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Optimal an extracellular alkaline protease production condition for *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa JM07 which produces an extracellular alkaline protease was isolated from the Yellow Sea in China. The cultural conditions were optimized for maximum enzyme production. Maximum enzyme activity was achieved when the bacterium was grown in soybean meal (3.5%, w/v), corn steep meal (1.5%, w/v), Na₂HPO₄ (0.4%, w/v), KH₂PO₄ (0.03%, w/v), Na₂CO₃ (0.02%, w/v), MgSO₄ (0.02%, w/v), CaCl₂ (0.2, w/v), at pH 7.0 and 20°C over 12 h incubation period. The enzyme had an optimum pH of around 10 and maintained its stability over a broad pH range between 7 and 12. Its optimum temperature is around 30°C, and exhibited a stability of up to 40°C. The enzyme activity was strongly inhibited by DFP, suggesting that it belongs to the family of serine proteases.

Key words: *Pseudomonas aeruginosa*, alkaline protease, by-product.

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes and in recent years, the use of alkaline proteases in a variety of industrial processes involving detergents, food, leather and silk has increased remarkably (Maurer, 2004). Currently, a large proportion of the commercially available alkaline proteases are derived from *Bacillus* strains (Tekin et al., 2012), although several fungal sources are being increasingly employed (Chellapandi, 2010). 30 to 40% of the production cost of industrial enzymes is estimated to be accounted for by the cost of the growth medium. Considering this fact, the use of cost-effective growth medium for the production of alkaline proteases from a microbe is especially important, because these enzymes account for approximately 60% of world-wide enzyme consumption (Deng, 2008). Large quantities of agricultural and agro-industrial residues are generated from diverse agricultural and industrial practices. These residues represent one of the most energy-rich resources on the planet (Nigam et al., 2001). For this purpose, soybean meal and corn steep meal were

recognized as a potentially useful and cost-effective medium ingredient. The principal objective of this study was to screen for the soybean meal and corn steep, and further to optimize the levels of the screened medium, for alkaline protease production by employing *Pseudomonas aeruginosa* JM07 along with some biochemical properties of the enzyme. This study is a component of a broader investigation into the influences of cheap raw materials such as soybean meal and corn steep, as determined using statistical methodologies.

MATERIALS AND METHODS

Microorganism and growth conditions

The organism used in this study was a *Pseudomonas* species, which was isolated from the Yellow Sea in China. This strain was determined to be a member of *Pseudomonas* according to its 16S rDNA and fatty acids profile (The data are not listed in this article). Bacterial protease was directly determined using a casein agar

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plate during cultivation. The culture media used was Luria-Bertani broth (LB), and the pH was adjusted to 7. The medium (200 ml) in 500 ml baffled flasks was inoculated with 1 ml of a 16 h old seed culture, and incubated at 20°C with shaking at 200 rpm for 12 h. The cell-free supernatant was recovered by centrifugation (6 000 rpm, 4°C, 30 min), and used for determining extracellular alkaline protease activity.

To find the best protease production condition, carbon sources including soluble starch, sucrose, glucose, lactose, fructose, maltose, corn steep meal and citric acid were added separately to a fixed concentration of 1.0 %. Nitrogen supplement including peptone (1.5%), fish meal (1.5%), tryptone (1.5%), yeast extract (1.5%), urea (1.5%), soybean meal (2.5%), cotton-seed meal (1.5%) and ammonium citrate (1.0%) were added separately. The medium that supports the best protease activity was applied in the following investigation of protease characteristics.

Protease assay and purification

Standard conditions for proteolytic activity assay was assayed using casein (Sigma) as the substrate. Enzyme solution (200 µl) was added to 200 µl of glycine-NaOH (pH 10.4) buffer containing 1% casein, and incubated at 30°C for 10 min. The reaction was stopped by adding 400 µl 20% w/v TCA solution. After the preparation stood at room temperature for 10 min, the entire mixture was centrifuged at 12,000 × g for 10 min. The acid-soluble material was estimated spectrophotometrically at 280 nm. The blank sample was made by adding TCA before the enzyme. The difference in optical density between the sample and the blank was used as the measure of proteolytic activity.

