The Bioscan 5(1) : 75 - 79, 2010
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES

OPTIMIZATION OF CULTURE CONDITONS FOR THE PRODUCTION OF β-MANNANASE FROM AN AGAR UTILIZING PAENIBACILLUS SP. MSL-9

SANGANKAL MANJULA, MANOHAR SHINDE[1] AND JUNNA LALITHA*
Department of Biochemistry, Gulbarga University, Gulbarga - 585 106, Karnataka, INDIA
[1]Department of Biotechnology, M. V. J. College of Engineering, Bangalore - 560 067, Karnataka, INDIA
E-mail: smgouda@rediffmail.com

KEY WORDS
Paenibacillus sp.MSL-9
β-mannanase
Agar, Guar gum
Agro-industrial wastes

Received on :
24.12.2009
Accepted on :
18.02.2010

***Corresponding author**

ABSTRACT
An agar utilizing *Paenibacillus* sp. MSL-9 from marine water was acclimatized to mannans for production of β-mannanase. The mannanase production was optimum at 44-48 h of incubation (0.74U/mg) in minimal mineral salts medium containing guar gum (0.3%, w/v) as carbon source, and combination of sodium nitrate (0.15% (w/v) and beef extract (0.15%, w/v) as a nitrogen source, at 30ºC and pH 8.0 respectively. The β-mannanase was inducible by mannan polymers. Simple sugars did not induce the enzyme and also suppressed the enzyme production when co-supplemented along with the mannans. Utilization of various agro-industrial wastes on mannanase production was also evaluated.

INTRODUCTION

The microbial degradation of plant cell wall not only is important in maintaining terrestrial and marine life but also is of growing industrial importance, particularly in the bio-energy and bioprocess sector (Ragauskas *et al.,* 2006). Some of the most diverse plant cell wall polysaccharides are those containing â-1, 4-linked mannosides. The mannosidic bonds are hydrolyzed by β-mannanase (mannan-endo-1, 4-mannosidase; EC 3.2.1.78) (Mc Cleary, 1988) of glycoside hydrolase's families (GH s) 5 and 26 (Hogg *et al.,* 2003), which catalyze the random cleavage of β-1, 4-mannosidic linkages in the backbones of mannan, galactomannans, glucomannans and galactoglucomannans (Naughton *et al.,* 2001), resulting in various oligosaccharides as major products. Bacterial β-mannanase studied to data exhibited hydrolytic activity against defatted copra meal, locust bean gum and konjac as mannan substrates (Sudathip *et al.,* 2008). Guar gum (GG), a polygalactomannan obtained from the seeds of *Cyamopsis tetragonalobus,* is a water soluble polysaccharide and because of its low cost and excellent viscosifying properties, guar gum and its derivatives are extensively used in industrial applications including food, oil recovery (Prodhomme *et al.,* 1989), personal care (Cheng and Yang., 1991), etc. Structurally, it has a back bone of β-1, 4-linked mannose units with α-1, 6-linked galactose units attached as side chains to almost every alternate mannose unit. One of the main uses of guar is in food industry where its bulking, stabilizing and water binding properties are exploited (Chiu *et al.,* 2001). It is also used in dietary supplements as an indigestible sugar in obesity treatment. Moreover, partially hydrolyzed guar gum prepared by β-mannanase (McCleary, 1979; McCleary and Matheson., 1983) not only has low viscosity but also has various health benefits such as improving conditions of constipation, a hypocholersterolemic effect and hypolipidemic effect and improved intestinal micro flora balance (Cummings *et al.,*1987; Ishihara *et al.,* 2000).

Interest in mannan degrading enzyme systems from different sources has increased in the past decade, especially because of their biotechnological applications (Singh *et al.,* 2003). Mannanase has been effectively used in pulp bleaching (Lahtinen *et al.,* 1995; Cuevas *et al.,* 1996), in clarification of fruit juices (Christgua *et al.,* 1994), in manufacturing of instant coffee, chocolate and cocoa liquor (Francoise *et al.,* 1996). In addition, mannanase are potentially applied in the pharmaceutical industry for the production of physiologically active oligosaccharides (Lin and Chen, 2004). Bacterial species known to actively produce β-mannanase include *Aeromonas hydrophila* (Ratto and Poutanen, 1988), *Bacillus sp* (Abe *et al.,* 1994; Araujo and Ward, 1990a, 1990b) *Enterococcus casseliflavus* (Oda *et al.,* 1993) and *Streptomyces* sp. (Takahashi *et al.,* 1994). However, very few reports on β-mannanase producing marine bacteria; *Vibrio* sp. Strain MA-138 (Tamura *et al.,* 1995) Owing to the above applications of mannanase, the present study was undertaken. In this report, the optimization of culture conditions for alkaline β-mannanase production by *Paenibacillus* sp. MSL-9 using guar gum as sole source of carbon and energy are presented. The *Paenibacillus* sp. MSL-9 is isolated as an agarase producer from a marine sample.

