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Full Length Research Paper

Strain improvement and optimization of the media composition of chitosanase-producing fungus *Aspergillus* sp. CJ 22-326

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A mutant *Aspergillus* sp. CJ 22-326-14 with higher production of chitosanase was selected by serial mutation procedure, and the optimization of medium components was performed using one-factor-at-a-time combined with orthogonal array design method. The results showed that chitosan, wheat bran and $(NH_4)_2SO_4$ were the most important components by one-factor-at-a-time experiments. And the optimal concentrations were obtained by orthogonal array design method as (g/L): chitosan 20, wheat bran 10, $(NH_4)_2SO_4$ 2, KH_2PO_4 2 and $MgSO_4 \cdot 7H_2O$ 0.5. After the strain improvement and optimization of the media, the chitosanase activity from *Aspergillus sp.* CJ 22-326-14 reached to 3.92 U/mL in 250 mL flasks, which was nearly 4-fold increase of chitosanase production at the parent lowest level.

Key words: *Aspergillus* sp., chitosanase, mutant, fermentation, optimization, orthogonal array design method.

INTRODUCTION

Chitosan, a D-glucosamine polymer, is present in the cell walls of a limited group of fungi (the genera Rhizopus, Absidia and Fusarium) in nature. It is usually prepared from chitin (crab or shrimp shell origin) by the artificial deacetylation in the presence of alkali, and its biological properties such as biocompatibility and antimicrobial activity have been investigated (Alfonso et al., 1995; Gao et al., 1995; Senel and McClurre, 2004). Chitosan is expected to be utilized as functional foods, medical supplies, and biologically active substances. Especially, the chitooligo- saccharides depolymerized from chitosan are also of demand because of its high solubility and improved absorption *in vivo*. Simultaneously, the chitooligosaccharides with different degrees of polymerization (DP), especially those with six residues or more, show strong physiological activities, such as antitumor activities (Suzuki et al., 1986; Suzuki et al., 1989), immunoenchancing effects (Suzuki, 1996), antifungal activity (Hirano and Nagao, 1989; Kendra et al., 1989) and antimicrobial activity (Jeon et al., 2001; Savard et al., 2002).

Chitosanase (EC 3.2.1.132) is an enzyme that catalyzes the endo-type cleavage of β-1,4-linkages between D-glucosamine (GlcN) residues in chitosan (Allan and Hadwiger, 1979). Chitosanases have been found from many microorganisms, including fungi, actinomycetes and bacteria; and they have been purified and characterized (Boucher et al., 1992; Fenton and Eveleigh, 1981; Izume et al., 1982; Seino et al., 1991; Yamasaki et al., 1993; Yoshihara et al., 1992). Chitosanases from individual organisms differ in their hydrolytic pattern. In most cases, bacterial chitosanases are inducible by the substrate chitosan and play a role in the degradation and utilization of exogenous chitosan. In contrast to bacterial chitosanases, there have been few reports on chitosanases from fungi. Chitosanase is secreted into the medium by the fungal strain belonging to the genera *Aspergillus* and two types of chitosanase ChiA and ChiB were purified and partially characterized from the culture supernatant of *Aspergillus* sp. CJ22-326 (Chen et al., 2005). However, the lower yield of chitosanase from the *Aspergillus* sp. CJ22-326 has been blocked the further industrial appliance.

The current study is aimed at the isolation of mutants of *Aspergillus* sp. for hyperproduction of chitosanase through

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UV-irradiation followed by NTG-mutagenesis. And the important factors such as carbon source and nitrogen source necessary to maintain such mutants with higher levels of chitosanase productivity were defined by one-factor-at-a-time and subsequently by orthogonal array design method.

MATERIALS AND METHODS

Materials

Chitosan (100% deacetylated) and chitin were purchased from local suppliers in China. A dichroism method (Domard, 1987) was applied in determining the acetyl content of the chitosan samples. All other reagents were of analytical or extra-pure grade and were obtained from commercial sources.

Microorganism

The wild type fungus used in this study is a new *Aspergillus* sp. strain isolated from a marine soil sample in China and stored at 4°C on potato dextrose agar (PDA) and sub-cultured once in every three months in our laboratory with the code CJ22-3 (Chen et al., 2005).