One protease unit was defined as the amount of enzyme that produces 1 µg of tyrosine in 1 min under the defined assay conditions. The standard curve of tyrosine was determined by the absorbance at 280 nm as a function of various concentrations of tyrosine, from 0 to 10 mg/ml.

All the following fractionations were done at 4°C, unless otherwise specified and the chromatography purification was monitored for protein by measuring absorbance at 280 nm.

Step 1. The culture was centrifuged at 4 000 rpm for 40 min at 4°C to remove cells and insoluble residues, and the culture supernatant was brought to 80% saturation with ammonium sulphate. The resulting precipitate collected by centrifugation (4 000 rpm) was dissolved in 25 mM borate buffer (pH 10.0), and it was then dialyzed against 2 L of the same buffer for 48 h, with four changes made at 12 h intervals.

Step 2. The dialyzed enzyme was loaded onto a cation exchanger (DEAE-Sephadex, Pharmacia, Sweden, 1.5×20cm) equilibrated with 25 mM borate buffer (pH 10.0). The flow rate was adjusted to 1 ml min⁻¹. The enzyme was eluted from the column using a linear NaCl gradient (0-1.0M) and the eluted fractions were collected. These fractions were collected and precipitated by the addition of 0.5 volume of chilled ethanol, and the enzyme precipitated removed by centrifugation at 4 000 rpm for 40 min.

Step 3. The protease solution prepared from step 2 loaded on to a Sephacryl S-200 gel filtration column (Pharmacia, Sweden, 1 cm × 80 cm) equilibrated with 25 mM borate buffer (pH 10.0). Fractions of the elution were collected at a flow rate of 0.2 ml min⁻¹. The active protease fractions were pooled and stored at -20°C.

Temperature and pH

The partially purified protease was incubated at various temperatures ranging from 0 to 50°C for 30 min. The protease activity was measured at 30°C in the following buffer systems: 0.1 M sodium acetate (pH 4.0-5.5); 0.1 M sodium phosphate (pH 6.0-7.5); 0.1 M

Tris-HCl (pH 8.0-9.0); 0.1 M glycine-NaOH (pH 9.5-11); and 0.1 M sodium carbonate (pH 11.5-12.0), respectively.

Chemical effects

The effects of various chemical reagents and metal ions on enzyme activity were examined by incubation the protease solution (in 25 mM borate buffer, pH 10.0) with different compounds at 20°C for 1 h, and the relative activities were determined under standard assay conditions.

Effects of detergent additives

The effects of various detergent additives on enzyme activity were examined by incubation of the protease solution (in 25 mM borate buffer, pH 10.0) with different compounds at 20°C for 1 h, and the relative activities determined under standard assay conditions.

Inhibition tests

The partially purified protease was incubated with different inhibitors under optimum reaction conditions. Residual activities in the presence of the inhibitors were compared with the controls without inhibitor.

RESULTS AND DISCUSSION

The organism

Several bacterial strains secreting alkaline proteases were screened and isolated from the Yellow Sea in China. Among these, one strain *Ps. aeruginosa* JM107 (The strain identification are not listed in this article) exhibited prominent clear zones around the colonies on casein agar plates indicating that it secretes significant amounts of protease. Some *Pseudomonas* species produce alkaline extracellular proteases, which are similar to those produced by *Bacillus* species. Furthermore, because *Ps. aeruginosa* can grow in alkaline, water-soluble oil, its enzymes must be adapted to function optimally under such extreme conditions. Therefore, *Pseudomonas* species may also have various industrial applications, including for the synthesis of a broad spectrum of organic molecules in non-aqueous solvents and detergents industry (Sugihara et al., 2002). So this organism was selected for further optimization of the extracellular protease production.

Enzyme production

There are several reports showing that different carbon sources have different influences on extracellular enzyme production by different strains (Chi et al., 2003). Therefore, effects of soluble starch, sucrose, glucose, lactose, fructose, maltose, corn steep meal and citric acid at the concentrations of 1% on protease production by *Ps. aeruginosa* JM07 were examined. The results in Figure 1

Figure 1. Effects of different carbon sources on protease production. The cells were cultivated in the production medium. All the data are given mean $\pm$ SD, $n=3$.

