

Production and Characterization of crude chitinase from *Trichoderma virens*

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Abstract

Chitinase hydrolyzes chitin, a biopolymer of N-acetylglucosamine which is being widely used in biological and agricultural research. Optimization of chitinase production by *Trichoderma virens* was conducted using batch cultures of *Trichoderma Minimal Medium (TM medium)* that consists of 2.0% (w/v) colloidal chitin as substrate. Fermentation was performed at agitation speed of 200 rpm and incubated at 30°C in 250 ml shake flasks. Optimum chitin concentration was obtained at 4.0% (w/v). The addition of a stimulant, methanol, at 0.2% (v/v) enhances chitinase production by 71.4%. Higher concentrations of methanol would inhibit chitinase production. Works for optimizing agitation speed was carried out in a 2.0 L batch culture using B.Braun Biostat-B bioreactor. The optimum conditions for chitinase productions were performed in the same bioreactor using disc turbine impellers (diameter of 5.5 cm) with air flow rate of 1.0 l/m and agitation speed of 250 rpm resulting in an increased chitinase activity of 0.447 U/ml compared to 0.052 U/ml in shake flask fermentation. This improvement of 88.4% might be due to efficient mixing and high oxygen transfer rate which is essential for fungal growth. Characterization of crude chitinase was carried out to determine the optimal conditions for the crude chitinase activity to hydrolyze chitin and the crude enzyme stability. The crucial physical factors affecting the optimum and stability of chitinase activity were pH and temperature. pH optimum was achieved at pH 4.0 in citrate phosphate buffer and chitinase was very stable at pH 3.0-7.0, with 350% of residual activity is retained at pH 7.0. Optimum temperature for chitinase activity was achieved at 50°C while its stability decreases with the increment of temperature. Only 37.5% of residual chitinase activity was retained at 70°C.

Keywords : Chitinase, *Trichoderma virens*, batch culture, optimization, characterization

1. Introduction

Chitinases (E.C. 3.2.1.14) are a group of enzymes that is responsible of hydrolyzing chitin polymer into its oligomers or monomers [1]. Chitinases are naturally produced by bacteria, yeasts, protozoa, plants, humans and animals where the enzyme is usually involved in natural protective mechanism [1-4]. Chitin polymer is the second most abundant naturally occurring polymer after cellulose and it exist as a structural polysaccharide of β -1,4-N-acetyl-D- glucosamine residues. This polymer is mainly derived from marine invertebrates such as shrimp, crabs, oysters and cuttlefishes. Chitin and its derivatives produced from enzyme hydrolysis are being widely

applied in the field of medicine, agriculture, food industry, biotechnology, waste water treatment and many industrial applications.

The commercial interest of utilizing chitin and its derivatives lead to the need of inexpensive, reliable source of active and stable chitinase preparations. Current limitation is the instability of enzyme in narrow pH and temperature range [5]. The aim of this study is to optimize chitinase production from locally isolated fungus *Trichoderma virens* UKM-1 in a 2.0 L bioreactor.

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2. Material and Methods

2.1 Fungus

Locally isolated fungal strain identified as *Trichoderma virens* UKM-1 was used as chitinase producer. The culture is grown on Potato Dextrose Agar. Upon maturation, spores were harvested with 1.0% (v/v) Tween 80 by lightly scraping the culture surface using a glass hockey stick. Spores were separated by centrifuging at 4000 rpm, 4°C for 30 minutes where the supernatant is discarded. Spores were resuspended with sterile distilled water to spore concentration of 1×10^5 spores/ml. Fungus stock is stored in beads systems at -80°C.

2.2 Fermentation Medium

The composition of *Trichoderma Minimal* medium [6] includes (g/L): colloidal chitin, 20; CaCl₂, 0.3; KH₂PO₄, 3.0 and MgSO₄, 0.3. The pH value of media was adjusted to pH 5.5 with 0.1 M NaOH or 0.1 M HCl prior to sterilization at 121°C, 15kPa for 20 minutes. 10% (v/v) of spore suspension were inoculated into medium. Working volume of 50 ml was used for 250 ml shake flask fermentation while for bioreactor fermentation the working volume is 1.5L.

