J Ind Microbiol Biotechnol (2007) 34:241–245
DOI 10.1007/s10295-006-0192-2

ORIGINAL PAPER

Optimization of extracellular alkaline protease production from species of *Bacillus*

Wei-Hua Chu

Received: 6 December 2005 / Accepted: 24 August 2006 / Published online: 15 December 2006

Abstract Thirty-five strains capable of secreting extracellular alkaline proteases were isolated from the soil and waste water near the milk processing plant, slaughterhouse. Strain APP1 with the highest-yield alkaline proteases was identified as *Bacillus* sp. The cultural conditions were optimized for maximum enzyme production. When the initial pH of the medium was 9.0, the culture maintained maximum proteolytic activity for 2,560 U ml^{-1} at 50°C for 48 h under the optimized conditions containing (g^{-1}): soyabean meal, 15; wheat flour, 30; K_2HPO_4, 4; Na_2HPO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.1; Na_2CO_3, 6. The alkaline protease showed extreme stability toward SDS and oxidizing agents, which retained its activity above 73 and 110% on treatment for 72 h with 5% SDS and 5% H_2O_2, respectively.

Keywords *Bacillus* sp. · Enzyme-producing condition · Extracellular alkaline proteases

Introduction

Proteases are one of the most important industrial enzymes accounting for nearly 60% of the total worldwide enzyme production [1, 3, 10]. Among the various proteases, microbial proteases play an important role in biotechnological processes accounting for approx. 59% of the total enzyme used [17]. Of these, alkaline proteases are very important industrial enzymes. They are used in the manufacture of detergents, food, pharmaceuticals and leather, in the production of protein hydrolysate, in the film industry, and by waste processing companies [6, 8].

In recent years a number of studies have been conducted to characterize alkaline proteases from different microorganisms including bacteria, moulds, yeasts and also mammalian tissues [7]. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications. In the present study, we report the isolation and selection of a bacterium (APP1), which is a potent producer of extracellular alkaline protease, and the optimization of culture conditions required for enzyme production by the isolate.

Materials and methods

Sample collection, isolation and screening

The soil samples were collected from sandy soil, milk processing plant, the drainage of a slaughterhouse and were diluted in sterile saline solution. A soil sample (1 g) was suspended in 3.0 ml of distilled water by vigorous vortexing. A 0.1-ml portion of the soil suspension was plated directly onto alkaline casein solid agar medium and incubated at 37°C. After a 48-h incubation, individual colonies were picked and purified by

W.-H. Chu (✉)
Laboratory of Microbiology, School of Life Science and Technology, China Pharmaceutical University, No. 24 Tongjiaxiang, Nanjing 210009, People's Republic of China
e-mail: chuweihua2002@yahoo.com.cn

streaking three times onto alkaline casein agar. Purified isolates were scored for extracellular protease production by streaking onto skim milk agar plates containing peptone (0.1%, w/v), NaCl (0.5%, w/v), agar (2.0%, w/v) and skim milk (10%, v/v). In situ protease production was demonstrated by the clearing of opaque milk proteins in the area surrounding isolated colonies growing on the surface.

Depending upon the zone of clearance, the high-yield protease strains were selected for further experimental studies.

Identification of selected isolate

Identification of selected isolate was studied based on different morphological, physiological and biochemical characteristics. The data was compared with standard description given in Bergey's Manual of Determinative Bacteriology [2].

Protease assay

Alkaline protease activity was determined using casein as a substrate at a concentration of 0.5% in 0.1 mol^{-1} glycine–NaOH buffer (pH 11.0) [14]. One unit of enzyme activity is defined as the amount of the enzyme resulting in the release of 1 μg of tyrosine per minute at 60°C under the standard assay conditions.

