International Research Journal of Natural and Applied Sciences
Vol. 3, Issue 11, November 2016 Impact Factor- 5.46
ISSN: (2349-4077)

Website: www.aarf.asia Email : editor@aarf.asia , editoraarf@gmail.com

ISOLATION AND IDENTIFICATION OF CHITOSANASE PRODUCING BACTERIA FROM MARINE SOURCE AND PHYSICOCHEMICAL PARAMETER OPTIMIZATION FOR ENZYMATIC PRODUCTION OF CHITOOLIGOSACCHARIDES

Arunkumar Ganesan[1], Bhanumathi Kannan[1]
[1]Dept of Zoology, Kamaraj College, Tuticorin, Tamil Nadu, Pincode -628003

ABSTRACT

Chitooligosaccharides (COS) is a derived oligosaccharides mixture produced from chitosan. COS possess various bioactivity of pharmacological importance. Enzymatic conversion of chitosan yields COS of defined degree of polymerization, rather than acid hydrolysis. In this study, chitosanase producing microorganisms, mainly bacteria was isolated from marine soil. About 25 isolates were screened for chitosanase production based on quantitative determination of reducing sugar using DNSA method. High enzyme activity exhibiting isolate was identified using 16S rDNA gene amplification, based on BLAST analysis, the organism was found to be Bacillus firmus. The enzyme production was maximum during 72 h incubation in static mode. The maximum enzyme activity was obtained at pH 9, temperature 50˚C. Thin Layer chromatography revealed the maximal enzymatic production of COS was observed during 3-7 h. Hence, this enzyme could be exploited for the production of COS.

Keywords: chitooligosaccharides, 16S rDNA, chitosan, D-glucosamine, alkalophilic chitosanase

Introduction

Chitosan is a non-toxic biopolymer found its application in various industry like pharmaceutical, food and wastewater treatment. Besides its application, certain physicochemical

properties like solubility in acid and high viscosity limits the application of chitosan (Lodhi *et al.,* 2014).The derivatives of chitosan are chitooligosaccharides (COS) which overcomes the limitations of chitosan in terms of solubility in water and low viscos nature. COS can be produced by chemical and enzymatic method. Chemical method produces COS of various degree of polymerization, whereas enzymatic method produces COS of same degree and length. COS found to be more active than chitosan and its biological activities was clearly elaborated (Xia *et al.,* 2010). Reports has demonstrated the hypocholesterolemic effect of COS (Kim & Rajapakse, 2005). Immunity enhancing and antitumor effects of COS was also evaluated (Suzuki *et al.,*1986). Enzymatic production of COS can be carried out by chitosanase, the enzyme produced by bacteria, fungi and certain plants, which acts on the β1-4 linkage between D-glucosamine residues in the partly deacetylated chitosan . It is absolutely necessary to isolate the chitosanase producing bacteria so that the production and purification of chitooligosaccharides can be simplified. Microbial source of enzyme is usually preferable because it can be produced in large quantum economically. Several authors reported the isolation of chitosanase producing bacteria, *Bacillus cereus* (Gao *et al.,* 2012), fungi *Metarhizium anisopliae* (Assis et al., 2010), *Chaetomium globosum* (Shehata and Aty, 2015). Antimicrobial effect of COS produced by *Pseudomonas* CUY8 chitosanase was reported (Wang *et al.,* 2007). Isolation of chitosanase producer from shrimp wastes was also demonstrated (Wangtueai *et al.,* 2006). Chitosanase producing *Bacillus sp* was isolated from seawater and soil (Zakaria *et al.,* 2012). Thermostable chitosanase gene from *Bacillus sp* strain CK4 was cloned and expressed in *E.coli* BL21(DE3) to produce thermostable chitosanase (Yoon *et al.,* 2000).

Infact, Chitosan is derived from chitin found in the exoskeleton of marine arthropods like crab and shrimp. The marine soil harbors the chitosan degrading microorganism which could be exploited for production of COS. Treatment of chitosan with *Bacillus cereus* D-11crude enzyme and purification by Ion exchange chromatography for COS production was explained clearly (Gao *et al.,* 2012). COS production using crude enzyme extract obtained from *Metarhizium anisopliae* was also carried out (Assis *et al.,* 2010).The aim of this study is to isolate and identify the chitosanase producing bacteria from marine environment and optimization of physico-chemical parameters like pH, temperature and incubation time for chitosanase enzyme production and to produce COS via enzymatic method.

