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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR ALKALINE PROTEASE FROM ISOLATED TRICHODERMA VIRIDE.

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ABSTRACT

The production and purification of alkaline protease from local strain of the Egyptian soil fungus *trichoderma viridi* was investigated. The enzyme which hydrolyze casein was produced at high level when fungus was grown for 6 days on a medium supplemented with 1.5% w/v starch as a carbon source, soybean flower (0.14% N_2) as a nitrogen source and initial pH 6.5 at 28°C. A 30.06 fold purified enzyme was obtained by acetone precipitation flowed by gel filtration using sephadix G-100. Protease was found to be highly active at pH 8.5 and 55°C, and stable up to 60 min at pH 7 and 50°C. Mutations Zn^{2+}, Mn^{2+} and Cu^{2+} activated the enzyme, whereas Co^{2+}, Fe^{2+} and k^{+} inhibited the enzyme activity. The activity of enzyme was found to be stable in liquid detergent (locally producer).

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes and are used in different industries like tannery, food industries, pharmaceutical, detergents and processes like waste treatment. The enzymes hydrolyze the peptide bonds in aqueous environments and used in silver recovery (Tunga *et al* 2003, Agrawal *et al* 2004). Pepsin was used in laundry detergents as early as 1913, but recently the use of alkaline proteases have increased (Baliey and Ollis, 1977).

Alkaline proteases are important due to both stability to several conditions such as temperatures of 50°- 60°C, high pH value and the presence of surfactants or oxidizing agents (Saad and Hamed 1997)).

Microorganisms are the most important sources for proteases production such as bacteria, moulds, and yeasts. Various species of *Trichoderma* have been studied in detail for production of alkaline proteases (Manonmani and Joseph, 1993). Other alkaline proteases producing includes *Aspergillus* sp (Nasuno and Ohara, 1972, Malacthi and Charkraborty, 1991) and Streptomyces (Saad, 1995).

The present studies reports the optimization of parameters for the production, purification of an alkaline protease by isolated *Trichoderma virdi* under optimum conditions.

MATERIALS AND METHODS

1- Microorganism:

Trichoderma viride was isolated from the Egyptian soil. The method involved plating 0.1 ml soil suspension on casein medium containing (g/L):

$(NH_4)_2 SO_4$, 1.4; KH_2PO_4, 2.0; $NaH_2 PO_4$, 6.9; $MgSO_4$ 7 H_2O, 0.3; dextrose, 1.0; peptone, 1.0; urea, 0.3 and agar, 20. pH adjusted to 7.3. The colony showing largest zone of protein hydrolysis after 6 days growth at 25°C was isolated and purified. The purified isolate was identified as *Trichoderma* viride by Plant Pathology Department, National Research Center. Egypt. This isolate was maintained on *PDA (*Patato dextrose agar*)*slants and stored at 4°C.

2- Inoculum preparation:

Spores form 6 days old culture was raised an PDA plate and incubated at 25°C. One disk of 4 mm diameter was used as an inoculum.

3- Enzyme production:

One disk (4 mm diameter) of PDA plate was inoculated to sterile 250 ml conical flask containing 50ml of casein medium and incubated at 28°± 2°C for 6 days with continuous shaking at 150 rpm.

4- Effect of some carbon and nitrogen sources on enzyme production:

The purpose of this experiment was to study the influence of sources of carbon and nitrogen on the production of protease enzyme, carbon sources instead of glucose were used as, starch, maltose, galactose, and sucrose, at 2%, and different nitrogen sources were used such as soybean flower, soluble casein, ammonium chloride, ammonium oxalate and ammonium sulfate.

5- Extraction and assay of protease

The contents of each flask containing grown culture mixed with 10 ml NaCl solution (1%) shakin for 1h. This proved to be suitable for maximum recovery (Agrawal *et al*, 2004). The suspension was filtered under vacuum to obtained the crude enzyme extract. Protease activity was assayed according to Hindazlotink *et al* (1983), in a reaction mixture containing: 1 ml of 1% (w/v) casein in 0.05M phosphate buffer (pH 5-8.5), 0.5 ml of culture filtrate. The reaction mixture was incubated in an incubator shaker at 50°C for 20 min. The reaction was terminated by the addition of 1ml trichloroacetic acid (TCA 10%). Complete precipitation of residual protein was obtained by immersing the tubes in an ince bath. The supernatant after centrifugation (6000 rpm, 20 min) was analysed for tyrosine liberated according to Greenberg (1957). A control sample was run containing the reaction mixture with added TCA at zero time.

One unit of proteolytic activity is defined as the amount of enzyme which liberated 1mg tyrosine/min under assay conditions.

The specific proteolytic activity was expressed as number of enzyme units per mg protein which was determined according to the method of lowry *et al* (1951).

6- Buffer's:

The following buffers were used in a 0.1M:

a- Citrate/ phosphate buffer (pH 4.0 to 5.5)

b- Sodium phosphate buffer (pH 6.5 to 7.5)
c- Tris-HCl buffer (pH 8.0 to 9.0)
d- Carbonate/ bicarbonate buffer (pH 9.0 to 10)
These buffers were prepared according to Gomeri method (1959).

7- Enzyme purification:

Purification was carried out at 4°C. The crude enzyme extract precipitated by organic solvent according to a modified technique of kaufman (1971), followed by sephadex G-100 column which had been equilibrated with 0.05M sodium phosphate buffer pH 7.3. The column was eluted with the same buffer until the density of effluent at 280 nm was zero. Fractions (2.ml each) were collected and the fractions with high protease activity were pooled and concentrated by Lyophilization.