2.3 Colloidal chitin preparation

20 g of commercial chitin from crab shell (Fluka) was stirred into 200 ml of concentrated HCl. Mixture was continuously stirred with slight heating of 40°C for 5 minutes. Cold distilled water was added in excess amount. Chitin was allowed to precipitate and washed with distilled water until pH reached neutral.

2.4 Assays

Chitinase activity was determined using DNS method [7] with modifications. 1.0 ml of sample supernatant was incubated with equal volume of 10% (w/v) colloidal chitin in 0.2mM phosphate buffer pH 6.5 and incubated at 50°C for one hour. Reaction was terminated by adding 1.0 ml NaOH 0.1M and boiled for 5 minutes. Enzyme suspension is obtained after centrifuging at 4000 rpm for 5 minutes. 1.0 ml of enzyme was mixed with 1.0 ml DNS solution and boiled for 5 minutes. 5ml of distilled water was added to solution and reading was observed at UV absorbance of 535 nm. One unit (U) of chitinase activity is defined as the amount of chitinase needed to catalyze the release of 1µmol of *N*-acetyl-*D*-glucosamine. Protein concentration was assayed using the method of Lowry [8].

2.5 Determination of optimum pH and temperature

The effect of pH on chitinase activity was determined by incubating enzyme at 50°C in varying composition of standard assay buffer between pH 1 to 13 (KCl-HCl buffer, pH 1-2; citrate buffer, pH 3 and 5; citrate-phosphate buffer, pH 3-7; sodium acetate buffer, pH 4-5; phosphate buffer, pH 6-8; KCl-NaOH, pH 12-13). The effect of temperature was determined by incubating enzyme in citrate-phosphate buffer of pH 4 at various incubation temperatures ranging from 30-70°C. Enzyme stability for each parameter was studied by prolonging the incubation period to 2 hours.

3. Results and Discussion

3.1 Shake flask Studies

3.1.1 Optimizing Substrate Concentrations

In this study, colloidal chitin that acts as carbon source was used as substrate. Optimum substrate concentration is crucial to avoid substrate inhibition or fungal cell wall utilization. Fungal cell wall itself contains chitin that may be used for chitinase production [6, 9, 10]. Chitinase can be produced even without the availability of substrate in minimal medium. Infact, chitinase activity is higher in fermentation flaks without colloidal chitin compared to fermentation in flasks with certain colloidal chitin concentrations. This shows that substrate competition occurs between fungal cell wall and colloidal chitin. The omission of nitrogen source in *Trichoderma* minimal medium caused fungal hyphae to be partially empty and broken at the tips. Fragmentation of hyphae releases chitinase into medium [11]. For insoluble substrate such as colloidal chitin, access to enzyme reaction sites on this polymer is restricted to enzyme diffusion. The number of enzyme binding active sites on the substrate exceeds the number of enzyme molecules, unlike in the case of soluble substrates like glucose [12]. The highest chitinase activity was obtained at chitinase activity of 0.109 U/ml in the presence of 4.0% (w/v) of colloidal chitin as presented in Figure 1.

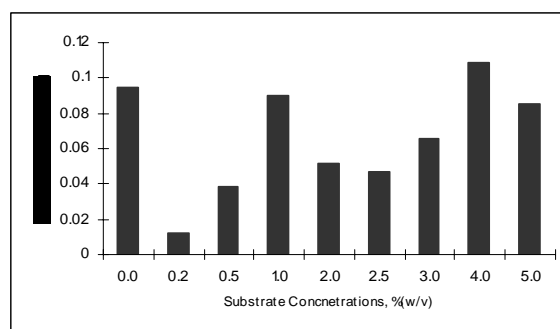


Figure 1: Effect of colloidal chitin concentration on chitinase production

3.1.2 Screening and optimizing stimulant

The complexity of fungal fermentation for chitinase production is caused by mycelia formations which affect the mixing and mass transfer limitations [11] and also the insoluble nature of chitin substrate. Felse, 2000 [13], reported that chitinase production by wild type fungus is much lower compared to bacterial fermentation. Stimulant is required to improve chitinase production as presented in Table 1.

