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Production of recombinant NPRC10 protease in 14-L fermentation scale

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ABSTRACT

Present investigation describes the production of neutral protease (NPRC10) by recombinant E. coli BL21 (DE3) through submerged culture in 14-L fermenter. Different cultural parameters such as aeration rate, agitation speed and inoculum size were optimized for the production of enzyme. It was found that the maximum production of NPRC10 was obtained after 44 h of batch fermentation at aeration rate of 4 L/min, agitation speed of 500 rpm and inoculum size of 1%. The highest activity of protease during the time course of batch fermentation was 103 unit/mL.

Keywords: *E. coli* BL21 (DE3), fermentation, neutral protease, NPRC10, recombinant enzyme

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INTRODUCTION

Proteases are a group of enzyme that hydrolyzes protein or polypeptide by acting through a specific peptide linkage, and yielding the peptide segment. Proteases naturally occur in all organisms and are the most important industrial enzymes [19]. Compared to plant or animal proteases, microbial proteases have many advantages because they have a longer shelf life and can be stored under less than ideal conditions for several weeks without significant loss of activity. Besides, the most of them are extracellular in nature and are directly secreted into the fermentation broth, thus simplifying downstream processing of the enzyme [8, 11]. Proteases have been widely used in various industrial applications such as food, pharmaceutical, chemical, weaving, washing detergent, leather, waste management and silver recovery. They account for approximately 40% of the total enzyme sale markets in these applications [9].

Recent approaches for increasing protease yield include screening for hyper-producing strains, cloning and overexpression, fed-batch, chemostat fermentation, and optimization of the fermentation conditions [8, 9]. In general, fermentation at the optimal condition increases the expression and production level of enzyme to many times in comparison with their naturally production. Thus, the studies focus on to find out the suitable conditions for fermentation processing in laboratory as well as in industry, play an important role for enzyme production in large scale.

The pET vectors have been recognized as one of the most efficient systems for producing recombinant proteins in *E. coli* [e.g. strain BL21(DE3)], and the advantages of this system have been discussed [3]. *E. coli* has been used for the overproduction of recombinant proteins, because of its ability to grow rapidly, its well-characterized genetics, and the availability of an increasing large number of expression vectors and mutant host cells [2].

We previously reported the cloning and expression of *nprC10* gene from *Bacillus subtilis* C10 in *E. coli* BL21(DE3) [17]. Further, this neutral protease (NPRC10) was purifed, biochemically characterized and its expression level was also enhanced in shaking flask culture [15]. In this work, we studied the conditions for efficient production of NPRC10 in 14-L fermenter which can be easily applied to produce the large amount of enzyme for several biotechnological applications.

MATERIALS AND METHODS

Bacterial strain and plasmid

Recombinant *E. coli* strain BL21(DE3) (Invitrogen) harboring expression pET200/D-TOPO vector which contains *nprC10* gene (accession number: FJ822054) was used in this study. The *nprC10* gene encoding neutral protease (NPRC10) of 42 kDa under the control of T7 promoter which initiates transcription by inducing with IPTG (isopropyl β-D-1-thiogalactopyranoside). The *nprC10* gene also contains the signal peptide-like sequence from nucleotides 161 to 250 and the mature peptide sequence from nucleotides 824 to 1723 [17].

Cultures and enzyme production

Initial culture. The stock was grown in 250-mL Erlenmayer flasks containing 50 mL of LB medium supplemented with 50 µg/mL kanamycin. The flasks were incubated at 37^{o}C on shaker at a rotation speed of 200 rpm for overnight to attain an optical density of 2.2 measured at 600 nm.

Cell biomass production. The culture was also performed in 50 mL LB medium at 37^{o}C and 200 rpm with 0.5% (v/v) inoculum size of initial culture for 64 hours.

Enzyme production. A 14-L fermenter (two six-blade Rushton-type impellers, BioFlo 110, New Brunswick Scientific, Edison, US) with 10 L of the modified HSG medium was used. Lactose at the final concentrations of 0.5% were added into the cultures when the OD600 reached at value of 2 to induce enzyme production instead of IPTG. The temperature of fermenter was then decreased and maintained at 20^{o}C. The pH was controlled to 7 by 3 N NaOH solution and 0.1% antifoam 204 solution (Sigma-Aldrich, St. Louis, MO) was added as needed [15, 16]. The range of aeration rates from 3-5 L/min, agitation speeds from 400-600 rpm, and inoculum sizes of biomass culture from 0.5-2% (v/v) were investigated to optimize enzyme production. Ten-milliliter samples were withdrawn during the time course of batch fermentation for the measurement of cell density and protease activity.