Enzyme production and optimization

Among the isolates, *Bacillus* sp. APP1 which exhibited a prominent clear zone was selected for the study of alkaline protease production. The isolate was maintained on TSB agar plate and stored at 4°C. The basal culture medium for the protease production contained (g^{-1}): K_2HPO_4, 4; Na_2HPO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.1; Na_2CO_3, 6, pH 7.5. Sodium carbonate solution was sterilized separately, and then added to the medium. The medium (100 ml) was inoculated with 1 ml of a 24-hour-old seed culture in 500 ml baffled flasks, and incubated at 37°C with shaking at 250 rpm for 48 h. The cell-free supernatant was recovered by centrifugation (8,000 *g*, 4°C, 20 min), and used for determining the protease activity.

To study the growth kinetics, 10% inoculum was used in 100 ml of sterile basic medium and incubated at 37°C for 24 h on environmental shaker (100 rpm). The microbial growth was monitored at 600 nm. The samples were collected and after centrifugation (6,000 rpm for 15 min), supernatant was retained for the enzyme assay. Potent strain was studied in detailed by changing the media conditions along with the control.

To optimize the culture conditions for maximum production of protease different parameters such as incubation periods (12, 24, 48 and 72 h), medium pH (5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0), temperature (37, 50, 65 and 70°C), carbon sources (wheat flour, glucose, lactose and maltose), nitrogen sources [peptone, soyabean meal, casein, $(NH_4)_2SO_4$] (Table 1), and surfactants and oxidizing agents were used. The effect of these parameters on protease production was recorded.

Results

Identification of the isolate

Five samples were collected and 35 microbial colonies secreting alkaline proteases were isolated from sandy soil, milk processing plant, the drainage of a slaughterhouse. The isolates were then purified, preserved and tested for their proteolytic ability. In preliminary screening the isolate APP1 showed better ability to hydrolyze skimmed milk casein (Fig. 1) and was finally selected for further optimization of the extracellular proteases production. The culture was maintained on slants containing LB medium with agar and stored at 4°C.

The characteristics of isolate APP1 are listed in Table 2. The isolate was an aerobic, spore forming, motile, rod-shaped bacterium that tentatively belonged

Table 1 Effect of different carbon and nitrogen sources on protease production by *Bacillus* sp. APP1

Carbon source	Concentration (%)	Protease activity (U ml^{-1})	Nitrogen source	Concentration (%)	Protease activity (U ml^{-1})
Soyabean meal	0.5	1,023	Wheat flour	2.0	678.4
	1.5	2,560		3.0	1,110.5
	2.0	1,364		4.0	829.0
Peptone	1.0	836.3	Maltose	0.5	1,025
Casein	1.0	650	Glucose	0.5	850
$(NH_4)_2SO_4$	1.0	160	Lactose	0.5	510

Cells were grown in the basal medium and supplemented with each nitrogen source or carbon source at 37°C for 48 h

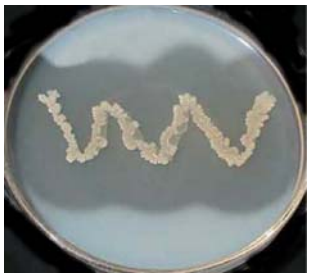

Fig. 1 The zone of hydrolysis of *Bacillus* sp. APP1 on milk agar

to the genus *Bacillus*. The sporangia were swollen with ellipsoidal subterminal spores.

Effect of incubation period on protease production

When the isolate *Bacillus* sp. APP1 was grown in liquid culture medium to determine optimum incubation time the study was carried out at 1–3 days incubation time and maximum enzyme formation (2,110 U ml^{-1}) was recorded with 2 days of incubation period. The biomass yield was found increase with an increase in the incubation period. The highest biomass yield (7.20 absorbance at 600 nm) was recorded after 4 days of incubation period and different growth characteristics also observed with different incubation period.