The fermentation medium used in this study consisted of carbon source and several macronutrients such as K^+ , Mg^{2+} and Ca^{2+} . Micronutrients are important in maintaining the structural configuration of enzymes [14]. However, Nawani, 2004 [15] commented that the increase of trace elements would decrease chitinase production. Both T2 and Absidia trace elements contains Zn^{2+} , Mn^{2+} and Fe^{2+} which were essential for microbial growth. Medium supplemented with T2 trace elements showed a slight increase in chitinase production compared to medium containing Absidia trace elements. This is because the presence of these trace elements reduced fragmentations and improves hyphae elongation. It was observed that the hyphae were less fragmented and longer compared to flasks containing Absidia trace elements. Absidia trace elements also contain Cu^{2+} and Co^{2+} . These heavy metal ions were suspected to interfere with the active binding sites of chitinase enzyme.

Methanol had been reported as the best type of alcohol being used as a stimulant to enhance primary and secondary metabolite productions such as citric acid or kojic acid. However, no reports had been found which studied the effect of alcohol in enzyme production. Fungal enzymes were known to be cell wall associated and enzyme activities were mainly found in the cell wall of mycelia in submerged fermentations [16]. Methanol promotes fungal cell wall permeability [17]. Therefore, with known occurrence of mycelia fragmentations, addition of methanol would further stimulate the excretion of chitinase into medium. Optimum methanol concentration is also crucial to avoid toxicity. Results in Figure 2 shows that only a slight addition of methanol would increase chitinase activity, while high methanol concentrations would decrease chitinase activity as high methanol concentrations would disturb fungal metabolism and mycelium morphology [17].

Table 1: Comparison of chitinase activity in different types of stimulants at 0.1% (v/v) concentration

Stimulant (0.1% v/v)	Max Chitinase Activity (U/ml)	Protein Concentration ($\mu\text{g/ml}$)
T2 trace elements	0.119	31.818
Absidia trace elements	0.055	196.364
Methanol	0.146	62.727

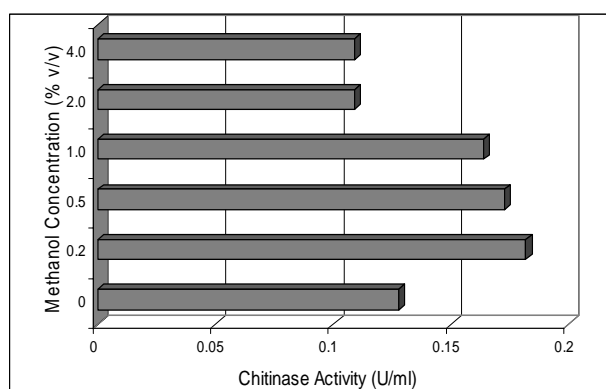


Figure 2: Chitinase activity in different methanol concentrations

3.2 Bioreactor fermentation

3.2.2 Agitation Speed

Further study on chitinase production was investigated in a 2.0L B. Braun Biostat-B bioreactor. Fermentations were carried out at a constant temperature of 30°C with air flow aeration of 1.0 l/m but at different agitation speeds of 200, 250, 300, 400 and 500 rpm. Result on the effect of different agitation speeds is depicted in Figure 3. Agitation play important role in fungal fermentation for mixing, efficient oxygen transfer and heat transfer [18]. In submerged bioreactor fermentation, agitation intensity may affect both fungal morphology and oxygen transfer. Agitation speed also causes morphological differences both in shake flask and bioreactor, resulting in different productivity of target enzyme [19]. Strong agitation would lead to free filaments formation while slow agitation would cause clumps and wall growth. Agitation speed of 250 rpm produces the highest chitinase activity with 0.447 U/ml. It was also observed that low agitation of 200 rpm formed clumps by the third day of fermentation. Wall growth was also observed on both oxygen and pH probes, baffles and impellers. By the fifth day of fermentation, fungus clumped on baffles and impellers. Clumping cause low oxygen transfer leading to poor chitinase production. Not only that, it would deprive fungus from oxygen. However, increase of agitation intensity of more than 250 rpm would not increase chitinase activity. This was due to strong mechanical force caused by impeller that may lead to mycelia damage [16]. In this study, at 300 and 400 rpm, chitinase production decrease steadily. However, at 500 rpm, chitinase production was slightly increased. It was suspected increase of chitinase was not related to chitin consumption. Instead, at this agitation speed, the mechanical force caused mycelia breakage, thus leading to excretion of chitinase by the damaged mycelia. Improvement made by each of optimization process is summarized in Table 2.