MATERIALS AND METHODS

Guar gum (GG) and Locust bean gum (LBG) were obtained from Sigma chemicals, USA. Copra meal, palm cakes were obtained from local market. All other chemicals were of analytical grade.

Bacterial strain

The bacterium was isolated from marine water sample as an agarase producer on MMS media supplemented with 0.1% agar as sole source of carbon and energy, described by Lakshmikanth *et al.,* (2006).The organism was identified as *Paenibacillus species* based on 16S rDNA sequence homology and the strain is designated as *Paenibacillus* sp. MSL-9 and the sequence was deposited in GenBank with an accession number FJ 859876.

Culture conditions

The *Paenibacillus* sp.MSL-9 had been isolated as described earlier as an agar utilizer in the laboratory on minimal-mineral salts medium (MMS) containing, (gL^{-1}); K_2HPO_4, 1.2; KH_2PO_4, 0.3; NH_4NO_3, 1.0; $MgSO_4$, 0.10; $FeCl_3$, 0.1; adjusted to pH 7.0, and agar 0.1% (w/v) as the only source of carbon and energy. Later, it was acclimatized to grow in MMS medium which was supplemented with GG 0.1% (w/v) as a sole source of carbon and energy. The modified MMS medium was used for standardizing the culture conditions and optimization for production of extra cellular mannanase by *Paenibacillus sp*. MSL-9. The medium contained (gL^{-1}) the following compositions, K_2HPO_4, 0.38; $MgSO_4$, 0.20; $FeCl_3$, 0.05; $NaNO_3$, 1.5; and Beef extract 1.5. The pH of the medium was adjusted to 8.0 and was supplemented with GG (0.1% w/v) as the only source of carbon and energy. The bacterial culture was maintained on MMS agar plates/slants supplemented with 0.1 (w/v) GG and was stored at 4ºC.

Cultivation of organism for enzyme production

Fifty ml aliquots of the MMS medium supplemented with 0.3% GG was dispensed in 250 mL Erlenmeyer flasks, and then sterilized at 121ºC for 15 min. After sterilization, the flasks were inoculated with 1mL of broth culture obtained from 3 days old culture; the flasks were then incubated in orbital shaker maintained at 30ºC; 160 rev min^{-1}. Thereafter, the contents of each flask were taken for analysis. Each treatment was carried out in triplicates and the results obtained throughout the work were arithmetic mean of three experiments.

Extraction of enzyme

At the end of incubation period, the bacterial cells were separated from the culture by centrifugation at 10,000 rev min^{-1} at 4ºC for 15 min in a cooling centrifuge. The clear supernatant thus obtained used as the crude enzyme source.

Assay of β-mannanase activity

The reaction mixture containing 0.5 mL of LBG (0.5%) in 20 mM tris buffer at pH 8.0 and 0.5 mL of enzyme solution was incubated in water bath at 40ºC for 15 min. The liberated mannose content was analyzed using dinitrosalicylate (DNS) reagent according to the method described by Miller, (1959). One unit of mannanase activity is defined as the amount of enzyme which produced 1μM of mannose under the assay conditions. The protein content of enzyme preparation was determined by the method of Lowry *et al.,* (1951) using Bovine serum albumin as standard.

