64.47
Protein	13.52

RESULTS AND DISCUSSION

Screening of mannan-degrading bacterial strains

Seven out of nine tested bacterial stains were able to hydrolyze galactomannan during growth on MS agar plates, zones of different sizes were cleared around the b-acterial species. The highest mannanase activity ratio 1.6 was reached by *B. amyloliquefaciens* 10 A 1 (Table 1). Therefore, this strain was selected for further studies mannanase activity has been reported in a wide variety of *Bacillus* species (Heck et al., 2005; Jiang et al., 2006), but few data are available on mannanase of *B. amyloliquefaciens*.

Effect of different substrates used as a carbon sources

Agro-industrial by-products are available in large amounts and they have been used for the production of several enzymes (EL-Helow and El- Ahwany, 1999; Howard et al., 2003). Figure 1 shows that several types of agro-industrial by-products were evaluated as substrates for mannanase production by *B. amyloliquefaciens* 10A1 in comparison to galactomannan (control). *B.amyloliquefaciens* grew well on various raw materials of commercial potential with significant differences in the rate of enzyme production. The large variation in mannanase yield may be due to the nature of cellulose or hemicellulose, presence of some components (activators or inhibitors) in these materials and variations in the substrate accessibility. Among these, potato peels were found to be the most suitable substrate for mannanase production. Enzyme activity (33.8 U/ml, 27.4 U/mg protein), almost 2.09 fold increase compared to galactomannan. Potato peels are agro-industrial by-products that could have good biotechnological potential. Nevertheless such waste was not tested extensively in pervious studies. Therefore, it was used for optimization studies as a carbon source replacing mannan.

Chemical composition of potato peels

The data presented in Table 2 show that potato peels contain considerable amounts of carbohydrate which stimulate the cells to express many hydrolytic enzymes (Priest, 1977). In addition it contains appreciable amounts of easily utilizable sugars which encourage growth initiation, and protein, which serves as essential nitrogenous compounds. Potato peels are renewable, cheap and widely available waste in Egypt. Utilization of waste potato peels to produce mannanase appears to be economic.

Effect of potato peel concentration

Figure 2 shows that mannanase activity increased as the concentration of potato peels increased and reached maximum activity (61.5 U/mg protein) in presence of 14 g/l of substrate which appeared to be the optimal level. Higher

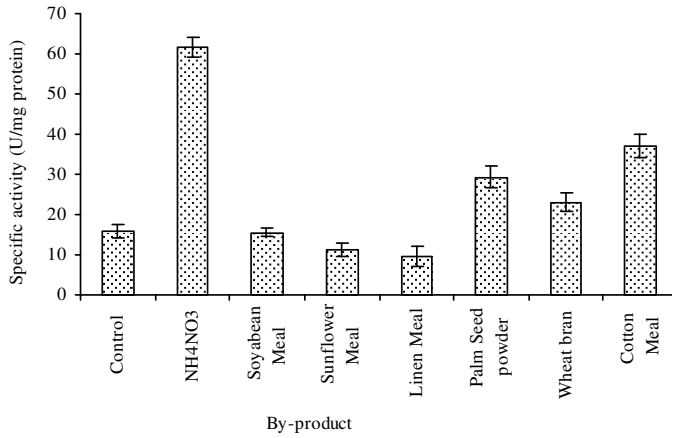

Figure 3. Effect of various agro-industrial by-products as a nitrogen sources on mannanase production by *B.amyloliquefaciens* during utilization of potato peels as carbon source. Control: no exogenous supply of nitrogen.

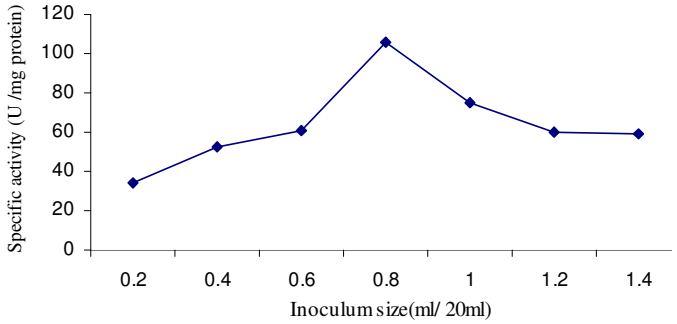

Figure 4. Effect of inoculum size on mannanase production by *B. amyloliquefaciens.*

concentrations were accompanied by lower enzyme activity as a result of releasing of hydrolysis end products that repress many catabolic operons (Magasanik and Neidhardt, 1987; Fisher and Sonenshein, 1991).