Materials and methods

Isolation of chitosanase producing bacteria

Soil samples were collected from Tuticorin coastal area. 10^{-6}, 10^{-7} dilutions were plated in nutrient agar plates containing 1% chitosan. Selected colonies were cultured in nutrient broth for 24 h and the culture broth was centrifuged at 6000*g* to remove the enzyme containing supernatant. Enzyme supernatant from selected colonies is tested for the release of glucosamine using Dinitrosalicylicacid method (Sadasivam and Manickam, 2005). One chitosanase unit (U) is defined as the amount of enzyme that liberated 1 µmol of D-glucosamine per minute under the assay conditions.

Identification of chitosanase producing bacteria

The selected chitosanase producing bacteria was identified by 16S rDNA amplification. Total DNA was extracted from overnight grown culture using DNA extraction kit (Himedia Laboratories Pvt. Ltd., Mumbai, India) and analyzed on 1% Agarose gel electrophoresis. Using the DNA as template, 16 S rDNA gene region was amplified using primers 16SF 5'AGAGTTTGATCCTGGCTCAG 3', 16SR 5'ACCTTGTTACGACTTCACCCCAG 3'. The thermocyclic conditions were initial denaturation at 94˚C for 2 min, followed by 30 cycles of denaturation at 94˚C for 1 min, annealing at 52 ˚C for 45 sec, extension at 72 ˚C for 1min and final extension at 72 ˚C for 10 min. The amplicon of size 1200 bp was sequenced and compared with several bacterial species using NCBI database. Phylogenetic tree was constructed using phylogenyfr software.

Optimization of physicochemical parameters

The enzyme was incubated at different pH 6-11, temperature 30 ˚C – 70 ˚C and enzyme activity was determined by estimation of reducing sugars by Dinitrosalicylic acid method. Culture was incubated at different time intervals 1d- 4d and enzyme activity was determined.

Production of chitooligosaccharides

Mixture of 1ml chitosan and 1ml enzyme was incubated at 50˚ C for various time intervals (1h, 2h, 3h, 4h, 5h, 6h). Qualitative estimation of COS production was done by Thin

Layer chromatography using n-propanol and 30% ammonia water (2:1) as solvent system and detected by spraying 0.1% ninhydrin reagent.

Results and Discussion

Several isolates were screened for the production of chitosanase enzyme. The isolate No.10 was exhibited maximum enzyme activity of 0.050 IU (Figure 1). The genomic DNA of isolate No.10 was produced amplification at 1200bp corresponding to 16S rDNA gene (Figure 2). On comparison of 1200bp sequence of chitosanase producer with 16S gene sequence of other bacterial species available in NCBI database, it exhibited 89% similarity with the bacteria *Bacillus firmus* and the phylogenetic tree was also plotted (Figure 3). The enzyme was found to be alkalophilic in nature with the activity, gradually increasing from pH 6 and attain maximum at pH 9 and then started decreasing gradually till pH11 and falls abruptly beyond pH 11(Figure 4). It is completely inactive in acidic below pH6. The enzyme was found to have broad temperature range from 30 ˚C to 70 ˚C with maximum at 50 ˚C, which revealed the enzyme is moderately thermostable (Figure 5). The enzyme production started at 1d (24h), increases and attain maximum at 3d (72h) and found to be static till 4d (96h) and started decelerating after 96h (Figure 6). The enzyme treated chitosan (1%), revealed the formation of chitooligosaccharides when compared to the raw chitosan in Thin Layer Chromatography (Figure 7). The chitooligosaccharides production initiated at 1h and slightly increased and stabilized up to 7h.