Media

All media were sterilized by autoclaving at 121°C for 15 min and the pH was adjusted before sterilization. The following culture media were employed throughout the work.

Potato dextrose agar (PDA) for slant and plate cultures

PDA contained (g/L): potatoes 200; glucose 20; yeast extract 1; and agar 20.

Selection media for slant and plate cultures

The selection media for slant composed of (g/L): soluble chitosan 10, glucose 20, yeast extract 1, $(NH_4)2SO_4$ 2, KH_2PO_4 2, $MgSO_4$ 0.5, and agar 20, pH 5.6.

Fermentation media for chitosanase production

Basal fermentation media were used as a fermentation medium for the enzyme production and composed of (g/L): soluble chitosan 10, yeast extract 1, KH_2O_4 2, $MgSO_4$ 0.5, pH 5.6. And the selection media was the basal medium with different carbon source (glucose, lactose, glucosamine, acetylglucosamine, chitosan, starch, wheat bran, dextrins, glycerol, molasses) and nitrogen source (urea, peptone, yeast extract, $(NH_4)2SO_4$, KNO_3, NH_4NO_3, $(NH_4)_2SO_4$).

Isolation and selection of mutants

UV irradiation

Four milliliter cell suspension (10^7 cells/ml) of the starting stain contained in a Petri dish was placed under an ultraviolet lamp (15 W, 2537 Å) with a distance of 15 to 30 cm, and was irradiated for different time intervals between 15 s and 30 min. The cell suspension was mixed by a magnetic stirrer during irradiation. Treated and untreated spores were diluted in sterile physiological saline, and 1 ml spore suspension was spread on to the single colony isolation medium to calculate the percentage survival. Samples with a death rate of 99.0% were subjected to subsequent isolation.

NTG (Nitrosoguanidine) treatment

1 ml of NTG solution (100 mg/ml, 0.2 M pH 6.0 phosphate buffer) was added in 1 ml cell suspension of the starting strain (~10^8 cells/ml). After incubation at 30°C on a rotary shaker of 120 rpm for different time intervals between 15 and 60 min, the mixture was diluted 1,000 times with sterile distilled water immediately. Treated and untreated spores were diluted in sterile physiological saline, and 1 ml spore suspension was spread on to the single colony isolation medium to calculate the percentage survival. Samples with a death rate of ca. 90% were subjected to subsequent isolation. The above treated suspension (100 µl) was spread on different screening plates. The plates were incubated at 37°C temperature for 5 days and the mutants were selected on the basis of clearance zones appeared on selection media.

Enzyme production in shake flask

All the cultivations were carried out in a 500 ml baffle flask with 150 ml of the culture medium containing respective amount of substrates. The flasks were inoculated with spores (approximately 10^7 cells/mL) from 7-day sold culture grown on PDA slant and incubated at 37°C with shaking at 150 rpm.

Fermentation in stirred tank bioreactor

The fermenter used was a 10 L mechanically stirred tank bioreactor (B. Braun Biostat C, Germany) with a working volume of 6 L. The fermentation media were sterilized before inoculum. The aeration rate in the bioreactor was maintained at 0.8 vvm with stirring at 170 rpm and the incubation temperature was 37°C.

Assay methods

The biomass was harvested at the end of fermentation, then they were centrifuged at 8 000 g for 10 min, then dried to constant weight at 60°C for sufficient time in laboratory vacuum ovens to obtain dry weight of the biomass. The supernatant was analyzed for extracellular enzyme activities and residual sugar. All the shake flask experiments were conducted in triplicates.

Unless indicated otherwise, chitosanase was assayed by measuring the reducing sugars liberated during the hydrolysis of chitosan with a DDA (degree of deacetylation) of 83%. The incubation mixture contained 1 ml of 0.5% soluble chitosan and 1 ml of diluted enzyme solution (pH 5.6). The incubation was carried out at 37°C for 15 min, with shaking. The amount of reducing sugar in the supernatant was measured using the modified dinitrosalicyclic acid (DNS) method (Ghose, 1969). One enzyme unit was defined as the amount of enzyme required to produce 1 µmol of reducing sugar as glucosamine per min.