Figure 2. Effects of different initial concentrations of corn steep meal, soluble starch and soybean meal on protease production. The cells were cultivated in the production medium.

showed that corn steep meal and soluble starch were the best carbon sources for protease production. The specific protease activity in the culture supernatant was 1218 U mg⁻¹ protein. This meant that strain used corn steep meal as sole carbon source for protease production (Figure 1). It is thought that corn steep meal is the best carbon source for fermentation industry due to its low cost and easily obtained material. Figure 1 also showed

that in the presence of other carbon sources, there was a reduction in protease production. This could be due to catabolite repression by high glucose available in the medium. However, increased yields of alkaline proteases were reported by several other workers who used different sugars such as lactose, maltose, sucrose and fructose (Jasvir et al., 2004; Canan et al., 2006). The results in Figure 2 indicated that the optimal concentration of

Figure 3. Effects of different nitrogen sources on protease production. Organic nitrogen concentrations used were peptone 1.5%; fish meal 1.5%; tryptone 1.5%; yeast extract 1.5%; urea 1.5%; soybean meal 2.5%; cotton-seed meal 1.5%. Inorganic nitrogen concentrations were ammonium citrate 1%. The cells were cultivated in the production medium. All the data are given mean $\pm$ SD, $n=3$.

corn steep meal for the maximum protease production by the JM07 was 1.5%. Under this condition, the specific protease activity reached 1835U mg⁻¹ protein. In contrast, the optimal concentration of soluble starch for the maximum protease production was 2.0%. Under this condition, the specific protease activity in the culture reached 1628U mg⁻¹ protein, suggesting that the JM07 cells grown in the presence of corn steep meal could produce more protease than those grown in the soluble starch.

It has been reported that effects of a specific nitrogen supplement on protease production differ from organism to organism although complex nitrogen sources are usually used for alkaline protease production (Kurmar et al., 1999). Figure 3 shows that soybean meal was stimulatory for alkaline protease production by the JM07 and substitution of soybean meal in the medium with other nitrogen sources including inorganic nitrogen sources decreased greatly the enzyme production. Specific protease activity in the presence of 2.5% soybean meal reached 2013U mg⁻¹ protein. The results in Figure 2 also indicated that the optimal concentration of soybean meal for the maximum protease production by the JM07 was 3.5%. The protease activity reached the highest, 2248U mg⁻¹.

Effect of pH and thermal on activity of the enzyme

The effects of pH on the protease activity toward casein were examined at various pH values at 30°C (Figure 4a). The enzyme was highly active between pH 8.0 and 12.0 with an optimum pH 10.0. The relative activities at pH 8.0 and 12.0 were about 78 and 75%, respectively of that at

pH 10.0. The high pH optimum is a common characteristic of alkaline proteases (Genckal et al., 2006; Nedra et al., 2007; Raja et al., 2006). The important detergent enzymes, subtilisin Carlsberg showed maximum activity at pH values of 8.0-10.0 (Hadj-Ali et al., 2007). The enzyme exhibited an optimum temperature of 30°C, with relatively high activity over a broad temperature range of 10-40°C. The enzyme activity at 10 and 20°C corresponded to 70 and 85% of the maximum activity at 30°C, which allowed lower wash temperatures when added to detergents. This protease is a true psychrophilic enzyme, both kinetically and thermodynamically adapted to cold, typical of the natural habitat of the bacteria, as proved by the following properties: low optimal temperature (30°C) when compared to not only the mesophilic subtilisin Carlsberg (60°C) but also to the Antarctic subtilisin S41 (40°C), a high relative activity at 0°C corresponded to 29% of the maximum activity at 30°C (Figure 4B).

Chemical effect

EDTA was examined for the effect on the activity of protease. At least 10 mM EDTA was required to inhibit protease activity more than 40%. This result indicated no requirement for metal cofactors. This is due to detergents containing high amounts of sequestering agents, which could chelate the metal ions and make them unavailable in the washing solution. The effects of metal ions on the activities of the protease were investigated. The protease was activated by Mg²⁺, K⁺, Ca²⁺, Ba²⁺, and Zn²⁺. Of metal ions, the Ca²⁺, Zn²⁺ are very strong activators. The activi-

Figure 4. (A) Optimum pH of the protease activity. Remaining activities were determined after incubating the enzyme in various buffers at different pH values. (B) Optimum temperature of the protease. The purified protease was incubated at various temperatures ranging from 0 to 50°C for 30 min.

