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Original paper

OPTIMIZATION OF *Bacillus* sp. K29-14 CHITINASE PRODUCTION USING MARINE CRUSTACEAN WASTE

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ABSTRACT

Chitin is present in large quantities in the marine crustacean waste disposed by seafood processing industries, making it very desirable as the substrate for producing chitinase, a hydrolytic enzyme of considerable interest in many industrial and agricultural applications. In our work, crustacean waste powder and its combination with colloidal chitin at different concentrations (0.5, 1.0, and 1.5%) were utilized to optimize the chitinase production by the bacterium, Bacillus sp. K29-14. The results showed that the chitinase production with the three different substrate concentrations was relatively constant in the range of 0.2 to 0.3 U/ml during 12 days cultivation, although there was a bit reduction after day 8. This activity profile seems to be similar to that of the protein content. Whereas the chitinase production on the media containing crustacean waste powder and its combination with colloidal chitin at the three concentrations showed that the highest activity (3.0 to 4.6 U/ml) was achieved on day 7 and 8. The specific chitinase activity with the waste powder at different concentrations of substrate (0.5, 1.0 and 1.5%) was increasing slowly during a nine-day cultivation. The optimal chitinase production (4.6 U/ml) was achieved with the combined substrate of 0.5% on day 8.

Key words: Marine crustacean waste, chitinase

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INTRODUCTION

Chitin is the second most abundant organic polymer in nature after cellulose (Shahidi, *et. al.* 1999). It is composed of *N*-acetylglucosamine (GlcNAc) monomers connected via β(1-4) linkages (Cohen-Kupiec & Chet, 1998; Shahidi, *et al.* 1999; Patil, *et al.*, 2000). The monomers are arranged in antiparallel (α), parallel (β), or mixed (λ) strands, with the α configuration being the most abundant (Gooday, 1994). Its chemical structure is very similar to that of cellulose, in which the C_2 positions of chitin are occupied by acetylamino group instead of hydroxyl groups (Felse and Panda , 1999; Patil, *et al.*, 2000) (**Fig. 1**).

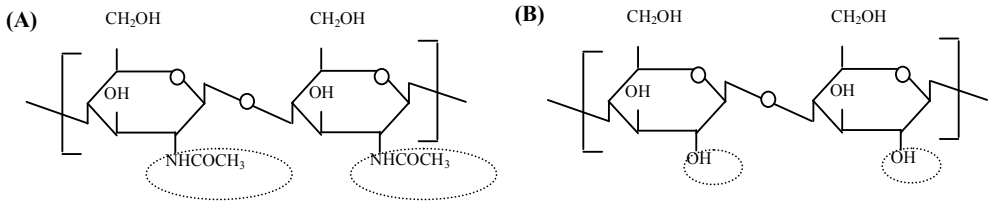

Fig. 1. Chemical structure of chitin (A), showing its similarity to cellulose (B). The difference is located on the C_2 positions as indicated with circulars.

Chitin is present in various organisms including marine invertebrates, insects, crustaceans, fungi, algae (Patil, *et al.*, 2000), zooplankton and several phytoplankton species (Svitil, *et al*. 1996). It was estimated that almost 10% of the global landings of aquatic products come from these chitin-rich organisms (Patil, *et al.*, 2000), making chitin possibly the most abundant in the marine environment (Svitil, *et al*. 1996). However, only 0.1 % of around 10^{11} tons chitin materials produced annually are being converted into valuable products (Toharisman, 2004). In the terrestrial environment, chitin also accumulates as crustacean waste disposed by the seafood processing industries. In the USA for example, 50-90% of the total solid waste landing is from the shellfish processing discards (Shahidi, *et al*. 1999).

The accumulation of the chitin waste in both marine environments seems to be a serious challenge for most of shellfish-producing countries, including Indonesia. The utilization of this waste for the purpose of chitinase production seems to be of highly considerable interest. This is due to the promising application of this enzyme in many industries and agriculture, including production of chitooligo-saccharides, biocontrol of plant pathogenic fungi and insects (biopestisides), preparation of protoplast, and production of single cell protein (Patil, *et. al.*, 2000; Toharisman, 2004). For such reason, we have currently been dealing with utilization of crustacean waste as the chitin source for chitinase production.

