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Production of NPRC10 protease by recombinant *Escherichia coli* through submerged culture in 40-L fermenter

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ABSTRACT

This work describes the production of neutral protease (NPRC10) by recombinant E. coli BL21 (DE3) through submerged culture in 40-L fermenter with working volume of 20 L. The parameters such as cell density, pH, inoculum size, and agitation speed were investigated for the production of enzyme. The results shown that the maximum production of NPRC10 was obtained after 34 h of batch fermentation at OD_{600} (cell density) of 2, inoculum size of 2% and agitation speed of 500 rpm with medium pH maintained at 7. The highest total activity of NPRC10 during the course of fermentation was approximately 76 unit/mL.

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

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INTRODUCTION

A protease (also termed peptidase or proteinase) is any enzyme that conducts hydrolysis of the peptide bonds in the protein molecule. Proteases can either hydrolyze specific peptide bonds, depending on the amino acid sequence of a protein, or hydrolyze a complete peptide to amino acids.

Proteases occur naturally in all living organisms. However, 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 [8-9]. Proteases are the most important industrial enzymes that execute a wide variety of functions and have various important biotechnological applications [13].

The pET vectors have been recognized as one of the most efficient systems for expression of recombinant proteins in *E. coli* [e.g. strain BL21 (DE3)] [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 reported the isolation and expression of neutral protease gene (*npr*C10) from *Bacillus subtilis* strain C10 in *E. coli* BL21 (DE3) [11]. The neutral protease (NPRC10) was biochemically characterized and its expression level was also improved in shaking flask culture [10]. The present work reports NPRC10 production in 40-L fermenter with appropriate conditions of submerged culture which can be applied to produce the large amount of enzyme.

MATERIALS AND METHODS

Bacterial strain and plasmid

E. coli strain BL21 (DE3) [F- *ompT hsdS*B (rB-mB-)] harboring expression vector pET200/D-TOPO (Invitrogen) was used in this work. The plasmid pET200/D-TOPO contains *npr*C10 gene (accession No. FJ822054) encoding 42-kDa neutral protease (NPRC10) under the control of T7 promoter. The *npr*C10 gene contains the signal peptide-like sequence from nucleotides 161 to 250 and the mature peptide sequence from nucleotides 824 to 1723 [11].

Cultures and enzyme production

The initial culture was grown in 250-mL Erlenmayer flasks containing 50 mL of LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl) supplemented with 50 µg/mL kanamycin. The flasks with inoculum size of 50 µL from the stock were incubated at 37^{o}C on shaker at a rotation speed of 200 rpm for overnight.

The biomass production was carried out in 14-L fermenter (BioFlo 110, New Brunswick Scientific, Edison, NJ, US) with 7 L working volume. The culture was grown in LB medium at 37^{o}C with agitation speed of 300 rpm, airflow rate of 3 L/min and inoculum size of 5% (v/v) from initial culture for 68 hours.

For enzyme production, a 40-L fermenter (BioFlo 510, New Brunswick Scientific Co., Edison, NJ, US) with 20 L of the modified HSG medium (3% glycerol, 0.7% yeast extract, 1.35% tryptone, 0.014% $MgSO_4.H_2O$, 0.15% KH_2PO_4, 0.23% K_2HPO_4, 0.5% glycine, 1.5% soluble starch and 10 mM Ca^{2+}, pH 7) was used. Lactose at the final concentrations of 0.5% was added into the culture when the OD_{600} reached a certain value to induce enzyme production. The culture was maintained at 20^{o}C after induction with airflow rate of 5 L/min. The pH was controlled at 7 by addition of 3 N NaOH solutions. The range of OD_{600} (cell density) from 1-4, inoculum sizes of biomass culture from 1-10% (v/v), and agitation speeds from 300-600 rpm were investigated to improve enzyme production. 0.1% antifoam 204 solution (Sigma-Aldrich, St. Louis, MO) was added as needed.

Analytical methods

Protease activity was determined by modified procedure 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

then centrifugated to obtain the supernatant. The tyrosine concentration of the supernatant was determined at the 750 nm wavelength in the SmartSpecTM Plus spectrophotometer (BioRad, 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.