Analytical methods

Protease activity was determined by slight modification based on the method of Anson (1938) using casein as the substrate [1]. One-milliliter of the supernatant was mixed with 2 mL of 2% (w/v) casein in Tris.HCl (50 mM, pH 7). The reaction was incubated at 55^{o}C for 10 min, 5 mL of 5% (w/v) trichloroacetic acid was then pipetted into the solution to terminate the reaction. The solution was incubated at room temperature for 20 min to precipitate the residue substrate, and centrifugated. The tyrosine concentration of the supernatant was determined at the 750 nm wavelength in the SmartSpecTM Plus spectrophotometer (Bio-Rad, USA). One unit of protease activity is defined as the amount of enzyme required to release 1 µg tyrosine per 1 mL per min under the standard assay conditions [16].

RESULTS

Effect of lactose on NPRC10 activity

Isopropyl β-D-1-thiogalactopyranoside (IPTG) was usually used to induce with high efficiency on expression of recombinant protein in *E. coli* but it must be removed by complicated methods from the induced products because of its toxicity. When at the efficient inducing concentrations, the cost of IPTG is approximate hundred fold of lactose [6, 7]. Studies on recombinant protein production using lactose as an inducer shown its high efficiency [6, 10, 14, 18, 24].

In our study, lactose was examined for its ability to induce the expression of the *nprC10* gene in shaking culture (50 mL volume in 250-mL Erlenmeyer flask) as a substitution for IPTG. The results indicated that a better expression effect for recombinant protease (approximately 57 unit/mL) was present when the lactose concentration used was 0.5% after 20 h of induction (Fig 1). Therefore, lactose concentration of 0.5% was used for further studies.

Biomass production

Recombinant *E. coli* BL21 (DE3) cells was grown at 37°C for 64 h. The profile of growth displayed a lag phase of 4 h, an exponential phase between hours 4 and 40, and a final, death phase. The growth stabilization of cells was maintained only for short time, and it is difficult to make prediction. Cell density increased continuously from 4 h to 40 h of culture, and reached a highest OD600 value of 6.6 (Fig 2). The study of production of extracellular NPRC10 was set up based on the growth profile of recombinant *E. coli* cells in batch fermentation with the time course from 20 h to 60 h.

Effect of aeration rates on NPRC10 production

The batch fermentation was carried out at 20°C with inoculum size of 0.5% and agitation speed of 300 rpm for 60 h to test the effects of aeration rates (3-5 L/min) on enzyme production. The profiles of NPRC10 specific activity are shown in table 1. It was found that the aeration rate of 4 L/min is the most suitable for the maximum production of NPRC10, 28 unit/mL after 40 h of culture. In contrast, at lower and higher aeration rates, the enzyme activities were all distinctly low.

These results suggest that selection of the aeration rate is necessary to attain high production of NPRC10 in this fermentation. Therefore, further experiments were conducted at air flow of 4 L/min as constant aeration rate.

Effect of agitation speeds on NPRC10 production

The effects of impeller agitation speeds of 400-600 rpm with 0.5% inoculum size and 4 L/min air flow on the production of NPRC10 are shown in table 2. Based on the maximum enzyme production, more than 76 unit/mL after 40 h of culture, 500 rpm was reputedly the best agitation speed. In different agitation speeds (400 and 600 rpm), highest NPRC10 yields were only from 28-51 unit/mL. The trend of enzyme production was similar in all batches of 14-L fermenter, showing a decline in NPRC10 activity after 40 h.

From these results, aeration rate of 4 L/min and impeller agitation speed of 500 rpm were chosen for the use in the next experiment.

Effect of inoculum sizes on NPRC10 production

The effect of inoculum size on NPRC10 production were investigated by using from 0.5-2% (v/v) of biomass culture. The results were shown in table 3 indicated the highest NPRC10 production (103 unit/mL) was obtained at 1% (v/v) inoculum size, after 44 h of culture in the 14-L fermenter with an agitation speed of 500 rpm and an aeration rate of 4 L/min, as compared to that at low or high inoculum sizes.

