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Identification of Chitosan-Degrading Microbes for the Production of Chitooligomer

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Abstract. A total of twenty bacteria were isolated from various kinds of seawater and soil samples collected along the beach of Kuala Terengganu. Liquid minimal media and solid minimal media were used for enrichment and direct plating isolation, respectively. Bacteria strains were isolated based on their ability to grow in liquid minimal media supplemented with 1% chitosan powder as the sole carbon and energy source. From 20 selected bacterial strains, only 10 cell cultures showed signs of inference of chitosan biodegradation. The activity of which was measured quantitatively for the reducing sugar produced in the growth medium. The strain isolated from Batu Buruk beach (L1) and from Chendering beach (BB2) showed a promising extent of biodegradation with the released reducing sugar concentration of 0.727 and 0.572 μmol/mL, respectively.

Keywords: Chitosan, chitosanase, Chitosanase-producing microorganisms, D-glucosamine, N-acetyl-D-glucosamine

1. Introduction

Chitosan is a linear polysaccharide of β-(1→4)-D-glucosamine. Chitosan is derived from chitin by deacetylation process. Normally, chitin itself is crustacean such as prawn and crabs. In aqueous solution, chitosan provides a positively charged macro-ion, and thus it is also known as soluble chitin. In nature, the polymer is partially acetylated and, in fact, the name chitosan describes a wide range of polymers with various proportions of D-glucosamine and N-acetyl-D-glucosamine residues. It has decomposability, good membrane forming state and biocompatibility functions. Chitosan enjoys multifunction polysaccharide. Recently, much attention has been given to converting chitosan to safe and functional chitooligosaccharides, because they show strong physiological activities, such as antitumor effects and antimicrobial activity (Aam and Berit, 2010).

Chitosanase (EC 3.2.1.132) or GlcN-ase is a hydrolytic enzyme acting on β-1,4-glycosidic linkage of chitosan to release chito-oligosaccharides. The difference between chitosanase and other hydrolytic enzymes, such as chitinase, lysozyme, and *N*-acetyl-β-D-glucosaminidase is rather obscure, especially in the case of enzymes that display activities toward multiple substrates. It is thought that the ability to hydrolyze a chitosan or pure deacetylated chitin (chitan) is an important criterion for classifying an enzyme as a chitosanase (Liu *et al*., 2009).

Chitosanases are produced by many organisms, including actinomycetes, fungi, plants and bacteria. Bacterial chitosanases have received special attention because they are important for the maintenance of the ecological balance and have been used to determine the mechanism of chitosan hydrolysis at both biochemical and molecular levels. Previously, several microorganisms including bacteria were reported to

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efficiently produce chitosanases to degrade chitosan to glucosamine oligomers. Furthermore, fungi and actinomycetes also produce chitosan oligomers from chitosan. The oligomers of D-glucosamine (GlcN) and of N-acetyl-D-glucosamine (GlcNAc) have interesting biological activities (Aam and Berit, 2010) including anti-tumor effects (Chui *et al.*, 2003; Jae *et al*., 1999), hypo-cholesterolemic effects (Makoto *et al*., 1996), anti-microbial activities (San Lang *et al*., 2008; Souad and Alain, 2002), disease-resistance responses, and as phytoalexin elicitors in higher plants.

The characteristic properties of chitosanase, chito-oligosaccharides and their derivatives thus have attracted interest from food and pharmaceutical industries since they can be used as edible additives, agricultural immunity controls and a promise of many other prospective applications such as prophylactic agents for liver diseases, atherosclerosis and hypertension (Makoto *et al*., 1996).

2. Materials and Methods

2.1. Isolation and Screening of Chitosanase-Producing Bacteria

The bacterial isolate used in this study were originally isolated from seawater and soil samples, which were collected along the coast of Kuala Terengganu, from Tok Jembal Beach, Batu Buruk Beach to Chendering Beach. Approximately 5 mL of collected water sample (or 5 g of soil samples) were shaked in 250 mL flask with liquid minimal media (pH 6.5) containing 1% chitosan powder as the sole carbon source. The mixtures were then incubated at 30°C for 2 days in an incubator shaker (150 rpm) before plating. Then, 0.1 mL of samples of diluted enrichment culture broth was transferred to solid minimal media followed by procedures of spread plate technique. For the direct plating method, 0.1 mL of collected water samples were spreaded onto the selective solid minimal media for bacteria growth. All single strong colonies on the plate were selected and maintained as slant cultures.