Effect of nitrogen source

Various inorganic nitrogen sources were tested in MS broth media containing 14 g/l potato peels. The concentration of each nitrogen compound was fixed at 0.105 g N l^{-1}. All nitrogen sources sustained bacterial growth. Ammonium nitrate appeared to be the best nitrogen source allowing the production of highest mannanase activity (61.5 U/mg protein), increased enzyme activity by 3.9 fold compared to the control (data not shown). Also ammonium nitrate was replaced one at a time by various agro-industrial by-products served mainly as nitrogen sources (Figure 3) to test their effect on enzyme production. The medium was supplemented with 1.4% (w/v) potato peels and the respective nitrogen sources at concentration 1% (w/v). Highest specific activity (35.12 U/ mg protein) was achieved by cotton seed meal. Considerable specific activities were also recorded by palm seed powder and wheat bran (29.2, 23.1 U/ mg protein) respectively. It is worthy to mention that omitting nitrogen source from the medium resulted in producing an enzyme of considerable activity (15.8 U/mg protein) indicating that potato peels could serve as carbon and nitrogen source. Moreover, a trial was performed to evaluate the utilization of potato peels (14 g/l) solely in water. Surprisingly, synthesized enzyme possessed an activity of 10.8 U/mg protein (data not shown).

Inoculum size

Inocula of different sizes (1 to 7%) of the total cultural volume were tried out with respect to mannanase production (Figure 4). The results indicate that protein concentration in the culture filtrate was positively affected by increasing the inoculums concentration. The maximum mannanase yield (105.7 U/ mg protein) was noted when the cultured medium provided with 4% inoculum. An increase in the inoculum size would ensure increased mannanase yield by *B. amylolequifaciens*. However, after a certain limit, the competition for the nutrients resulted in a decrease of the metabolic activity of the organism. With optimum inoculum size, there was a balance between biomass synthesis and availability of nutrients that supports production of enzyme (Nampoothiri et al., 2004).

Optimization of initial pH and temperature

The effect of different initial pH values ranging from 5 to 8 on mannanase production was studied. Adjustment of the pH was carried out using 1 N HCl/NaOH. Initial pH of the medium profoundly affected the mannanase production. Medium adjusted at pH 7.0 favored maximum enzyme production of 105.7 U/mg protein (data not shown). This observation supports the finding that extracellular enzymes are produced in high titre with pH optima at the growth pH (McTigue et al., 1994). The results obtained are similar to those observed by other authors Heck et al. (2005). Incubation temperature is characteristic of an organism and profoundly affects the enzyme yield (Ramesh and Lonsane, 1987). *B. amyloliquefaciens* exhibited a highest titre of mannanase (105.7 U/mg protein) at 35°C (data not shown). Similar observations were obtained by Mendoza et al. (1994) and El- Helow and Kattab (1996).

Regulation of mannanase expression

Addition of various carbohydrates (0.2 g/l) to the enzyyme production medium containing potato peels in order to eva-

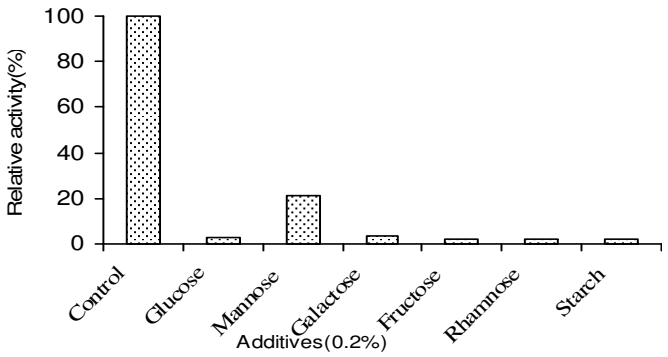

Figure 5. Effect of addition of carbon sources on mannanase production by *B. amyloliquefaciens*.

luate its induction or repression effect on mannanase production were tested (Figure 5). The highest activity was exhibited by potato peels and the association of additional carbon source with potato peels was accompanied by severe inhibitory effects on enzyme production. Such results may be due to the catabolic repression processes when easily assimilated carbon sources were added (Biswas et al., 1990).

Conclusion

To the best of our knowledge, this is the first report on the high-level production of 1,4-β-mannanase by *B. amyloliquefaciens* 10A1 from potato peels.

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