Effect of physic-chemical parameters on growth and production of mannanase

The influence of temperature on growth and production of extra cellular mannanase was studied at 20, 25, 30, 35, 37, 40, 45, and 50ºC. Mannanase production was also investigated at initial pH adjusted between 6.0 and 10.0 and incubated at 30ºC. The growth of the bacterium at different incubation periods was measured spectrophotometrically by monitoring the absorbance at 660 nm and mannanase activity was assayed in culture filtrates simultaneously. The effect of carbon source on the production of mannanase was studied using the MMS medium supplemented with 0.3% of the carbon sources. In other trials mannan was replaced by equal amounts of different carbon sources viz., simple sugars such as, galactose, glucose, mannose, maltose, lactose, or complex polysaccharides such as, soybean meal, palm cake, copra meal, LBG, GG, potato peels and defatted meal of palm cake and copra meal as natural substrates that might be useful for production of the enzyme on a commercial scale. The defatting of palm cake and copra meal was carried out according to the method of Lin and Chen (2004) using n-hexane. The effect of nitrogen sources on the growth and production of â-mannanase was studied by supplementation of different N-sources to the medium containing GG (0.3% w/v) as sole source of carbon. Ammonium nitrate (0.3%) used as nitrogen source in the basal medium was replaced by inorganic nitrogen sources (0.1-0.5%), ammonium chloride, ammonium sulphate, sodium nitrate, potassium nitrate, or urea and organic nitrogen sources (0.1-0.5%), peptone, beef extract, or yeast extract, each at a time. The culture conditions were maintained as stated earlier.

RESULTS

Physico-chemical factors influencing mannanase production

The extracellular mannanase production by *Paenibacillus* sp. MSL-9 was observed after 12 h of incubation in MMS-medium supplemented with GG and reached maximum at 48 h of incubation. At longer incubation periods the activity gradually decreased (Fig. 1). The pH of the culture filtrate decreased with prolonged incubation. However, it was still in the basic range. The effect of initial pH on growth and mannanase production was studied. Medium adjusted to pH 8.0 favored maximum enzyme production of 0.72 U/mg (Fig. 2). The influence of temperature on growth and enzyme production was studied at 20, 25, 30, 35, 37, 40, 45, and 50ºC. The bacterium grew at temperatures ranging from 20º to 50ºC. Both growth and mannanase (0.74 U/mg) production was found to be maximum at 30ºC (Fig. 3). All the carbon sources tested were found to support the growth of *Paenibacillus* sp. MSL-9. (Table 1**).** Among commercial mannans, LBG (0.3% w/v) was found to induce maximum production of mannanase (0.919 U/mg), followed by GG (0.74 U/mg). The defatted palm cake and copra meal favored the growth but comparatively less mannanase was produced. The simple sugars supported the growth but did not induce the mannanase production.

The effect of nitrogen sources on the production of β-mannanase was studied using the culture medium supplemented with GG (0.3% w/v) as sole source of carbon. Growth and protein content of the culture filtrate were differently affected by the nature of nitrogen source (Table 2). all the tested nitrogen sources were able to support the production of β-mannanase. Mannanase production was found to be maximum when the bacterium was grown on either sodium nitrate or beef extract. The MMS medium supplemented with sodium nitrate and beef extract as nitrogen source in the fermentation medium yielded maximum mannanase activity (5.738 μ/mg).

DISCUSSION

The mannan degrading enzyme systems have been described in a great variety of bacteria and fungi species and it was found to be inducible. The reports on marine isolates producing β-mannanase are only few (Tamura *et al.,* 1995). Although previous research regarding β-mannanase production from marine bacteria has been reported, little information on the optimization of its production is available. The *Paenibacillus* sp. MSL-9 was isolated as an agarase producer from a marine sample. The isolate MSL-9 grew at a broad range of pH from 5.0 to 10. Most of the marine isolates producing β-mannanase reported till date are reported to grow optimally at pH of 6.5 and 7.5 (Tamura *et al.,* 1995). The *Paenibacillus* sp. MSL-9 grew optimally at pH 8.0. Simple sugars supported the growth of the bacterium, but did not induce the mannanase production. The bacterium grew well and showed mannanase production when galactomannans were used as sole source of carbon and energy. The bacterium grew on raw materials of commercial potential with significant differences on the rate of enzyme production. The large variation in mannanase yield may be due to the nature of cellulose or hemicelluloses, presence of some components (activators or inhibitors) in these materials and variations in the substrate accessibility (Mona *et al.,* 2008). The mannanase production seems to be more when LBG is used as a sole source of carbon. Similar results are reported in *Bacillus subtilis* (Mendoza *et al.,* 1994), *Bacillus* species (Araujo and Ward, 1990a), *Alkalophilic Bacillus species* (Akino *et al.,* 1987) and *Enterococcus casseliflavus* (Oda *et al.,* 1993). GG is an indigestible galactomannans because of its highly substituted structure which may be the reason for decrease in mannanase production when compared to LBG. The source plant for GG, *Cyamopsis tetragonalobus* are grown mainly in north-western India and Pakistan, hence, GG could serve as a cheap carbon source for the production of mannanase. The co-supplementation of simple sugars with guar gum did not induce mannanase production as they would cause catabolite repression. This indicates that the enzyme is inducible in this bacteria and the synthesis of which is repressed by presence of other simple carbon sources. The mechanisms that govern the formation of extra cellular enzymes are influenced by the availability of precursors for protein synthesis. Furthermore, the nitrogen source can significantly affect the pH of the medium during the course of fermentation (Lin and Chen, 2004). Sodium nitrate along with beef extract served as very good combination of nitrogen source. In conclusion, the results