Residual sugar was estimated gravimetrically by DNS method (Ghose, 1969). A double beam UV/Vis scanning spectrophotometer (Unico UV2800, Shanghai, China) was used for measuring the % color intensity.

Experimental design

Media compositions experiments were designed to meet the nutri-

Table 1. Comparison of enzyme activities of *Aspergillus* sp. CJ 22-326 and positive mutants by UV irradiation.

Strain	Biomass (g/L)	Chitosanase activity (U/mL)	Increase rate (%)
Original (CJ 22-326)	2.97 ± 0.32	0.97 ± 0.02	100
CJ 22-326-02	2.67 ± 0.44	1.03 ± 0.09	106
CJ 22-326-05	2.71 ± 0.62	1.56 ± 0.23	161
CJ 22-326-09	3.01 ± 0.15	1.60 ± 0.14	165
CJ 22-326-11	3.11 ± 0.24	2.23 ± 0.37	230
CJ 22-326-14	3.02 ± 0.41	1.68 ± 0.54	173
CJ 22-326-16	2.78 ± 0.38	1.87 ± 0.09	192
CJ 22-326-17	2.84 ± 0.34	1.71 ± 0.16	176
CJ 22-326-25	2.54 ± 0.23	0.99 ± 0.12	103

tional demands of the mutants of *Aspergillus* sp. The carbon source, nitrogen source and mineral source were regarded as correlated factors of the culture medium, in particular carbon and nitrogen sources. According to the results of 'one–factor-at-a-time' experiments, theorthogonal array design method L9 (3^3) was selected to optimize the medium compositions in shake flask cultures.

RESULTS

Mutagenesis and screening for chitosanase hyperproduction

In general, strain improvement is considered one of the main factors involved in the achievement of higher titers of industrial metabolites (Podojil et al., 1984). Random mutagenesis and fermentation screening have been reported as an effective way to improve the productivity of industrial microbial cultures (Parekh et al., 2000). The most widely used mutagens are nitrosoguanidine (NTG), methylmethane sulfonate (MMS), ethylmethane sulfonate (EMS), N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and ultraviolet (UV) irradiation (Baltz, 1999).

Aspergillus sp. CJ22-326 was subjected to successive UV-irradiation mutagenic with followed by NTG treatment. The screening media gave fairly reliable indication of increased chitosanase activities. After each mutagenic treatment, enzyme production by the selected mutants was assessed in shake flask cultures and the most promising strain was further subjected to next mutagenic treatment. In the first mutation experiment, different doses of UV light exposure were given to the spore suspension of *Aspergillus* sp. CJ22-326. UV exposure for 60 s with the 15 W lamp at a distance of 15 cm has resulted in 8% survival rates of the cells. One of the mutants, *Aspergillus* sp. CJ 22-326-11 showed 2.3-time higher (2.23 U/mL) activities than the parent strain *Aspergillus sp.* CJ22-326 (Table 1). The further mutation of *Aspergillus sp.* CJ22-326-11 strain led to isolation of another 8 mutants capable of producing the high levels of chitosanase on selection medium followed by NTG treatment. The highest enzyme-producing mutant was the mutant strain *Aspergillus* sp. CJ 22-326-14 with the 3.4-times higher (3.32 IU/ml) activities than the parent strain *Aspergillus* sp. CJ22-326 (Tables 1 and 2).

The high-production hereditary feature of strain *Aspergillus sp.* CJ22-326 was tested to be stable (data not shown) and thus it was used as a production strain in the following fermentations. Su et al. (2006) obtained a mutant strain with higher chitosanase yield by mutation the wild strain *Bacillus sp.* S65 with nitrogen ion beam, and at the same time the fermentation time was shortened significantly which greatly increased efficiency.