RESULTS AND DISCUSSION

Biomass production

E. coli cells containing *npr*C10 gene was grown at 37^{o}C for 68 h. The profile of cell growth exhibited a lag phase of 1 h (data not shown), an exponential phase between hours 2 and 44, and a final, death phase. The phase of growth stabilization was quite short, and difficult to predict. Cell density increased from 2 h to 44 h of culture, and reached a maximum OD_{600} value of approximately 7 (Fig 1). Our investigations on production of extracellular NPRC10 in 40-L fermenter were designed based on the growth profile of recombinant *E. coli* cells.

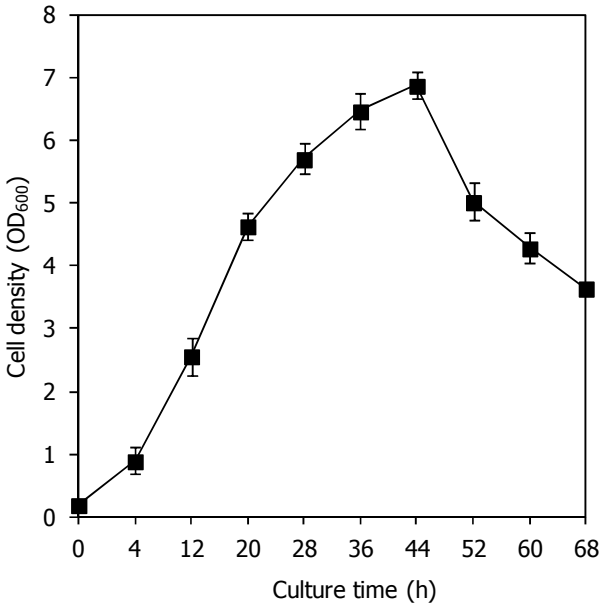

Figure 1. Growth profile of recombinant *E. coli* BL21 (DE3) cells in batch culture

Effect of cell density on NPRC10 production

The value of OD_{600} for induction plays a crucial role for maximizing expression of recombinant proteins [7]. The effect of cell density was evaluated at different stages of growth (OD_{600} from 1 to 4) in fermenter with 1% inoculum size, 400 rpm agitation speed and initial pH 7. The hydrolytic activity of NPRC10 was analyzed after 24 h of lactose induction at 20^{o}C. The results shown the maximum NPRC10 production (about 40 unit/mL) was observed when the cell density reached an OD_{600} value of 2. The enzyme activity strongly decreased at OD_{600} values of ≥3 (Fig 2).

Isopropyl β-D-1-thiogalactopyranoside (IPTG) was usually used to induce expression of recombinant protein in *E. coli* but it must be removed from the induced products because of its toxicity. When at the efficient inducing concentrations, the cost of IPTG is approximate hundred

fold of lactose [5-6]. Therefore, many studies used lactose as a substitution for IPTG and shown its efficiency [12, 16].