Effect of nitrogen and carbon sources on the protease production

Among the organic nitrogen sources used, soyabean meal had the significant effect on the production of the extracellular protease, and high level of the production was achieved when the cells were grown in a medium containing 1.5% (w/v) soyabean meal (2,560 U ml^{-1}). However, casein (650 U ml^{-1}), peptone (863.6 U ml^{-1}) and $(NH_4)_2SO_4$ (160 U ml^{-1}) exhibited poor effect on the protease production in *B. subtilis* APP1, as the yields were less than approx. 25.4, 33.7 and 6.25% in comparison with soyabean meal, respectively. Among the various carbon sources tested, the addition of 3% (w/v) wheat flour was observed to be the effective concentration for protease production (1,110.5 U ml^{-1}). In addition to wheat flour, we also examined the effect of other carbon sources on the protease yield. It was observed that significant improvement in protease yield was obtained with supplementation with 0.5% (v/v) liquid maltose (1,025 U ml^{-1}). However, glucose (850 U ml^{-1}) and lactose (510 U ml^{-1}) showed decrease in protease yields, and reduced approx. 17 and 50%, respectively. Following the optimization, the highest yield (1,110.5 U ml^{-1}) was achieved in *B. subtilis* APP1.

Optimal pH and temperature of the enzyme

The growth and enzyme production was studied at pH 5.0–12.0. It was optimum at pH 9.0 (1,810 U ml^{-1}). Increased alkalinity was not favorable up pH 10, as both growth and enzyme production were reduced. Further, the reduction in enzymatic level was more pronounced than that in growth pattern at lower pH (Fig. 2). Temperature is one of the most important factors affecting the enzyme production. The results

Table 2 Characteristics of *Bacillus* sp. strain APP1

Cell morphology Vegetative cells	Motile rods, ends rounded, singular in general, but also occurring in pairs
Presporal forms	Extensive subterminal swelling prior to spore formation
Sporangia	Subterminal swelling
Spores	Ellipsoidal (0.6–0.8 by 1.2–1.4 μm)
Biochemical and cultural	
Growth temperature (°C)	Maximum, 65–70; minimum, 10–15
Gram reaction	Positive
Nutrient agar (pH 7)	Growth
Nutrient agar (pH 7) plus 2% NaCl	Moderate growth
Nutrient agar (buffered at pH 9.5–10.5)	Excellent growth
Nutrient agar (pH 10) plus 7.5% NaCl	Growth
Pigment formation on glucose or tyrosine agar (pH 10)	Negative
Starch hydrolysis (pH 10)	Positive
Gelatin hydrolysis (pH 10)	Strong liquefaction
Casein hydrolysis (pH 10)	Positive
Lipid digestion on olive oil agar (pH 10)	Positive

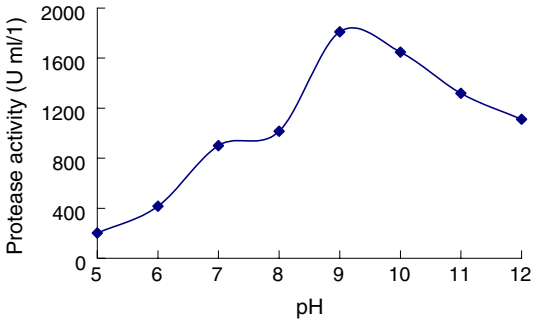

Fig. 2 Production of protease by *Bacillus* sp. APP1 at different pH

referred to a positive relationship between protease production and incubation temperature up to 50°C, while the activity decreased rapidly above 65°C (Fig. 3).

Effect of surfactants and oxidizing agents on the enzyme activity

The protease produced by *Bacillus* sp. APP1 was stable not only toward the non-ionic surfactants like Triton-X-100 and Tween-20 but also toward strong anionic surfactant like SDS. Especially, it showed high stability against SDS and retained approx. 73% of its activity even after treatment with 5% SDS for 72 h. Further, the enzyme exhibited an enhanced activity on treatment for 72 h with 5% hydrogen peroxide (Table 3).