Time profile of growth and chitosanase production by wild-type and mutant *Aspergillus* sp. CJ22-326-14 strain in defined medium

The profile of chitosanase production by *Aspergillus sp.* CJ22-326-14 mutant and the wild strain *Aspergillus sp.*CJ22-326 cultured in shaken flasks is shown in Figure 1. Compared to the parent strain, mutant *Aspergillus sp.* CJ22-326-14 produced more chitosanase activity at all intervals of time with highest activity detected on 96th hour of incubation. This activity was about 3 times more than that secreted by parent strain *Aspergillus* sp. CJ22-326.

Effect of different carbon sources and concentration on growth and chitosanase production

During the microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but also as important energy source (Dunn, 1985). In order to improve the chitosanase production, single factor experiments various carbon sources of 1% (w/v) concentration including glucose, lactose, glucosamine, acetylglucosamine, chitosan, starch, wheat bran, dextrins, glycerol and molasses using the same other nutrients as the production medium was conducted in defined media tested individually in fermentation flask. The results after 72 h are shown in Figure 2. From the Figure 2, results apparently showed that chitosan gave the highest production of the chitosanase when it was used as the

Table 2. Comparison of enzyme activities of *Aspergillus sp.* CJ 22-326-11 and positive mutants by NTG treatment.

Strain	Biomass (g/L)	Chitosanase activity (U/mL)	Increase rate (%)
Original (CJ 22-326-11)	3.11 ± 0.12	2.23 ± 0.07	100
CJ 22-326-13	2.83 ± 0.23	2.90 ± 0.40	130
CJ 22-326-14	3.02 ± 0.15	3.32 ± 0.13	149
CJ 22-326-18	2.76 ± 0.21	2.60 ± 0.17	116
CJ 22-326-21	2.95 ± 0.17	2.84 ± 0.10	128
CJ 22-326-27	2.92 ± 0.20	3.05 ± 0.21	137
CJ 22-326-31	3.17 ± 0.28	3.21 ± 0.23	144
CJ 22-326-33	3.05 ± 0.10	3.18 ± 0.22	143
CJ 22-326-35	3.14 ± 0.18	2.95 ± 0.09	132

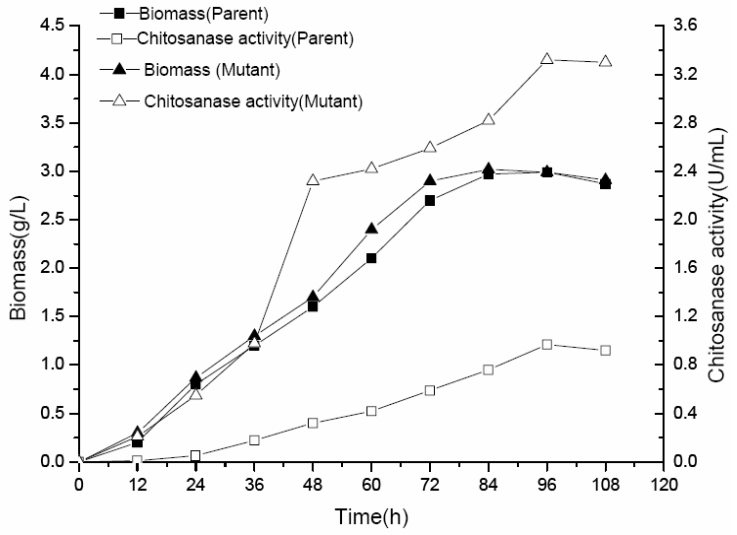

Figure 1. Time profile of growth and chitosanase production by wild-type and mutant CJ22-326 strains in shaken flasks.