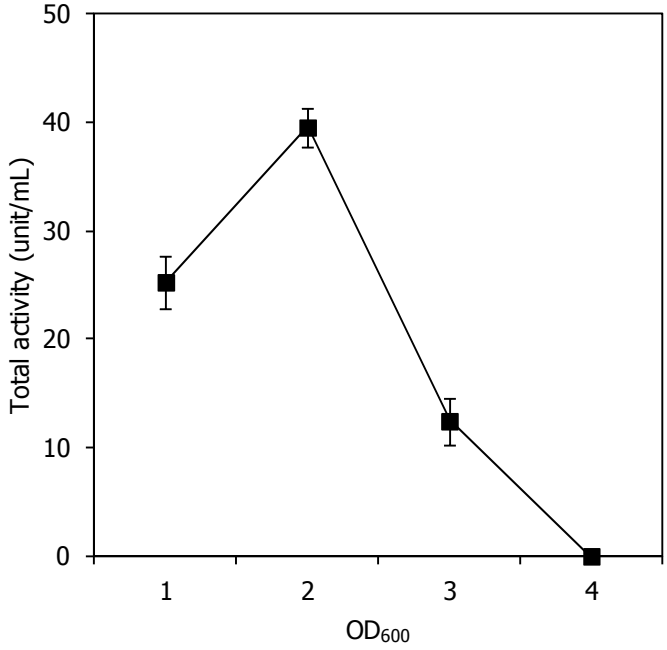

Figure 2. Effect of cell density on NPRC10 production

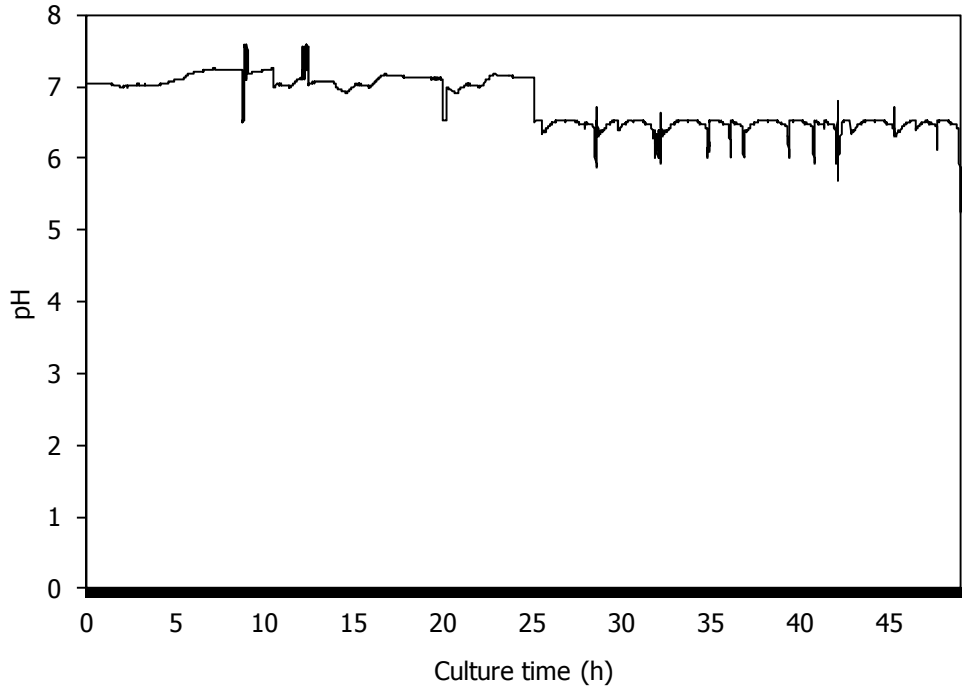

Figure 3. Online profile of pH was automatically recorded by fermenter in initial pH way with Advanced Fermentation Software (AFS)

Effect of pH on NPRC10 production

The influence of pH on NPRC10 production was determined in the both initial pH and controlled pH ways during the course of fermentation with 1% inoculum size, 400 rpm agitation speed. Induction for enzyme production was performed after cell density reached an OD_{600} value of 2. The online profile of initial pH way shown a pH value of about 7 was maintained between hours 1 and 25, and then it was about 6.5 from 25-50 h of induction (Fig 3). NPRC10 production increased when pH 7 to be controlled and reached a maximum value of approximately 61 unit/mL after 38 h, whereas protease yield was lower in initial pH way (Table 1).

According to Paranthaman et al (2009), microbial strains depend on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product formation [14].