In the previous work, it was reported about the use of cructacean waste as the substrate for the production of various extracellular enzymes, including chitin deacetylase (Fawzya, *et al*., 2004), chitinase (Uria, *et al.* 2005), and chitosanase (Chasanah, *et al*., 2005). In particular, it was found that optimal chitinase production was achieved by using crustacean waste powder and its combination with colloidal chitin, as reported by Uria, *et al.* (2005). As the follow up of the previous work, we present our data here about chitinase production on such two forms of chitin at different concentrations using *Bacillus* sp. K29-14. This is intended to know the concentration of chitin substrate needed for the optimal chitinase production.

The bacterium, *Bacillus* sp. K29-14 used in this research belongs to thermophile, a microbial group capable of growing at elevated temperatures. There are many advantages associated with thermophile in industrial and biotechnological applications including (i) lower fermentation/feedstock cost, (ii) decreased viscosity of culture broth, (iii) sterility requirements not as stringent as for mesophiles, (iv) low biomass leading to high product, many of its enzymes secreted, (v) simplified recovery of volatile

products, (vi) thermal stability of its enzymes, (vii) resistance of thermophilic enzymes towards detergent and denaturants, and (viii) relatively higher specific activities in comparison to mesophilic enzymes (Berguist, *et al.*, 1987). That is the major reason why such bacterium was chosen in our work.

MATERIALS AND METHODS

Preparation of chitin substrates for enzyme assay.

In our experiment, chitinase production was observed towards the substrate, marine crustacean waste powder as well as its combination with colloidal chitin. Each substrate was prepared at three following concentrations: 0.5%, 1.0%, 1.5%.

Preparation of chitin powder from marine crustacean waste.

The crustacean waste powder used in this experiment was prepared from swimming crabs waste obtained from Cirebon, West Java. Preparation of the crustacean waste powder was carried out according to the method modified from (1997) (Oktavia, *et al.*, 2005). At first, the crab waste was immersed in 2% of NaOH for around 12 hours. The NaOH-treated waste was rinsed several times until the pH reached netral value, and then dried under the sunlight. The dried waste was treated with 3.5% NaOH at 70^{o}C for 2 hours in order to remove the attached meat (deproteination). Then demineralisation of the deproteinated waste was done with 15% HCl for 1 hour. The pH was adjusted up to 7 via several times of rinsing with water. After being dried under the sunlight, the dried waste was homogenized by blender, resulting in chitin powder, which was called crustacean waste powder in our experiment.

Preparation of colloidal chitin.

Colloidal chitin was made from commercially available chitin powder (Sigma) based on the method as described by Arnold & Solomon (1986). Twenty grams of the chitin powder (Sigma) was mixed with 400 ml of concentrated HCl, and then kept overnight in the cool room. Then the pH of the resulting suspension was neutralized by adding 10 N NaOH. After keeping the suspension overnight in the refrigerator, it was centrifuged (5.000 rpm for 30 min) and washed with 2 liters distilled water for desalting. The resulting colloidal chitin was ready to use. The waste along with the colloidal chitin prepared from chitin powder (Sigma) was used as the substrates for chitinase production.

Optimization of chitinase production.

The production host used during the experiment was the thermophilic bacterium, *Bacillus* sp. K29-14 obtained from Laboratory of Microbiology and Biochemistry, Research Centre for Biotechnology, Bogor Agricultural University. The characteristics of this bacterium had already been reported by Tanuwidjaja (1999). Production of chitinase from the bacterium was performed by growing it at 55^{o}C in the 150-ml broth Minimal Synthetic Medium (MSM) containing 0.1% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.1% NaCl, 0.7% $(NH_4)_2SO4$, 0.05% yeast extract. To optimize chitinase production, the bacterial cultivation was carried out with crustacean waste powder at various different concentration (0.5, 1.0, and 1.5) and then culture sampling was conducted regularly everyday for 6 days. In addition, chitinase production was also observed towards the mixture of colloidal chitin and crustacean waste powder.

Extraction of chitinase.

To isolate extracellular chitinase, 10-ml cell culture was centrifuged at 10.000 rpm, 4°C for 25 minutes. The resulting cell-free supernatant was obtained, which was used further for activity assay.

Assays of chitinase activity and protein content.