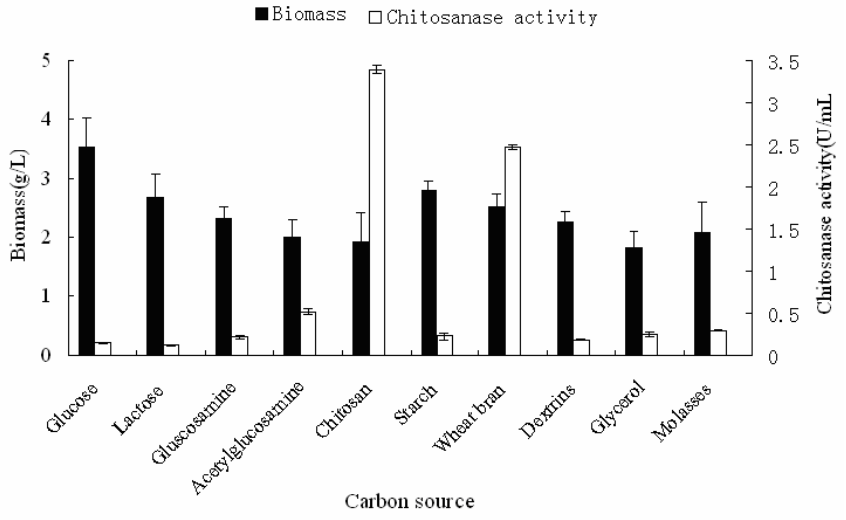

Figure 2. Effect of carbon sources on chitosanase production and cell growth in 72 h duration.

main carbon source in the medium. This suggested that chitosanase is the typical inducible enzyme, and chitosan is the most favorable inducer for protein and enzyme synthesis, when acetylglucosamine and glucosamine are presented as the carbon source. Chitosanase activity is also high in the wheat bran medium, which also indicate that the components in wheat bran could satisfy the nutrition requirement of the mutant strain.

Considering the abilities of influencing the chitosanase induction, the complex carbon sources chitosan and wheat bran were chosen to investigate their influences on the production of chitosanase.

The influence of chitosan content on the chitosanase production of *Aspergillus* sp. CJ22-326-14 is summarized in Figure 3. Relatively better yields were obtained with chitosan concentration from 5 to 25 g/L, and the highest enzyme activity was observed with 20 g/L of chitosan. Our results also showed that increasing of chitosan concentration beyond 20 g/L decreased the chitosanase production. This was probably due to the increasing broth rheology and decreasing the dissolved oxygen with further substrate chitosan addition.

The use of agricultural residues in microbial fermentations is being encouraged due to their ease of availability and inexpensiveness (Pandey et al., 2001), and they contain carbon, nitrogen, vitamin and minerals that are utilized by microbes. Substitution of glucose in the base medium with wheat bran (10g/L) resulted in a sustained secretion of chitosanase (2.95 U/l). But as the wheat bran level increased, the fermentation began to resemble to semi-solid state fermentation, hence lower concentration of wheat bran would be favorable to the chitosanase secretion and production by the *Aspergillus* sp. strain.

Effects of different nitrogen sources and concentrations on growth and chitosanase production

Figure 4 shows that the response levels when various nitrogen sources were added as nitrogen sources to the basal medium at a concentration of 2.0 g/L. Results indicated that some of the nitrogen sources in the medium, especially the inorganic ones, showed a positive effect on chitosanase activity when 1% chitosan and 2 whet bran as the carbon source. The best result was 3.11 U/mL obtained in the presence of $(NH_4)_2SO_4$ after 72 h fermentation. The following suitable nitrogen sources were beef extract, and $(NH_4)_2SO_4$ combined with beef extract, with the chitosanase activity of 2.06 and 2.36 U/mL, respectively. And the results of 'one–factor-at-a-time' experiments showed that chitosanase production from *Aspergillus* sp. CJ22-326-14 was highest when the culture medium contained 2 g/L of $(NH_4)_2SO_4$ (data not shown). Simultaneously the high chitosanase production using $(NH_4)_2SO_4$ is also an important feature of *Aspergillus* sp. CJ22-326-14 cultures, since a simplified purification strategy can be applied. In the case of proteins used as N-sources, a protein contamination (non-totally consumed during the fungal culture) could be found at the end of the fermentation.

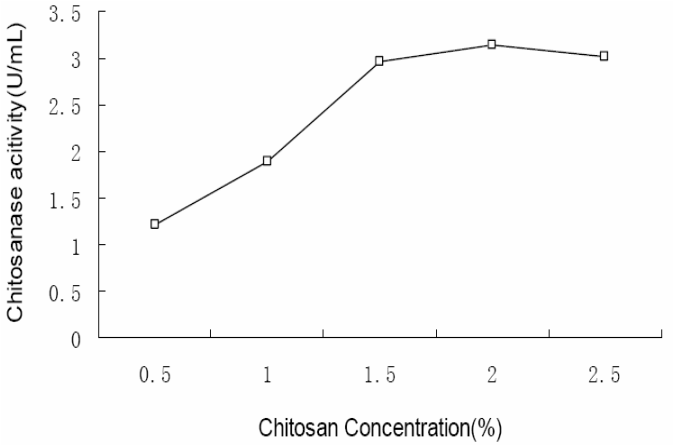

Figure 3. Effect of different chitosan concentration on chitosanase production.