Table 2: Improvement in chitinase production after each optimization process

		Chitinase Activity (U/ml)	% of Improvement in chitinase production compared to unoptimized conditions
<i>Shake flask fermentation</i>			
Unoptimized	2.0% (w/v) colloidal chitin	0.052	-
<i>Optimization</i>			
Colloidal Chitin Concentration	4.0% (w/v) colloidal chitin	0.109	52.3
Inducer selection	0.1% (v/v) different types of inducers	0.146	64.4
Methanol Concentration	0.2% (v/v) methanol	0.182	71.4
<i>Bioreactor Study</i>			
Effect of agitation speed	250 rpm	0.447	88.4
	200 rpm	0.076	31.6
	300 rpm	0.109	52.3
	400 rpm	0.064	18.8
	500 rpm	0.146	64.4

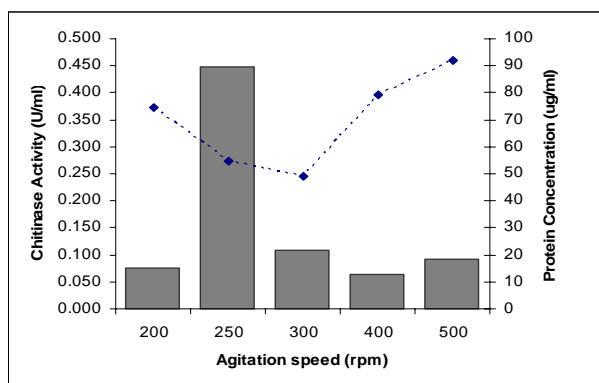


Figure 3: The direct effect of different agitation speed towards chitinase activity and protein concentration

3.3 Characterization of Crude Chitinase Enzyme

3.3.1 Effect of pH upon crude chitinase activity

Crude chitinase activity was found to be the most active citrate phosphate buffer compared to other buffer as shown in Figure 4. It was found that crude chitinase activity was optimum at pH 4 with more than 80% of its relative chitinase activity was retained at acidic condition of pH 3 and pH 5 as in Figure 5. This result was similar to chitinases produced by *Penicillium aculeatum* and *Verticillium lecanii* [1, 2, 20]. At pH value 6, enzyme activity started to decline giving relative chitinase activity of only 18.5% while at pH 7.0 only 3.7%. Eventhough chitinase activity declined, crude chitinase was found to be stable. At pH 3, 100% of its activity was retained and up to 350% of crude chitinase activity was retained at pH 7 as illustrated in Figure 6.

3.3.2 Temperature Optimum and Stability

The activity of crude chitinase was found to be optimum and stable at 45-50°C as presented in Figure 7. This result is similar to purified chitinase of *B. cereus* 6E1 *Chi36* as reported by Wang *et al.*, 2001 [2]. This study also shows that chitinase is unstable at temperature above 50°C as shown in Figure 8. At temperature 70°C, only 37.5% of its residual activity was retained.

4. Conclusion

Chitinase production by *Trichoderma virens* in *Trichoderma* minimal medium at optimum condition is improved by 88.4% in bioreactor compared to unoptimized condition in shake flasks. Optimization of substrate was conducted in shake flask where 4.0% (w/v) of colloidal chitin was the optimum substrate concentration. Other optimization works done in shake flasks include stimulant screening and optimization. It was found methanol was the best stimulant at 0.2% (v/v). Further optimization conducted in a 2.0L Biostat B bioreactor with optimum agitation speed of 250 rpm resulting in 88.4 % improvement. Crude chitinase produced was found to be optimum at pH 4 and at 50°C. Crude chitinase was stable at pH nearing neutral but, crude chitinase stability decreases as temperature increases from 50-70°C.