The chitinase activity was assayed based on the method of Spindler (1997). Enzyme solution (150 µl) was added to the mixture consisting of 300 µl of 0.1% colloidal chitin and 150 µl of phosphate buffer pH 7.0. After incubation at 55°C for 10 min, the reaction mixture was subjected to a refrigerated centrifugation at 10.000 rpm for 5 min. The resulting supernatant (200 µl) was added with 500 µl of H_2O and 1000 µl of Schales reagent, which was followed by boiling it for 10 min. After cooling, the absorbance of the mixture was measured at 420 nm. One unit of the chitinase activity was defined as the amount of enzyme which yield 1 µmol of reducing sugar as *N*-acetylglucosamine (GlcNAc) equivalent per minute. The protein content of the crude chitinase was estimated according to the Lowry method with bovine serum albumin as the standard (Bollag and Edelstain, 1991).

RESULTS AND DISCUSSION

Optimization of chitinase production was carried out by cultivating *Bacillus* sp. K29-14 in the media containing crustacean waste powder at three different concentrations (0.5, 1.0, and 1.5%). In addition to that, chitinase production was also observed towards the mixture between crustacean waste powder and colloidal chitin at such three different concentrations. The results were summarized in **Fig. 2** and **3**.

Figure 2A-C indicates the profile of chitinase activity and protein amount for the substrate containing crustacean waste powder. The chitinase activity with three different concentrations of the substrate (0.5, 1.0, and 1.5%) were relatively constant in the range of 0.2 to 0.3 U/ml during a 12-day cultivation, although there was a bit reduction after day 8. This activity profile seems to be similar to that of the protein amount, suggesting that other proteins might also be excreted, in which chitinase was a large part in the protein mixture. It was assumed that the continuous production of chitinase was due to the enough availability of chitin in the growth medium, as the result of the slow rate of chitin biodegradation during the cultivation. Subsequently, the slow rate is probably due to the low solubility of crustacean waste powder in the medium.

However, the chitinase production in our experiment was probably induced by the chitin added into the growth medium. It was assumed that chitin and its degradation products played a role as an inducer system in stimulating the production of chitinase, as reported for *Metarhizium anisopilae* (Felse and Panda, 1999). This case was also similar to that reported by Frandberg & Schnurer (1994) that the products of chitin degradation, mainly *N,N′*-diacetylchitobiose ($GlcNAc_2$), induced the chitinase production by *Bacillus pabuli* K1. Especially when chitin is added into the medium, it is partially hydrolyzed by extracellular chitinases into oligomers, mainly dimers of GlcNAc ($GlcNAc_2$), which is hydrolyzed further by β-*N*-Acetylglucosaminidase (GlcNAcase) to form the monomers, GlcNAc. Then these monomers are taken up via the phos-photransferase system (Park, *et al.*, 2000).

The chitinase production with the combined substrate (crustacean waste powder and colloidal chitin) at the concentration of 0.5 and 1.0% (Fig. 2D-F) showed that the highest activity (3.0 to 4.6 U/ml) was achieved on day 7 and 8, indicating that the optimal production of

chitinase was on those days. This subsequently suggests that the presence of colloidal chitin in the growth medium could increase the chitinase production, which significantly appeared on day 7 and 8. It was assumed that the activity increase on those days was due to the higher solubility of colloidal chitin in the growth medium, making it more accessible by chitinase in comparison to the waste powder.

In relation to the protein content, as shown in Fig. 2D-E, it seems that the increase in the activity was not always followed with the increase in the protein content. In particular in **Fig. 2D**, it was found that the protein content on day 8 was relatively low at the high level of chitinase activity. This indicates that a significant portion of total protein released into the growth medium was chitinase. This phenomenon appears to be different from the substrate of 1.5%, in which the high chitinase level was not followed with increasing the protein amount. This means that the reduced portion of chitinase in the total protein secreted at the higher concentration of chitin.

Figure 3 describes the specific chitinase activity at different concentrations of substrate (0.5, 1.0 and 1.5%). The specific activity level on the waste powder was increasing slowly during nine days of cultivation (**Fig. 3A**). Subsequently, it was found that the activity at the lower concentration (0.5%) was a bit higher than that at the higher ones (1.0 and 1.5%). It appears that it took a longer time for chitinase to degrade the chitin in the waste powder, due to the low solubility of the powder in the growth medium. The phenomenon was different from that on the combination of colloidal chitin and crustacean waste, as shown in **Fig. 3B**. The optimal specific activity for this combined substrate (0.5, 1.0 and 1.5%) was achieved on day 8. Then the specific activity significantly declined after day 8.