Discussion

Alkaline proteases are generally produced by a wide range of microorganism including bacteria, moulds, yeasts and also mammalian tissues. Among bacteria, *Bacillus* sp. are specific producers of extracellular proteases [19]. *Bacillus*-derived alkaline proteases are the major industrial workhorses and the recent trend

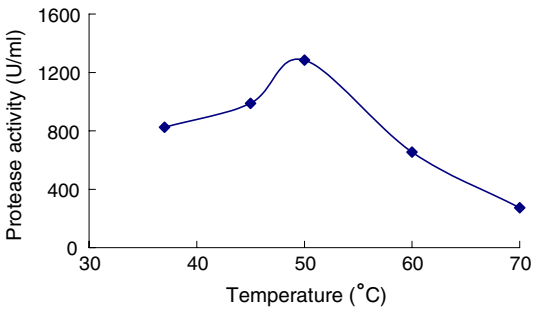

Fig. 3 Production of protease by *Bacillus* sp. APP1 at different temperature

Table 3 Effect of oxidizing agents and surfactants on protease activity from *Bacillus* sp. APP1

Surfactants/oxidizing agents	Concentration (%)	Remaining activity (%)
None	–	100
Triton-X-100	1	102.5
Tween-20	1	113
SDS	1	98.5
	5	73
H_2O_2	1	102
	5	110

The protease was preincubated with oxidizing agents and surfactants for 72 h period at room temperature and the remaining activity was measured using the standard protease assay. Residual activity was determined as percentage of control with no additions

toward the use of alkaline proteases from these sources in different process applications like detergents, tanning, food, waste treatment and peptide synthesis has increased remarkably because of their increased production capacities, high catalytic activity and high degree of substrate specificity [12, 13]. Alkaline proteases are generally produced by submerged fermentation. 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. In addition, no defined medium has been established for the production of proteases from different microbial sources. In this paper, we reported a new strain of *Bacillus* sp. APP1, which produced high levels of an extracellular alkaline protease during growth on alkaline medium.

Production of alkaline proteases is highly dependent on the ratio of carbon and nitrogen sources. Since soybean meal is an inexpensive and readily available substrate [5], it was to be a possible candidate for the cost-effective production of an extracellular protease when used as culture medium ingredient. Results showed that soyabean meal was an effective medium ingredient for the protease production by *Bacillus* sp. APP1 among the nitrogen sources tested. However, the addition of casein and peptone showed no or little effect on the protease production in *Bacillus* sp. APP1. This result was consistent with some other *Bacillus* species [4, 9]. And the addition of $(NH_4)_2SO_4$ also showed no effect on the production of protease of *Bacillus* sp.

APP1. This result was somewhat different from the other organism [15, 21].

Wheat flour and maltose were found most favorable sources for the enzyme production. Though not much favorable, glucose was also constructively utilized for alkaline protease production by *Bacillus* sp. APP1. However, lactose exhibited a negative effect on the protease production. Contrary to this result, Mabrouk et al. [16] reported the enhancement of protease production in *B. licheniformis* ATCC 21415 by the addition of lactose, but a lowered yield was observed with the addition of maltose to the culture medium.

The protease from *Bacillus* sp. APP1 showed stability and compatibility not only toward the non-ionic surfactants like Triton-X-100 and Tween-20 but also toward strong anionic surfactant like SDS. Kobayashi et al. [11] reported that an alkaline protease from *Bacillus* sp. KSM-K16 retained approx. 75% activity on treatment with 5% SDS for 4 h. Earlier reports on the stability of alkaline proteases toward oxidants had indicated that an alkaline protease from *Bacillus* sp. RGR-14 showed 40% loss in enzyme activity with 1% H_2O_2 [18], while a subtilisin-like protease from *Bacillus* sp. KSMKP43 lost little or no enzyme activity on treatment with 10% H_2O_2 for 30 min [20]. Comparing these results, the *Bacillus* sp. APP1 protease exhibited a significant compatibility and stability toward both surfactants and oxidizing agents.