Results of orthogonal array method

The orthogonal array design technique is a traditional method that has been successfully applied to improvement of culture media for fermentation process and provides the relationships among various factors, and the order of the significant factors for the optimum results (Bakhtiari et al., 2006; Krishna et al., 2005; Sreenivas et al., 2004).

In the present study, L9 (3^3) design was applied to screen the significant factors according to preliminary experiments. The three factors chitosan, wheat bran and $(NH_4)_2SO_4$ and their relevant levels are shown in Table 3. In the course of optimization experiments, the fermentation temperature, rotation speed, and fermentation period were set at 30°C, 150 rpm, and 72 h, respectively. The effects of those factors on chitosanase production were calculated and the analysis results were shown in Tables 3 and 4. The order of effects of factors on chitosanase production was chitosan > $(NH_4)_2SO_4$ > wheat bran. On the basis of the intuitive analysis, the optimization results were as follow: the maximal production of chitosanase could be obtained when the concentrations of the culture medium composition were (g/L): chitosan 20, wheat bran 10, $(NH_4)_2SO_4$ 2, KH_2PO_4 2 and $MgSO_4 \cdot 7H_2O$ 0.5.

The validation experiments were conducted to obtain the maximum yields of chitosanase activity by *Aspergillus* sp. CJ22-326-14 using the above optimal culture compositions with triplicate experiments. And the mean values of the chitosanase activity were 3.92 ± 0.45 U/mL which is much higher than those in basal media culture, suggesting that the chitosanase activity has been further improved

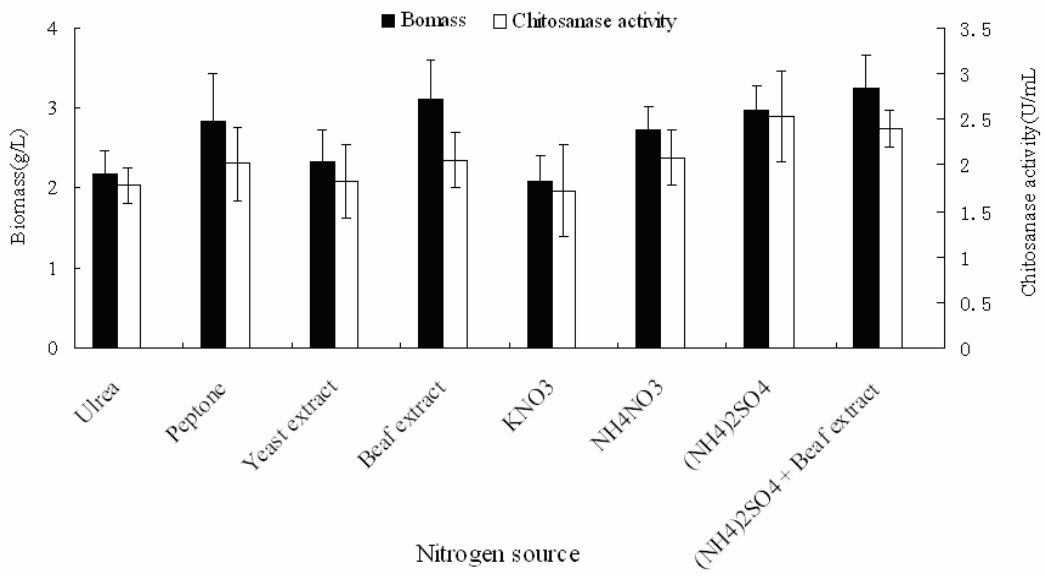

Figure 4. Effect of nitrogen source on chitosanase production in 96 h duration.