It is clear from **Fig. 3** that the specific activity with the combined substrate on day 8 was significantly higher than that with waste powder itself. It seems that it was easier for chitinase to degrade colloidal chitin, in which the structural compactness was reduced. Another possible reason is that the main product of colloidal chitin, *N*,*N*-di-acetylchitobiose or $(GlcNAc)_2$ induced both chitinase and GlcNAcase as reported by Shimahara, *et al.* (1992) for *Bacillus licheniformis*.

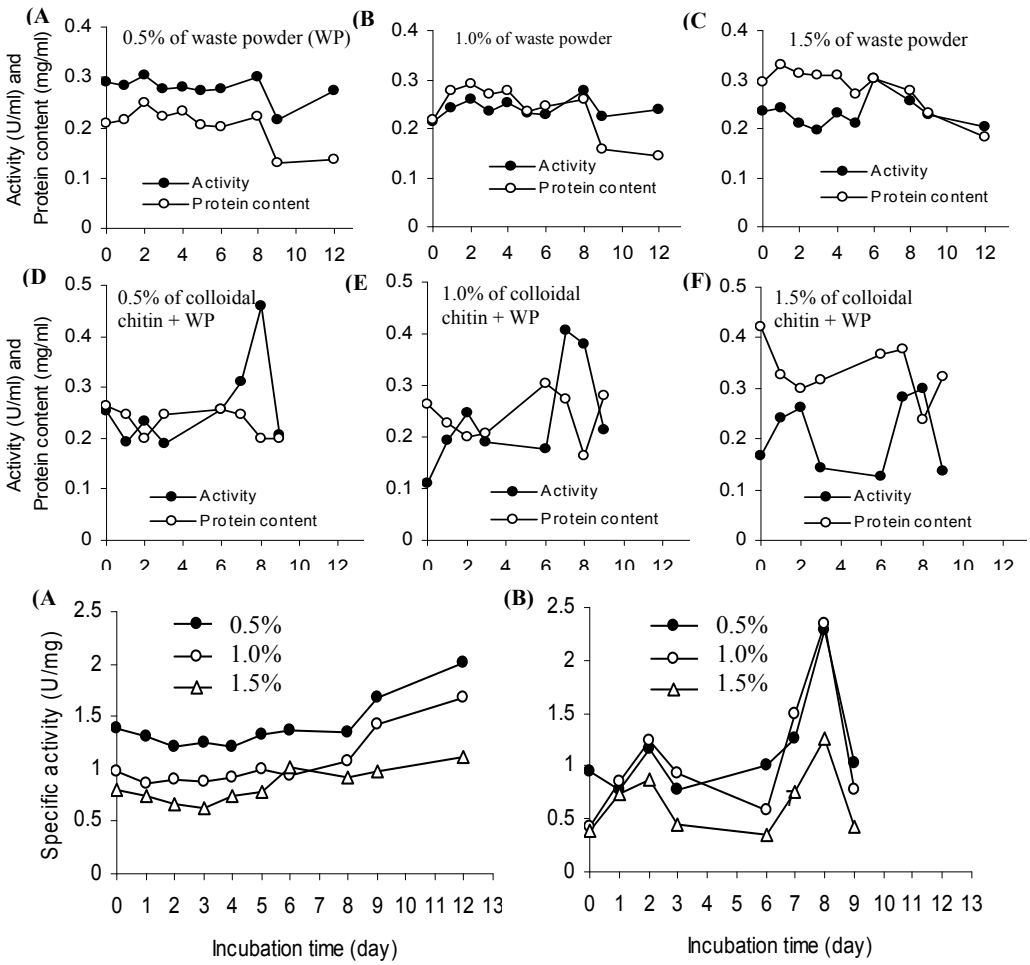

Fig. 3. Spesific activity profile of the chitinase produced by *Bacillus* sp. K29-14 in the media containing two forms of chitin source, in this respect crustacean waste powder (A) and the mixture of colloidal chitin and crustacean waste powder (B).

CONCLUSION

The chitinase production with crustacean waste powder at different concentrations of substrate (0.5, 1.0 and 1.5%) was increasing slowly during a nine-day cultivation. Among the two forms of substrate with three different concentrations, the combined substrate (crustacean waste powder and colloidal chitin) of 0.5% was found to be the best substrate for optimal chitinase production by *Bacillus* sp. K29-14. Subsequently, the optimal production with such substrate was achieved on day 8. For further work, it is necessary to overexpress the enzyme in the suitable production host, *Escherichia coli*, which was followed by the purification and characterization of the chitinase in order to know its properties.

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