Conclusions

In conclusions, the available evidence suggests that strain APP1 is a novel *Bacillus* sp. which can produce alkaline proteases. The optimum conditions for protease production was when cultivated for 48 h at 50°C with pH 9.0 in a medium containing (g^{-1}): soyabean meal, 15; wheat flour, 3; K_2HPO_4, 4; Na_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.1; Na_2CO_3, 6.

References

1. Beg KB, Sahai V, Gupta R (2003) Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor. Process Biochem 39:2003–2209
2. Bergey DH, Holt JG (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams & Wilkins, Baltimore
3. Ellaiah P, Adinarayana K, Rajyalaxmi P, Srinivasulu B (2003) Optimization of process parameters for alkaline protease production under solid state fermentation by alkalophilic *Bacillus* sp. Asian J Microbiol Biotechnol Environ Sci 5:49–54
4. Fen QP, Shen JM, Gao Y (1994) Study on the selection of high-yield alkaline protease producing strain by UN-treatment of protoplasts 1: research on the selection of original strain and the condition for enzyme production. J Lanzhou Univ (Nat Sci) 30(4):83–87
5. Gattinger LD, Duvnjak Z, Khan AW (1990) The use of canola meal as a substrate for xylanase production by *Trichoderma reesei*. Appl Microbiol Biotechnol 33:21–25
6. Godfrey T, Reichelt J (1985) Industrial enzymology: the application of enzymes in industry. Nature, London
7. Griffin HL, Greeve RV, Cotta MA (1992) Curr Microbiol 24:111–117
8. Gupta R, Beg QK, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15–32
9. Joo HS, Kumar CG, Park GC, Paik SR, Chang CS (2003) Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties. J Appl Microbiol 95:267–272
10. Kalisz HM (1988) Microbial proteinases. Adv Biochem Eng Biotechnol 36:1–65
11. Kobayashi T, Hakamada Y, Adachi S, Hitomi J, Yoshimatsu T, Koike K, Kawai S, Ito S (1995) Purification and properties of an alkaline protease form alkalophilic *Bacillus* sp. KSM-16. Appl Microbiol Biotechnol 43:473–481
12. Kumar CG, Takagi H (1999) Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17:561–594
13. Kumar CG, Malik RK, Tiwari MP (1998) Novel enzyme based detergents: an Indian perspective. Curr Sci 75:1312–1318
14. Kumar CG, Tiwari MP, Jany KD (1999) Novel alkaline serine proteases from alkalophilic *Bacillus* sp.: purification and characterization. Process Biochem 34:441–449
15. Lee W, Young-Jecho, Gyu-Mok S, Choi C (1992) Characteristic and action pattern of alkaline protease produced from *Bacillus* sp. CW-1121. Korean Biochem J 24:537–542
16. Mabrouk SS, Hashem AM, El-Shayeb NMA, Ismail AS, Abdel-Fattah AF (1999) Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415. Bioresour Technol 69:155–159
17. Maugh T (1984) Need a catalyst? Design an enzyme. Science 223:269–271
18. Oberoi R, Beg QK, Puri S, Saxena RK, Gupta R (2001) Characterization and wash performance analysis of an SDS-stable alkaline protease from a *Bacillus* sp. World J Microbiol Biotechnol 17:493–497
19. Priest FG (1977) Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol Rev 41:711–753
20. Saeki K, Hitomi J, Okuda M, Hatada Y, Kageyama Y, Takaiwa M, Kubota H, Hagihara H, Kobayashi T, Kawai S, Ito S (2002) A novel species of alkalophilic *Bacillus* that produces an oxidatively stable alkaline serine protease. Extremophiles 6:65–72
21. Shumi W, Hossain MDT, Anwar MN (2004) Production of protease from *Listeria monocytogenes*. Int J Agric Biol 6:1097–1100