Chitosanase enzyme is an industrially important enzyme used in the production of chitooligosaccharides has gained attention recently (Gao *et al.,* 2012; Assis *et al.,* 2010; Shehata and Aty, 2015; Wangtueai *et al.,* 2006; Zakaria *et al.,* 2012). In this study, chitosanase producing bacteria was isolated from marine soil and identified using 16S rDNA gene. BLAST analysis revealed that the bacterium was *Bacillus firmus*. The enzyme was found to be alkalophilic in nature and active at pH 9 which is different from the results given by other reports, where the optimum pH was in the range of pH 4.0 -8.0 (Somashekar and Joseph, 1996). However, *Penicillium janthinellum* chitosanase has activity in a range of pH 7-9 which supports our result (Nguyen *et al.,*2014). The enzyme is thermostable in a temperature range of 40˚ C - 60˚ C with maximal activity at 50˚ C which is better than chitosanase isolated from *Penicillium janthinellum* and *Chaetomium globosum* which was stable at 25˚ C -50˚ C and 40˚ C respectively (Nguyen *et al.,*2014; Shehata and Aty, 2015). The enzyme production was maximum after 72h incubation at

37˚ C, this explains the time required by the bacteria to utilize the chitosan as carbon source and the enzyme production is inducible by chitosan. Maximal production of enzyme from *Bacillus cereus* D11 was achieved at 30˚ C (Gao *et al.*, 2012) for 3 days. TLC image clearly revealed the production of COS production begins after the completion of 1h and attains maximum after 3h and continues till 7h and hence this enzymatic production of COS is more advantageous than other chitosanase reported, in which 18h incubation was required (Gao *et al.*, 2012).Hence, this enzyme could be used for the industrial scale production of COS.

References

Assis, C.F., Araujo, N.K., Pagnoncelli, M.G.B., Pedrini, M.R.S., Macedo, G.R. and Santos, E.S. (2010), "Chitooligosaccharides enzymatic production by Metarhizium anisopliae", Bioprocess Biosystem Engineering, http://doi 10.1007/s00449-010-0412.

Gao, X.A., Zhang, Y.F., Park, R.D., Huang, X., Zhao, X.Y., Xie, Jiao. and Jin, R.D. (2012), "Preparation of chitooligosaccharides from chitosan using crude enzyme of Bacillus cereus D-11", Journal of Applied Biological Chemistry,55(1), 13-17.

Kim, S.K.and Rajapakse, N. (2005), "Enzymatic production and biological activities of chitosan oligosaccharides (COS): a review", Carbohydrate Research,62, 357-368.

Lodhi, G., Kim, Y.S., Hwang, J.W., Kim, S.K., Jeon, Y.J., Je, J.Y., Ahn, C.B., Moon, S.H., Jeon, B.T. and Park, P.J. (2014), "Chitooligosaccharides and its derivatives: Preparation and Biological Applications", BioMed Research International, http://dx.doi.org/10.1155/2014/654913.

Nguyen, A.D., Huang, C.C., Liang, T. and Nguyen, V.B. (2014), Carbohydrate Polymers,108, 331-337.

Sadasivam, S., Manickam, A. (1996),Biochemical Methods, New Age International, Second Edition.

Shehata, A.N. and Aty, A.A.A. (2015), "Improved production and partial characterization of chitosanase from a newly isolated Chaetomium globosum KM651986 and its application for chitosan oligosaccharides", Journal of chemical and pharmaceutical research, 7(1), 727-740.

Somashekar, D. and Joseph, R. (1996), " Chitosanases- Properties and Applications: A review", Bioresource Technology,55, 35-45.

Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S. and Suzuki, M. (1986), " Anti-tumor effect of hexa-N-acetylchitohexose and chitohexose", Carbohydrate Research, 151,403-408.

Wang, Y.W., Zhou, P., Yu, J., Pan, X., Wang, P., Lan, W. and Tao, S. (2007), " Antimicrobial effect of chitooligosaccharides produced by chitosanase from Pseudomonas CUY8", Asia Pacific Journal of Clinical Nutrition,16 (1), 174-177.

Wanhtueai, S., Worawattanamateekul, W., Sangjindavong, M., Naranong, N. and Sirisansaneeyakul, S. (2006), " Isolation and screening of chitosanase producing microorganisms", Kasetsart Journal of Nat. Sci, 40, 944-948.

Xia, W., Liu, P., Zhang, J. and Chen, J. (2010), "Biological activities of chitosan and chitooligosaccharides", Food Hydrocolloids, http://doi:10.1016/j.foodhyd.2010.03.003.