Screening for chitosanase production was carried out by using selective liquid minimal media. Samples were taken out after 24 hours of incubation and the analysis of chitosanase activity was performed by detecting the reducing sugars released. The bacteria strain that produced the highest chitosanase activity was selected for subsequent characterization and chitosanase production.

2.2. Growth Conditions

Isolation of bacteria strains which has the ability to produce chitosanase was done using solid and liquid minimal media. The culture was grown at 30°C on a 150 rpm rotary shaker in 250 mL flasks containing 100 mL medium. The liquid minimal media was prepared to contain 1% chitosan powder, 0.05% $MgSO_4.7H_2O$, 0.07% $K_2HPO_4.3H_2O$, 0.03% KH_2PO_4, 0.5% NaCl and 1.2% $(NH_4)_2SO_4$, and were autoclaved (121ºC for 15 min at 15 psi). Liquid minimal cultures were supplemented with yeast extract to a final concentration of 0.3% (w/v).The pH of 6.5 was attained by adding NaOH 1 M. Oxoid bacteriological agar (15 g/L) was added prior to autoclaving.

2.3. Characterization and Identification of Bacteria

The bacteria strains which released highest reducing sugars (see 2.6) were selected for characterization using biochemical tests and morphological analyses. Gram stain test, endospore stain test and acid fast stain test were performed and the morphology of strain were determined through observation on selective agar medium. The tests were observed under 1000x immersion oil objective of a light microscope. The results of staining and culturing were combined with the results of biochemical tests to definitively identify the bacteria. The biochemical tests evaluate the metabolic properties of a bacterial isolate. After a number of biochemical tests have been performed, the combined test results forms a biochemical pattern for an isolate, which is unique for each species.

2.4. Analytical Methods

A volume of 5.0 mL of samples were pipette out aseptically from conical flasks in a shaker at 3-hour intervals for 24 hours growth period for bacteria. Subsequently, the sample was transferred into a falcon tube and spinned at 4000 rpm for 15 minutes. The supernatant was used for growth analysis and chitosanase assay.

2.5. Growth Analysis

A volume of 1.0 mL samples were pipette out periodically at 3-hour intervals for 24 hours period for bacteria growth determined by measuring turbidity on a Shimadzu UVmini-1240 UV-Vis Spectrophotometer at 680 nm wavelength.

2.6. Chitosanase Assay

Activity of the enzyme was measured by quantitatively the reducing sugars produced from chitosan. Each assay was carried out in triplicates, by incubating 0.2 mL of the enzyme solution with 1 mL of 0.3% (w/v) chitosan in 50 nM phosphate buffer of a pH 7, at 37^{o}C for 30 min. The reaction was terminated by heating it at 100^{o}C for 15 min. The resulting adducts of reducing sugars were analyzed and measured spectrophotometrically at 540 nm wavelength. The amount of reducing sugar produced was measured by modified dinitrosalicyclic acid method (Fink *et al*., 1991). One unit of chitosanase activity is defined as the amount of enzyme required to release 1 µmol of detectable reducing sugars at 37 °C in 1 min.

3. Results and Discussion

3.1. Isolation and Screening of Chitosanase-Producing Bacteria

It is anticipated that the colonies which grew well and healthy in liquid minimal media would produce chitosanase. The results showed that L1 strain and BB2 strain released quite a reasonably high concentration of reducing sugar (i.e. 0.727 µmol/mL and 0.572 µmol/mL respectively). Figure 1 summarizes all of the microbial chitosanases that have been isolated in this work.

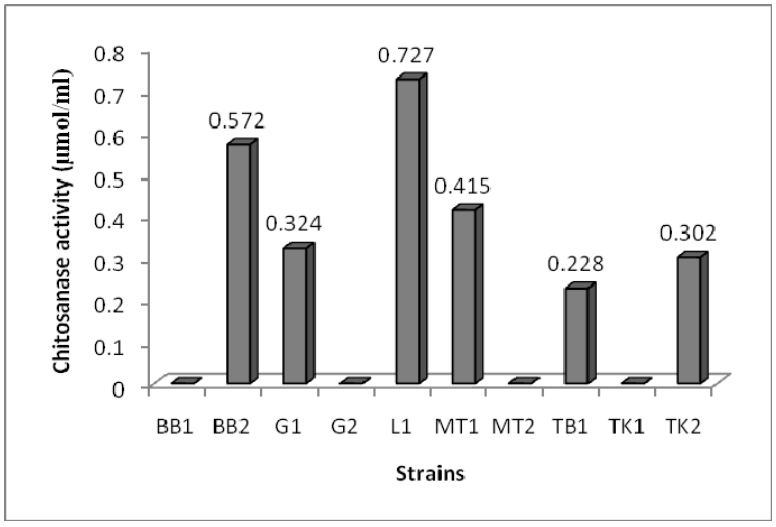

Fig. 1: Chitosanase activity for ten strains of bacteria in chitosan biodegradation.