Table 1: Effect of different carbon sources on β-mannanase production

Carbon sources (0.3 %, w/v)	Activity (U/mg)
Galacto mannans	
Guar gum	0.740
Locust bean gum	0.919
Palm cake	0.129
Defatted palm cake	0.462
Copra meal	0.210
Defatted copra meal	0.572
Potato peels (oven dried)	0.540
Simple sugars	
Glucose	0.002
Galactose	0.001
Mannose	0.002
Sucrose	0.001

Table 2: Effect of various nitrogen sources on β-mannanase production

Nitrogen sources (0.3% in the medium)	activity (μ/mg)
Inorganic nitrogen sources	
Sodium nitrate	0.741
Ammonium nitrate	0.367
Ammonium sulfate	0.519
Ammonium chloride	0.124
Potassium nitrate	0.550
Urea	0.494
Organic nitrogen sources	
Peptone	0.427
Yeast extract	0.864
Beef extract	1.020
Combined form	
Sodium nitrate + Peptpone	2.312
Sodium nitrate + Yeast extract	1.850
Sodium nitrate + Beef extract	5.738

0.15% each in the medium.

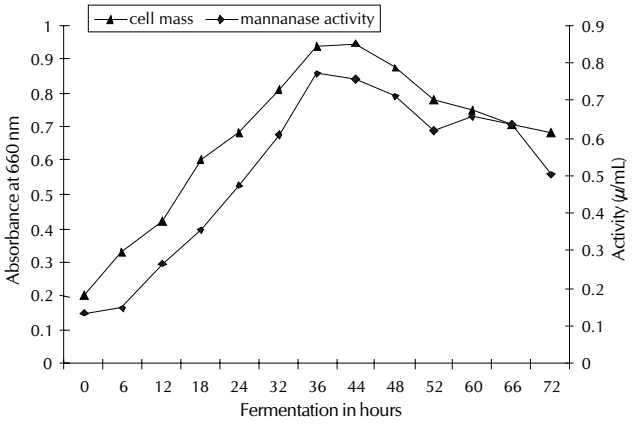

Figure 1: A typical growth profile and mannanase production by *Paenibacillus sp.* MSL-9

demonstrated the production of mannan degrading enzymes using GG and agro-wastes like palm cake, copra meal and potato peels as substrates which offer significant benefit due to cheaper cost and abundant availability. From the results observed, it can be concluded that production of mannan degrading enzymes are associated with growth of the bacterium, *Paenibacillus* sp. MSL-9 and the alkaline mannanase secreted by later may be useful in industrial procedures, food processing and in biobleaching. Further, purification and char-