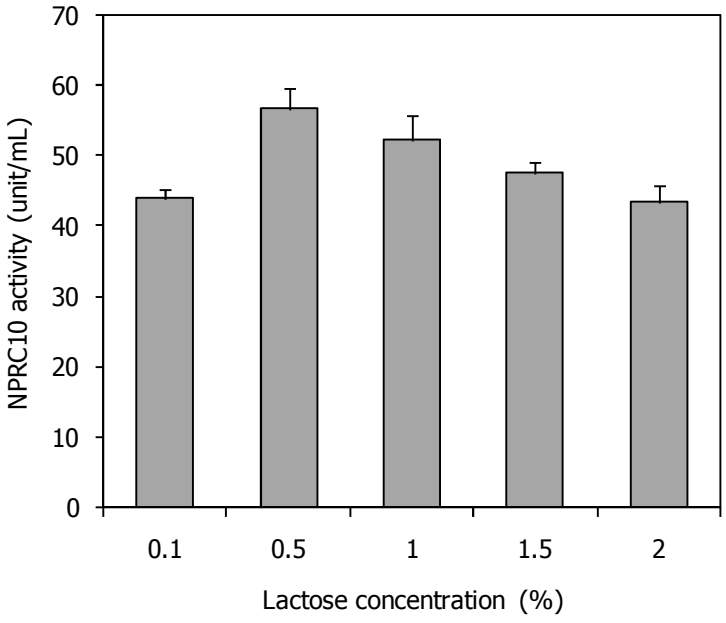

Figure 1. Effect of lactose as an inducer on NPRC10 production

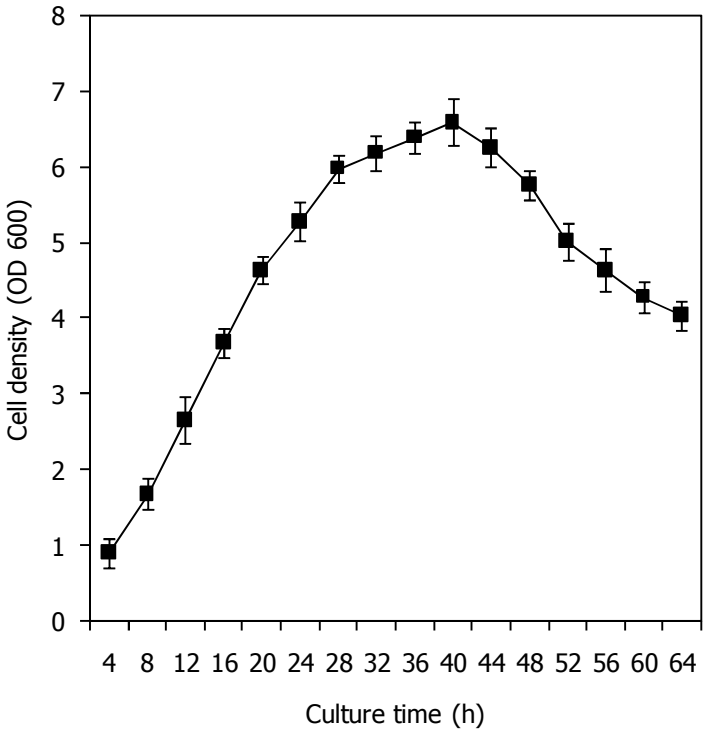

Figure 2. Growth profile of recombinant *E. coli* BL21 (DE3) in batch culture

Table 1. Effects of different aeration rates on NPRC10 production

Culture time (h)	NPRC10 activity (unit/mL)		
	3 L/min	4 L/min	5 L/min
20	4.04 ± 1.23	7.92 ± 1.15	7.17 ± 1.25
24	5.47 ± 1.10	9.28 ± 1.20	8.05 ± 1.56
28	8.81 ± 0.95	11.42 ± 2.06	10.30 ± 1.22
32	12.55 ± 1.85	13.75 ± 1.54	11.88 ± 1.79
36	15.06 ± 1.71	19.80 ± 1.29	14.75 ± 1.49
40	14.63 ± 1.44	28.38 ± 1.56	19.34 ± 1.15
44	12.22 ± 1.19	20.77 ± 1.74	16.09 ± 1.36
48	10.71 ± 1.43	16.49 ± 1.32	13.86 ± 1.64
52	9.42 ± 0.96	14.55 ± 1.07	10.44 ± 1.34
56	8.22 ± 1.12	13.74 ± 1.09	9.22 ± 1.88
60	7.05 ± 1.55	12.50 ± 1.16	8.41 ± 1.24