Table 1. Effect of pH on NPRC10 production

Induction time (h)	Total activity (unit/mL)	
	Initial pH = 7	Controlled pH at 7
22	27.05 ± 2.25	36.88 ± 1.55
26	41.11 ± 1.81	49.56 ± 2.25
30	41.83 ± 2.20	53.22 ± 2.19
34	42.36 ± 1.48	56.25 ± 1.72
38	49.97 ± 2.42	60.89 ± 2.61
42	50.25 ± 1.26	59.11 ± 1.65
46	50.56 ± 1.46	57.20 ± 1.34
50	42.16 ± 1.83	52.46 ± 1.59

Table 2. Effect of inoculum sizes on NPRC10 production

Induction time (h)	Total activity (unit/mL)			
	1%	2%	5%	10%
22	51.05 ± 2.23	36.88 ± 1.50	17.29 ± 2.21	23.33 ± 1.56
26	53.38 ± 1.89	49.56 ± 2.25	23.28 ± 1.87	39.83 ± 2.29
30	56.99 ± 2.21	53.22 ± 2.10	26.46 ± 2.26	45.54 ± 2.13
34	58.01 ± 1.45	57.09 ± 1.76	29.05 ± 1.49	54.94 ± 1.71
38	57.86 ± 2.45	64.85 ± 2.64	40.06 ± 2.45	51.10 ± 2.66
42	56.35 ± 1.28	60.11 ± 1.68	54.99 ± 1.28	47.28 ± 1.65
46	53.12 ± 1.49	59.29 ± 1.34	61.63 ± 1.44	43.36 ± 1.33
50	50.30 ± 1.83	52.46 ± 1.55	45.85 ± 1.86	35.22 ± 1.58

Effect of inoculum sizes on NPRC10 production

Inoculum size was also an important factor for enzyme production. Therefore, its effect on NPRC10 production was investigated by transferring 1-10% (v/v) of biomass culture into the 40-L fermenter, and operated in an agitation speed of 400 rpm and pH controlled at 7. Lactose induction was started after cell density of various inoculum sizes reached an OD_{600} value of 2. The results were shown in table 2 indicated the highest NPRC10 production (approximately 65 unit/mL) was obtained at 2% (v/v) inoculum size after 38 h of induction. High inoculum size (5 or 10%, v/v) contains a large volume of biomass production medium, thus it can cause an unfavourable influence for enzyme production.

Effect of agitation speeds on NPRC10 production

One of the most important parameters in aerobic fermentations is the dissolved oxygen concentration. Typical methods of controlling the dissolved oxygen level in fermentation include variation of the agitation speed [4] or air flow rate [15]. In this work, the effect of various agitation speeds from 300-600 rpm, with 2% inoculum size, optimum cell density (OD_{600} = 2) and pH 7 on the NPRC10 production is shown in table 3. Based on the maximum enzyme production, approximately 76 unit/mL after 34 h of culture, 500 rpm was found to be the optimum agitation speed. Whereas at different agitation speeds (300, 400 and 600 rpm), highest NPRC10 productions were in the range of 62-65 unit/mL.

Table 3. Effect of agitation speeds on NPRC10 production

Induction time (h)	Total activity (unit/mL)			
	300 rpm	400 rpm	500 rpm	600 rpm
22	27.46 ± 2.20	36.88 ± 1.50	59.47 ± 2.20	10.11 ± 1.50
26	45.57 ± 1.80	49.56 ± 2.20	61.65 ± 1.80	13.27 ± 2.20
30	46.87 ± 2.20	53.22 ± 2.10	68.36 ± 2.20	18.03 ± 2.10
34	57.17 ± 1.40	57.09 ± 1.70	75.99 ± 1.40	30.02 ± 1.70
38	61.52 ± 2.40	64.06 ± 2.60	72.90 ± 2.40	46.39 ± 2.60
42	56.35 ± 1.20	60.11 ± 1.60	65.08 ± 1.20	52.12 ± 1.60
46	45.77 ± 1.40	59.29 ± 1.30	59.12 ± 1.40	64.75 ± 1.30
50	32.96 ± 1.80	52.46 ± 1.50	55.81 ± 1.80	55.66 ± 1.50

CONCLUSION

Recombinant *E. coli* BL21 (DE3) culture was successfully established in 40-L fermenter. The suitable cultural conditions for high yield of NPRC10 were an impeller agitation speed of 500 rpm, an inoculum size of 2% (OD_{600} = 2) and pH 7. Our results indicate the potential for the production of NPRC10 by an *E. coli* expression system.

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