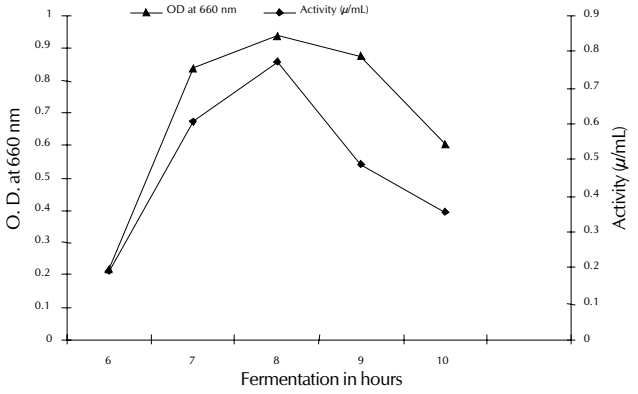

Figure 2: Effect of pH on growth and mannanase production by strain MSL-9

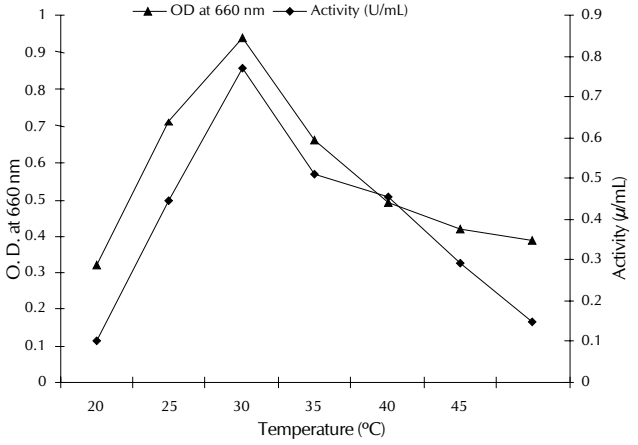

Figure 3: Effect of temperature on growth and production of mannanase by MSL-9.

acterization of the mannanase is under progress and results will be published in near future.

ACKNOWLEDGEMENTS

The work was partly supported by grants in aid by University Grants Commission, New Delhi, India. (Project No.F.No.31-286/2005 (SR) dt: 31-03-2006). Thanks are also due to Gulbarga University, Gulbarga for providing laboratory facilities.

REFERENCES

Abe, J., Hossain, Z. M. and Hizukuri, S. 1994. Isolation of b-mannanase producing microorganisms. *J. Ferment. Bioengineer*. **3:** 259–261.

Akino, T., Nakamura, N. and Horikoshi, K. 1987. Production of β-mannanase and â-mannosidase by an alkalophilic *Bacillus* sp. *Applied Microbiology and Biotechnology*. **26:** 323-327.

Araujo, A. and Ward, O. P. 1990a. Hemicelluloses of *Bacillus species*: preliminary comparative studies on production and properties of mannanase and galactanases. *J. Appl. Bacteriol*. **68:** 253–261

Araujo, A. and Ward, O. P. 1990b. Mannanase component from *Bacillus pumilus*. *App. and Env. Microbio*. **56:** 1954–1956.

Cheng, C. G. and Yang, V. C. 1991. Editors. Cosmetic and Pharmaceutical Applications of Polymers. New York: Plenum Press. pp. 117.

Chiu, C. W., Henley, M. J., Zallie, J. P. and Jeffcoat, R. 2001. Bulking agents and processes for preparing them from food gums, United States Patent No.6, 229,924, issued to National Starch and Chemical Investment Holding Corporation (Wilmington, DE).

Christgua, S., Andersen, L. N., Kaupinen, S., Heldt- Hansen, H. P. and Dalboege, H. 1994. Purified enzyme exhibiting mannanase activity; Application in oil, paper, pulp, fruit and vegetable juice industry and in carrageen extraction. Patent NOVO-Nor disk, 9425576, 10 Nov 1994.

Cuevas, W. A., Kant linen, P., Tanner, B. B. and Leskiner, S. 1996. Purification and characterization of novel mannanase used in the pulp bleaching. In: Srebotnik, E. and Mesner, K. (Eds), Biotechnology in the pulp and paper industry. pp. 123-6. Facultas-universi tatsverlag, Vienna, Austria.

Cummings, J. H., Branch, W. and Jenkins, D. J. A. 1987. Colonic response to dietary fiber from carrot, cabbage, apple, bran and guar gum. *Lancet*. **1:** 5-9.

Francoise, V. C., Ghakis, C., Dupont., Morosoll, R. and Kluepfel, D. 1996. Improved production of mannanase by *Streptomyces lividans*. *Applied and Env. Microbio*. **63:** 56-58.

Hogg, D., Pell, G., Dupree, P., Goubet, F., Martin-Orue, S. M., Armand, S. and Gilbert, H. J. 2003. The modular architecture of Cell *Vibrio japonicas* mannanase in glycoside hydrolase's families 5 and 26 points to differences in their role in mannan degradation. *Biochem. J.* **371:** 1027–1043.

Ishihara, N., Chu, D. C., Akachi, S. and Junej, L.R. 2000. Preventive effect of partially hydrolyzed guar gum in infection of *Salmonella enteritidis* in young and laying hens. *Poult. Sci.* **78:** 689-697.