Table 1. Effects of various chemical reagents on the activity of enzyme.

Material	Concentration (mM)	Relative activity (%)	Material	Concentration (mM)	Relative activity (%)
EDTA	10	60±2.5	Tris	1(v/v)	98±2.6
	20	50±2.1	Borax	1(v/v)	95±2.1
ZnCl ₂	2	121±1.8	Triton X-100	1(v/v)	92±1.7
CaCl ₂	1	128±1.5	DFP	1	0
KCl	1	105±2.2	PMSF	1	95±1.8
MgCl ₂	1	112±1.7	Aprotinin	1	92±2.3
BaCl ₂	1	108±2.5	LBTI	1	96±2.5
AgNO ₃	1	42±1.3	SBTI	1	94±2.9
	10	0	Bestatin	1	78±1.5
SDS	1(v/v)	43.8±1.9	Cystatin	1	73±1.6
Tween-20	1(v/v)	104±2.5	TLCK	1	100
Tween-80	1(v/v)	108±2.3	TPCK	1	100

ties (28 and 21%) of the protease in the presence of Ca²⁺ (1mM) and Zn²⁺ (2mM) were higher than that observed in the control solution. In contrast, the protease activity was completely inhibited by Ag⁺ (10 mM). The data is show in Table 1.

Effects of detergent additives

Enzymes are usually inactivated by surfactants. In the presence of 1% w/v SDS (anionic surfactant), the protease retained 43.8% of its activity. As for non-ionic surfactants, 1% v/v Tween-20 or Tween-80 increased activity about 4-8%. The other detergent additives exhibited the least inhibitory effect upon the enzyme. Since the enzyme has been shown to be stable under different surfactants,

the protease could be suggested to be suitable for the wash industry. The data is show in Table 1.

Protease inhibitors

The effect of natural and synthetic inhibitors on the partially purified protease was also investigated. Enzyme activity was strongly inhibited only by DFP among the serine protease inhibitors tested, which included PMSF, aprotinin, LBTI and SBTI. In addition, bestatin and cystatin exhibited the least inhibitory effect upon the enzyme. It was also interesting to note that the trypsin selective reagent TLCK and chymotrypsin alkylating TLCK did not inhibit enzyme activity. The data is also show in Table 1.

Conclusion

Alkaline proteases are generally produced by a wide range of microorganism including bacteria, moulds and yeasts (Ahmed et al., 2010). Alkaline proteases are the major industrial workhorses and the recent trend toward the use of alkaline proteases from these sources in different process applications like detergents, food and wool treatment has increased remarkably because of their increased production capacities, high catalytic activity and high degree of substrate specificity (Kurmar et al., 1999). Alkaline proteases are generally produced by submerged fermentation (Ahmed et al., 2010). In commercial practice, the optimization of medium composition is done to balance between the various medium components, thus minimizing the amount of unutilized components at the end of fermentation. Research efforts have been directed mainly toward evaluating the effect of various carbon and nitrogen nutrient cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the medium and optimization of environmental and parameters such as pH, temperature, aeration and agitation (Ahmed et al., 2010). In this paper, we reported a new strain of *Ps. aeruginosa* JM07, which produced high levels of an extracellular alkaline protease during growth on the medium containing soybean meal (3.5%, w/v), corn steep meal (1.5%, w/v), Na_2HPO_4 (0.4%, w/v), KH_2PO_4 (0.03%, w/v), Na_2CO_3 (0.02%, w/v), MgSO_4 (0.02%, w/v), CaCl_2 (0.2, w/v), at pH 7.0 and 20°C with shaking at 200 xg for 12 h.

The partially purified protease was inhibited by DFP, suggesting that it is a member of the serine protease family. The protease had an optimum pH of around 10, which is a typical characteristic of alkaline proteases (Nedra et al., 2007). In comparison with the overall enzymatic properties of alkaline proteases from other sources (Chellapandi, 2010; Deng, 2008; Kurmar, 1999), the protease proved to have an excellent stability against pH, detergents additives and chemical detergents. The high enzyme activity in alkaline pH at moderate temperature (30°C) and stability under harsh conditions such as surfactants and heavy metals indicate that the purified enzyme is suitable for industrial purposes, especially cold wash industry.

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