Table 3. Orthogonal array design and the response of the dependent variable chitosanase produced by *Aspergillus* sp. CJ 22-326-14 in shake flask culture.

Run	Independent variables (g/L)			Dependent variable
	A Chitosan	B Wheat bran	D $(NH_4)_2SO_4$	Chitosanase (U/mL)
1	1(20)	1(5)	1(2)	3.51 ± 0.35
2	1(20)	2(10)	2(3)	3.25 ± 0.36
3	1(20)	3(15)	3(4)	3.20 ± 0.15
4	2(25)	1(5)	3(4)	2.98 ± 0.27
5	2(25)	2(10)	1(2)	3.22 ± 0.19
6	2(25)	3(15)	2(3)	3.01 ± 0.30
7	3(30)	1(5)	2(3)	2.43 ± 0.24
8	3(30)	2(10)	3(4)	2.87 ± 0.38
9	3(30)	3(15)	1(2)	2.72 ± 0.11
K1	9.96	8.92	9.39	
K2	9.21	9.34	8.95	
K3	8.01	8.93	8.85	The order for Chitosanase: Chitosan > $(NH_4)_2SO_4$ >Wheat bran
k1	3.33	2.97	3.12	
k2	3.07	3.11	2.99	
k3	2.67	2.96	2.95	
R	0.66	0.15	0.17	

Table 4. Analysis results of orthogonal experiment.

Source	Sum of square	DF	F-ratio	Significance
A	6.722	2	3.159	**
B	1.552	2	0.729	*
C	0.184	2	0.086	
D	0.054	2	0.025	
Error	8.51	8		

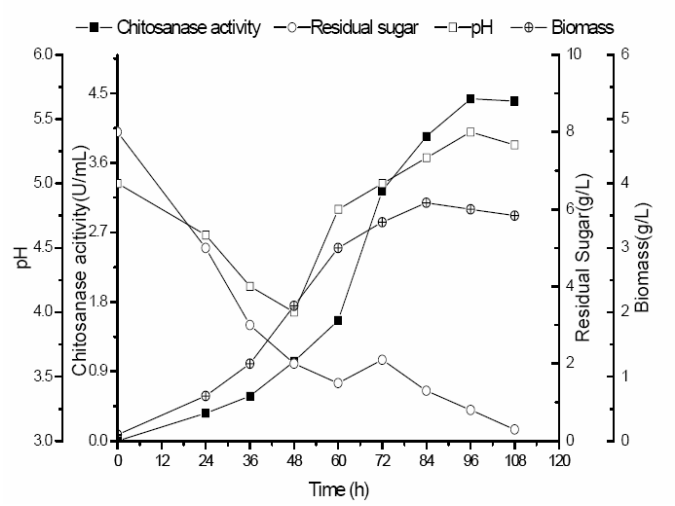

Figure 5. Time profile of batch fermentation in 10 L fermenter using the optimized medium.

with orthogonal experiment design.

Figure 5 showed the batch profile of chitosanase production, biomass growth, residual sugar content and pH in fermenter under optimized condition. As shown in Figure 5, minor chitosanase activity can be detected during the first 24 h and significant increase in chitosanase activity as observed after 24 h cultivation. The maximum chitosanase activity reached 4.11 U/mL at 96th hour cultivation and a slight decrease in chitosanase activity was observed after that time. This can be attributed to the difference of fermentation parameters in scale up of the fermentation process, such as dissolved oxygen, cell viability, metabolite and biomass.

Conclusion

Although optimization of fermentation media and operating parameter for the submerged culture of various enzyme production have been severally documented, few reports are available in literature regarding the strain improvement and optimization of nutrition composition for chitosanase production. In the present study, a mutant, *Aspergillus* sp. CJ22-326-14, capable of showing hyper-productivity of chitosanase was isolated using successive mutation procedures UV-irradiation followed by NGT treatment of the spores. The enzyme production was markedly enhanced by serial optimization of medium composition with one-factor-at-a-time combined with orthogonal array design method. Further investigation should emphasize on the influence of the cultivation parameters in the fermenter for large scale production of chitosanase.

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