Lahtinen, T., Kristoo, P. and Paioheim, M. 1995. Mannanase in softwood Kraft pulp bleaching. Sixth international conference on Biotechnology in the pulp and paper industry, 11-15 June. pp. 4-101.

Lakshmikanth, M., Manohar, S., Patnakar, J., Vaishampayan, P., Shouche, Y. and Lalitha, J. 2006. Optimization of culture conditions for the production of extracellular agarases from newly isolated *Pseudomonas aeruginosa* AG LSL-11. *World J. Microbiol. Biotechnol.* **22:** 531–537.

Lin, T. C. and Chen, C. 2004. Enhanced mannanase production by submerged culture of *Aspergillus niger* NCH-189 using defatted copra based media. *Process Biochem*. **39:** 1103-1109.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biological Chemistry*. **193:** 265-275.

Mc Cleary, B. V. 1979. Modes of action of β-mannanase enzymes of diverse origin on legume seed galactomannans. *Photochemistry*. **18:** 757-763.

Mc Cleary, B. V. 1988. Carob and guar galactomannans. *Methods in Enzymology*. **160:** 523–527.

Mc Cleary, B. V. and Matheson, N. K. 1983. Action patterns and substrate binding requirements of β-mannanase with monosaccharide and mannan-type polysaccharides. *Carbohydrate Research*. **119:** 191-219.

Mendoza, N. S., Arai, M. and Kawaguchi, T. 1994. Isolation of mannan-utilizing bacteria and the culture conditions for mannanase production. *World J. Microbiol. Biotechnol*. **10:** 51-54.

Miller, G. L. 1959. Use of dinitrosalicyclic acid reagent for determination of reducing sugar. *Analytical Chemistry*. **31:** 426-428.

Mona, E. M., Mabrouk Amani, M. D. and El, Ahwany. 2008. Production of â-mannanase by *Bacillus amylolequifaciens* 10A1 cultured on potato peels. *African J. Biotechnol*. **7(8):** 1123-1128.

Naughton, P. J., Mikkelsen, L. L. and Jensen, B. B. 2001. Effects of nondigestible oligosaccharides on Salmonella *enterica Serovar Typhimurium* and nonpathogenic *Escherichia coli* in the pig small intestine *in vitro*. *Applied and Env. Microbiol*. **67:** 3391–3395.

Oda, Y., Komaki, T. and Tonomura, K. 1993. Production of β-

mannanase and β-mannosidase by *Enterococcus casseliflavus* FL 2121 isolated from decayed konjac. *Food Microbiol*. **10:** 353-358.

Prodhomme, R. K., Constein, V. and Knoll, S. 1989. Advances in Chemistry Series 89.Washington, DC: ACS Publishers; 25. [3] Brode, G. L., Goddard, E. D., Harris, W. C., Salansky, G. A. In: Gebelein.

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., Frederick, W. J., Jr, Hallett, J. P., Leak, D. J, Liotta, C. L., Mielenz, J. R., Murphy, R., Templer, R. and Tschaplinski, T. 2006. The path forward for biofuels and biomaterials. *Science***. 311:** 484-489.

Ratto, M. and Poutanen, K. 1988. Production of mannan-degrading enzymes. *Biotech. Lett* **.10:** 661-664.

Singh, S., Andreas, M. M. and Bernard, A. P. 2003. *Thermomyces lanuginosus* properties of strains and their hemicelluloses. FEMS Microbiology Reviews. **27:** 3-16.

Sudathip, T., Suttipun, K., Dietmar, H. and Sunee, N. 2008. Selection and characterization of mannanase-producing bacteria useful for the formation of prebiotic manno-oligosaccharides from copra meal. *World J. Microbiol. Biotechnol*. **24:** 1425–1433.

Takahashi, H., Yang, S. I., Fujiki, M., Kim, M., Yamamoto, T. and Greenberg, N. A. 1994.Toxicity studies of partially hydrolyzed guar gum. *J. Am. Coll. Toxicol***. 13:** 273-278.

Tamura, Y., Araki, Amagoi, H., Mori, H. and Morishita, T. 1995. Purification and characterization of an extra cellular â-1, 4-mannanase from a marine bacterium, *Vibrio* sp. Strain MA-138. *Applied and Env. Microbiol*. **61:** 4454-4458.