Table 2. Effects of different agitation speeds on NPRC10 production

Culture time (h)	NPRC10 activity (unit/mL)		
	400 rpm	500 rpm	600 rpm
20	7.92 ± 2.32	53.85 ± 1.44	9.47 ± 1.78
24	9.28 ± 2.72	58.48 ± 1.67	11.38 ± 1.84
28	11.42 ± 3.144	60.65 ± 1.70	14.42 ± 2.18
32	13.75 ± 2.51	64.62 ± 2.28	20.21 ± 2.66
36	19.80 ± 2.02	71.91 ± 2.75	40.29 ± 2.93
40	28.38 ± 2.61	76.31 ± 2.45	50.92 ± 3.25
44	20.77 ± 3.15	62.24 ± 2.90	39.47 ± 2.50
48	16.49 ± 2.27	58.71 ± 2.06	30.84 ± 2.48
52	14.55 ± 1.83	57.58 ± 2.68	26.64 ± 2.52
56	13.74 ± 2.14	55.25 ± 2.24	21.00 ± 1.85
60	12.50 ± 2.45	48.34 ± 1.83	19.22 ± 1.47

Table 3. Effects of different inoculum sizes on NPRC10 production

	NPRC10 activity (unit/mL)		
Culture time (h)	0.5% (v/v)	1% (v/v)	2% (v/v)
20	15.32 ± 2.80	85.68 ± 2.40	19.21 ± 3.00
24	19.21 ± 3.50	88.62 ± 2.90	24.00 ± 3.20
28	23.00 ± 4.30	89.47 ± 3.20	34.25 ± 3.30
32	28.02 ± 3.80	92.39 ± 3.60	40.00 ± 3.80
36	32.53 ± 3.20	95.56 ± 3.30	50.00 ± 3.10
40	39.39 ± 4.40	97.74 ± 4.60	53.75 ± 4.20
44	43.49 ± 4.80	102.68 ± 4.00	62.50 ± 4.80
48	49.59 ± 4.80	103.25 ± 3.70	58.00 ± 4.10
52	43.85 ± 3.20	100.71 ± 4.90	50.00 ± 4.20
56	38.16 ± 3.40	95.49 ± 3.40	45.00 ± 3.20
60	31.15 ± 2.80	90.34 ± 3.90	38.62 ± 2.80

DISCUSSION

The *nprC10* gene from *Bacillus subtilis* strain C10 was cloned into *E. coli* BL21(DE3) expression vector pET200/D-TOPO under the control of T7 promoter. Protease activity was found in the fermentation broth when induced cells were incubated for 20 h. Similar secretion of foreign proteins by *E. coli* has been observed in other studies [12, 25].
One of the most important parameters in aerobic fermentations is the dissolved oxygen concentration (DO) expressed as the percentage of the saturation value at atmospheric pressure. Oxygen is an essential element for aerobic growth and is very difficult to supply due to its low solubility [20]. Typical methods of controlling the dissolved oxygen level in fermentations include variation of the agitation speed [5] or air flow rate [23]. Acoording to Sargantanis and Karim (1996), the aeration rate that yielded the maximum total β-lactamase from *B. subtilis* was around 4 L/min out of a tested aeration range of 0.5-7.3 L/min in 1.5-L fermenter [20]. Lee et al. (1999) optimized curdlan production from *Agrobacterium* species in a 5-L fermenter at different agitation speeds (300-700 rpm) [13]. Their results shown that at a higher agitation speed (600 rpm), the highest curdlan production (64.4 g/L) was obtained in 120 h of a batch fermentation. Another report shown the highest yield of L-phenylalanine on glycerol was 5.6 g/L at agitation speed of 400 rpm and aeration rate of 8 L/min [12].

Tunga et al. (1999) indicated that the inoculum density does not have unlimited effect on fermentation processes. It has some optimum value depending upon the microbial species and fermentation processes [22]. Sivakumar et al. (2006) reported 5 mg/mL were inoculated in 250-mL Erlenmeyer flask for the maximum production of L-glutaminase from *Streptomyces rimosus* strain LG-10 [21]. Chae et al. (2000) shown that 5% (v/v) inoculum size is the most suitable amount for chloramphenicol acetyltransferase (CAT) production in *E. coli* JM105 at 30°C, 1 vvm air flow, and 450 rpm stirrer speed, with an initial working volume of 2 L [4].

In conclusion, recombinant *E. coli* BL21 (DE3) culture was successfully established in 14-L fermenter. The suitable cultural conditions for high yield of NPRC10 were an aeration rate of 4 L/min, an impeller agitation speed of 500 rpm and an inoculum size of 1%. Our results indicated the potential for the production of NPRC10 by an *E. coli* expression system.

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