3.2. Further Characterization and Identification of Bacteria Strain L1

The major concerns of selection of bacteria for the production of chitosanase are always the high specificity activity and good yield of high degree polymerized chitooligomers because they are very important for industrial application. The selected strains of chitosanase producers (L1 strain) were subjected to further characterization. Bacterial isolate L1 was subjected to a taxonomic analysis as described in *Bergey's Manual of Systematic Bacteriology*, and was identified as a member of the genus *Bacillus.* This genus often occurs in subsurface waters and in the soil environment (Nelson *et al*., 1988). Among the bacteria, the *Bacillus sp.* is particularly useful for the chitosanase production (Yu *et al*., 2009). Chitosanases with different activities and characteristics were produced by *Bacillus sp*. isolated from plants and soil, including *Bacillus cereus* S1 (Kurakake *et al.*, 2000). According to the result of the GEN III OmniLog® Identification System, L1 strain is most closely related to *Bacillus cereus*.

3.3. Correlation Between Growth of *Bacillus Cereus* L1 with Chitosanase Released

Chitosanase activity was measured every 3-hour interval over 24 hours growth period. Figure 2 shows the plot of the growth measured at A_{680nm} of *Bacillus sp* LI on 1% chitosan in a 100 mL liquid minimal media.

During growth the chitosanase activity was measured in the growth medium. Bacterial strain *Bacillus* sp. L1 produced highest chitosanase during exponential phase of the growth.

However, enzymes in nature have different specificities on chitosan depending on the different degrees of deacetylation. The plot was quite linear during exponential growth of *Bacillus sp* LI, as shown in Figure 2, indicating that the rate of chitosanase production was proportional to the growth rate. The fact that 90% deacetylated chitosan is more susceptible to hydrolysis than 100% deacetylated chitosan may suggests that *N*-acetylglucosamine residues in the chitosan are important in the recognition mechanism of the substrate by the enzyme (Jae *et al*., 1999). Thus the ability to degrade 100% deacetylated chitosan is an important characteristic to use to categorize enzymes as chitosanases.

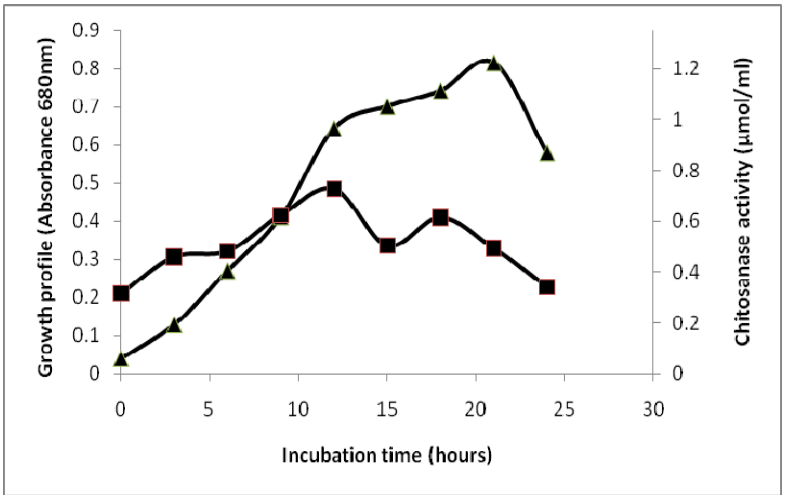

Fig. 2: Growth and chitosanase activity by locally isolated *Bacillus sp* L1 in minimal media 1% chitosan as sole source of carbon **(▲: *Bacillus sp*. L1 growth, ■: chitosanase activity)**

4. Conclusion

The major concerns of selection of bacteria for the production of chitosanase are always the high specificity activity and good yield of chitooligomers because they are very important for industrial application. Bacterial chitosanases have received special attention because they are important for the maintenance of the ecological balance and have been used to determine the mechanism of chitosan hydrolysis at both biochemical and molecular levels. Compared to the numerous reports on the primary structure and function of chitinases, information on chitosanases is still relatively limited. The significance of this study is to isolate the bacterium that constitutively produces chitosanase at the highest level in a culture medium by applying the appropriate isolation technique of bacteria. This study had also determined the useful bacteria for the production of chitosanase.

5. Acknowledgements

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