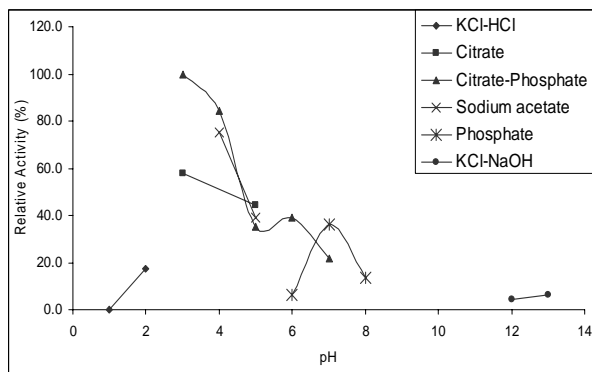


Figure 4: Effect of pH on chitinase activity. Crude chitinase was incubated at 50°C for 1 hour with 10%(w/v) colloidal chitin in KCl-HCl buffer (pH1-2), Citrate buffer (pH 3,5), Citrate-Phosphate buffer (pH 3-7), sodium acetate buffer (pH 4-5), phosphate buffer (pH6-8) and KCl-NaOH (pH 12-13).

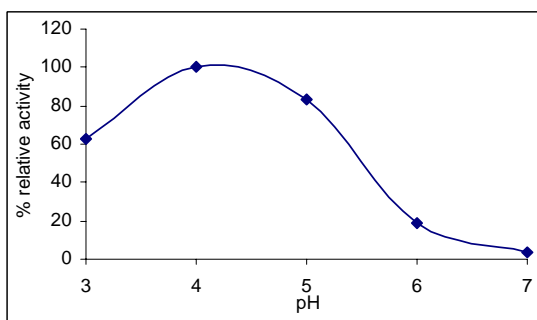


Figure 5: Effect of pH on chitinase activity at 50°C

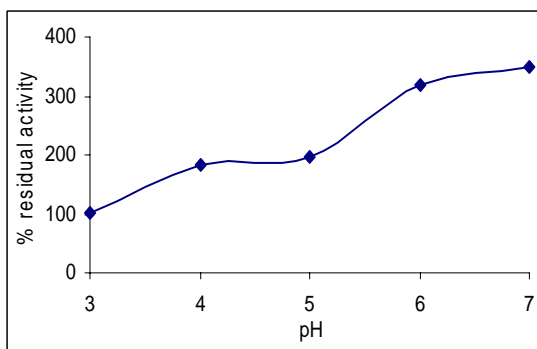


Figure 6: Percentage of residual chitinase activity in various pH at 50°C

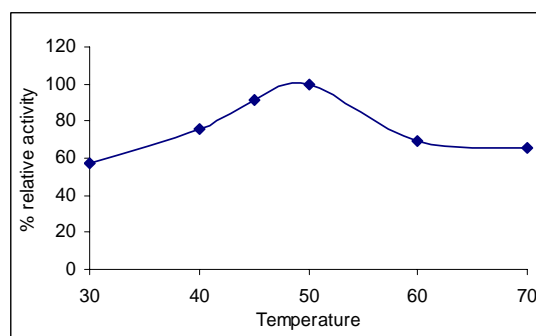


Figure 7: Effect of temperature on chitinase activity at pH 4.

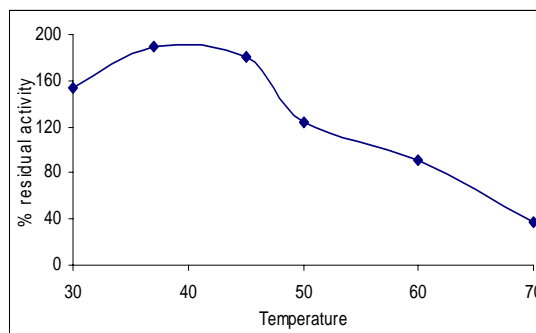


Figure 8: Percentage of chitinase activity in various temperatures at 50°C

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