Yoon, H.G., Lee, K.H., Kim, H.Y., Kim, H.K., Shin, D.H., Hong, B.S. and Cho, H.Y. (2002), " Gene Cloning and Biochemical Analysis of Thermostable Chitosanase (TCH-2) from Bacillus coagulans CK108", Bioscience, Biotechnology, and Biochemistry, 66(5), 986-995, DOI: 10.1271/bbb.66.986.

Zakaria, M., Zakaria, S.N., Musa, M., Hamilin, H. and Zulkifly, N.A.H. (2012), " Identification of chitosan degrading microbes for the production of chitooligomer", International conference on chemistry and chemical Engineering,38.

.

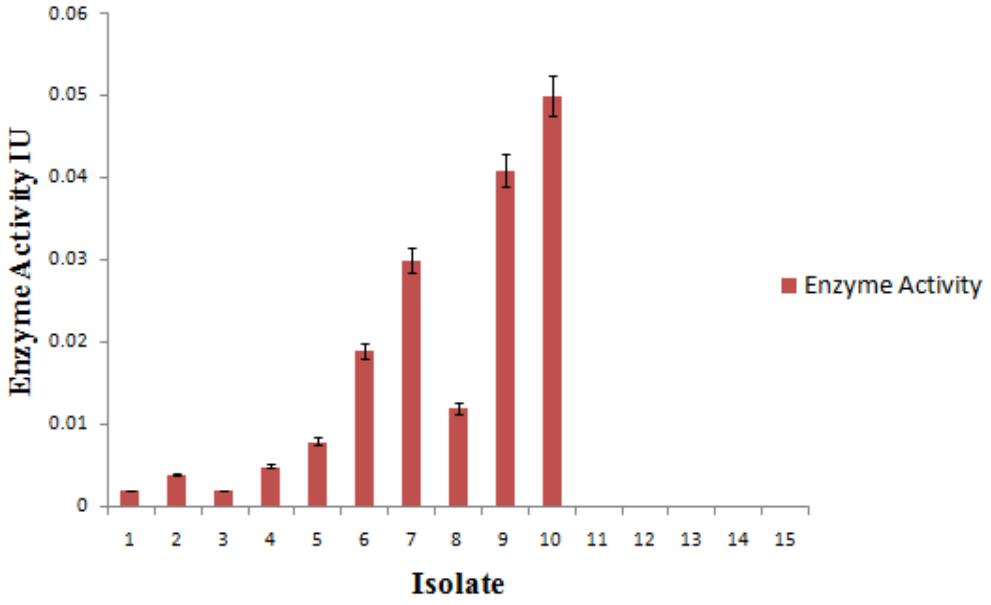

Figure 1. Screening of potent chitosanase producing bacteria

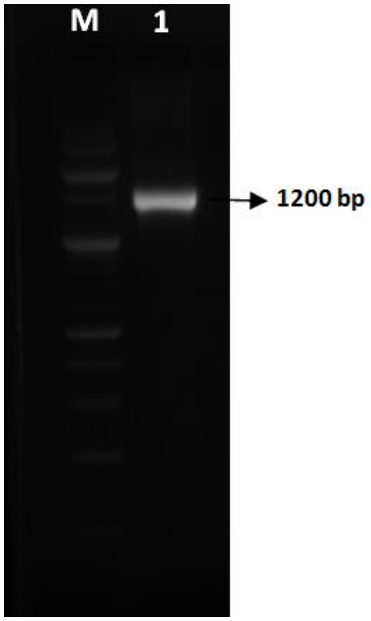

Figure 2. PCR amplification of 16 S rDNA

Lane M: 100bp DNA ladder, Lane 1: 1200bp PCR amplicon

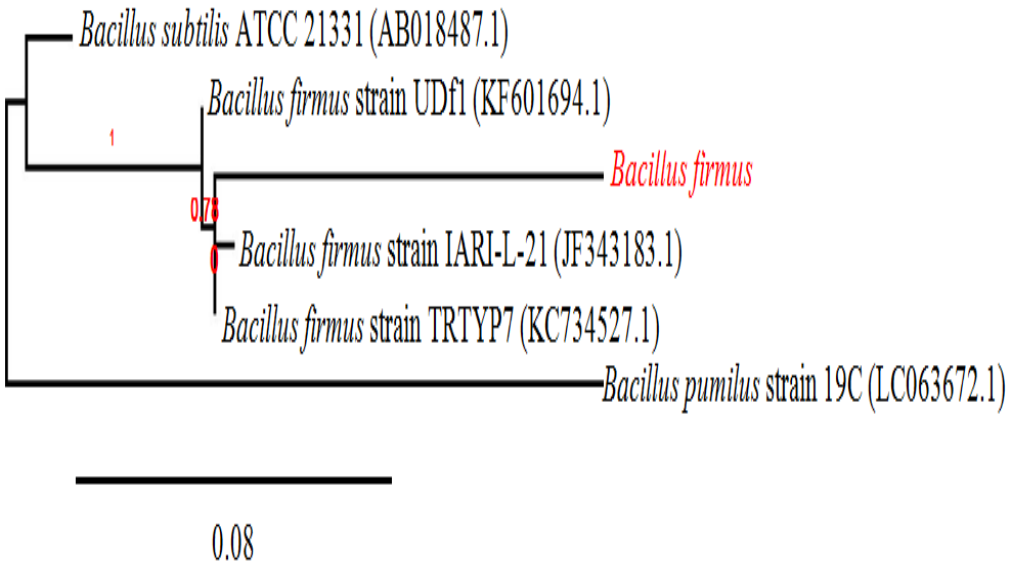

Figure 3. Phylogenetic tree analysis of chitosanase producing bacteria

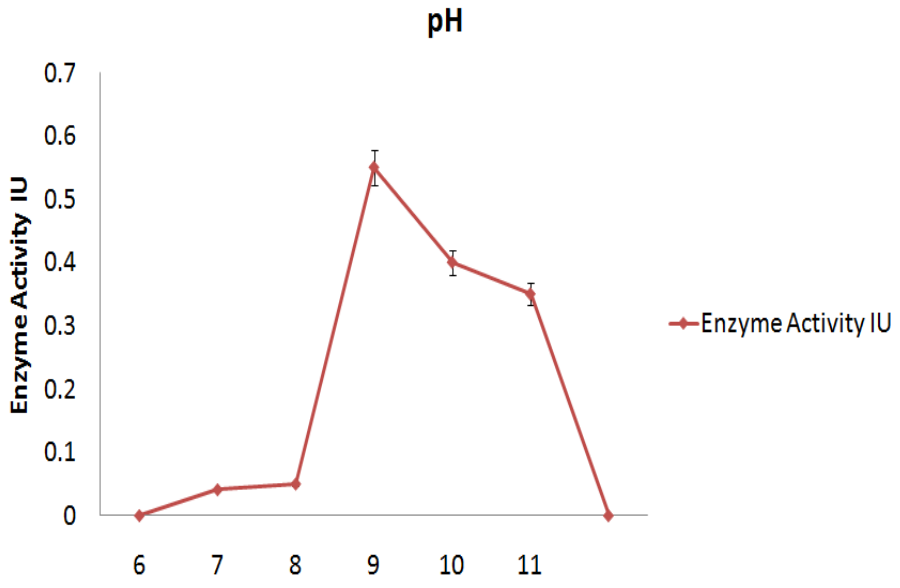

Figure 4. Effect of pH on chitosanase production

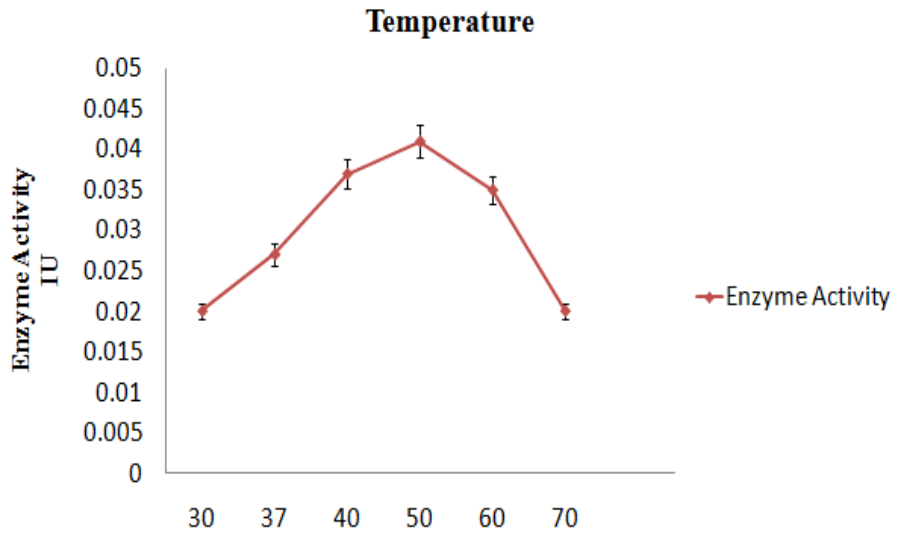

Figure 5. Effect of temperature on chitosanase production

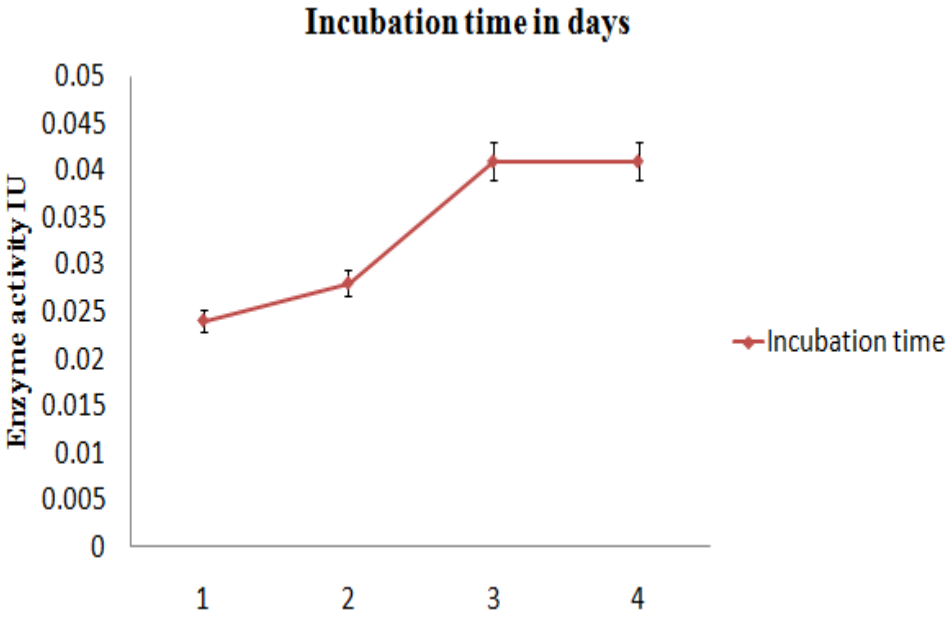

Figure 6. Effect of incubation time on chitosanase production

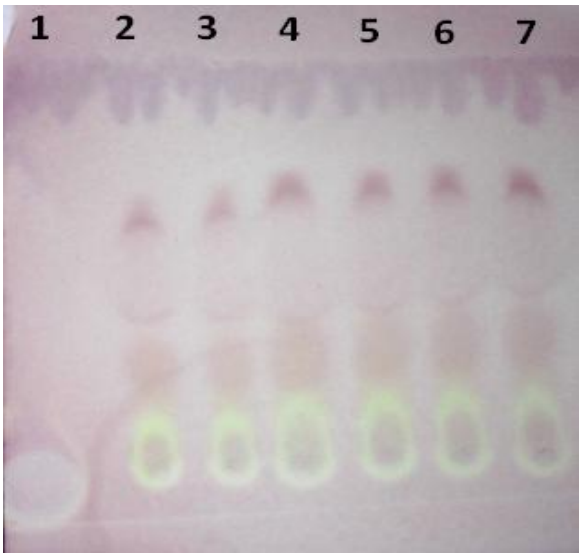

Figure 7. TLC of chitooligosaccharides

Lane 1: raw chitosan, Lane 2: enzyme treated chitosan 1h, Lane 3: enzyme treated chitosan 2h, Lane 4: enzyme treated chitosan 3h, Lane 5: enzyme treated chitosan 4h, Lane 6: enzyme treated chitosan 5h, Lane 7: enzyme treated chitosan 6h