8- Optimisation of assay parameters:

8.1- Effect of pH on purified enzyme activity and stability:

Activity of the purified enzyme was measured at different pH values. The pH of the reaction mixture was adjusted by using one of the buffers mentioned above. To carry out the pH stability, 0.5 ml of purified enzyme solution incubated with 1ml of each of the mentioned buffers were for 10, 15, 20, 30, 40, 50 and 60min. at 50°C then add the substrate and the relative activities were measured.

2.8.2- Effect of temperature on enzyme activity and stability:

The activity of purified protease was determined by incubating the reaction action mixture at different temperature from 25° to 70°C. To check the enzyme stability against temperature, the enzyme was incubated at variable degrees of temperatures (30°, 35°, 40° and 70°C) for different times from 10 min to 1hr, then the relative activities were measured.

8.3- Effect of different metalions on the purified enzyme:

Purified alkaline protease was pre-incubated for one hr with different metal ions including Mn^{2+} ($MnSO_4.7H_2O$), Fe^{2+} ($FeSO_4\ 7H_2O$), K^+ (KCL); Co^{2+} ($CoCL_2$); Zn^{+2} ($Zn\ SO_4.7H_2O$) and Cu^{+2} ($CuSO_4.SH_2O$) at a final concentrations of 0.1M at pH 8.5 and temperature 55°C and the remaining activities determined.

8.4- Effect of detergent on the enzyme activity:

To examine the effect of detergrent on purified alkaline protease activity, our suggested formula contained (w/v): Sodium dodecyl benzene sulfonate (SDBS) 8%, sodium tripolyphosphae (STPP), 20% neutral liquid silicate 8%, triethanolamine oleat 5% and water to 100. There materials were obtained from the local market. The test contained two concentrations of the enzyme 1 and 2% in a final concentration. This detergent were kept at room temperature (30°C) for 48 hr and the activities were determined.

RESULTS AND DISCUSSION

1- Optimization of production parameters:

The optimization of enzyme production was initiated at different pH values of the medium. The culture exhibited maximum activity after 6 days at pH 6.5 (sodium phosphate buffer, 0.1M) (Table 1). The influence of the addition of various carbohydrates at 2% on the protease production is shown in Table (2). These data indicated that the starch and glucose were the best carbon sources respectively, while sucrose repressed enzyme synthesis by 87.7%. The most suitable starch concentration on protease production is presented in Table 3. The data shows that 1.5% of starch increased the enzyme production of by 23.5% than 2% of starch.

Table 1: Effect of initial pH of the medium on proteolytic production by *Trichoderma viride*.

Initial pH	Activity u/ml	Initial pH	Activity u/mL
5.0	3000	7.0	9353
5.5	7000	7.5	9000
6.0	9200	8.0	5200
6.5	9400	8.5	4100

Table 2: Effect of carbon sources on proteases production.

Carbon source	Activity u/ml	Carbon source	Activity u/ml
Glucose	8300	Sucrose	1020
Maltose	7100	Glycerol	6000
Galactose	6500	Starch	8500

The concentration of each carbon sources 2%

Table 3: Effect of different concentrations of starch on protease production.

Starch%	Activity u/ml	Starch%	Activity u/ml
0.25	2900	2.00	8500
0.50	5600	2.5	8010
1.00	9400	3.00	7020
1.50	10500		

The effect of different sources of nitrogen (each of them contained 0.07% nitrogen) on the protease production is shown in Table 4. The production of protease was slightly enhanced by most of the nitrogen sources tested with exception of soybean flower and soluble casein are the best nitrogen sources which showed the production of protease up to 10500 and 8300 u/ml respectively, while urea decreased the production to 86 u/ml.

The effect of the different concentrations of (N_2) of soybean flower on protease production are shown in Table 5. The *Trichoderma viridi* strain produced higher proteases activities after 6 days when initial soybean used contained 0.14% nitrogen.

Table 4: Effect of nitrogen sources on protease production.

Nitrogen source	Activity U/ml	Nitrogen source	Activity U/ml
Casien	8300	Amm phosphate mohonhydrate	960
Soybean flower	10500	Sod. Nitrate	770
Amm. Chloride	1206	Pot. Nitrate	475
Amm. Oxalate	1165	Urea	86
Amm. Sulfate	1050		

Casein (0.07%N_2) as a control

Table 5: Effect of concentration of (N_2) of soybean flower on protease production.

N_2%	Activity u/ml
0.07	10500
0.14	13600
0.28	10000
0.56	8350

2- Partial purification of protease enzyme:

The purification sequences and results are summarized in Table 6. The active acetone precipitated fraction 30-60 (v/v) contained the activity was purified by sephadex G-100 gel filtration to obtaine a 7.2 yield, 30.06 purification fold and specific activity 1986 u/mg protein.

Table 6: Purification of alkaline protease.

Purification step	Total protein (mg)	Total activity (u)	Sp. Activity u/mg	%of Recovery	Purification Fold
Crude filtrate	280	18500	66.07	100	1.0
Partial Purification by acetone 90%	64.2	15000	233.6	81.08	3.536
Sephadex G-100	7.2	14300	1986.1	-	30.06

3- Effect of pH values and temperatures on enzyme activity and stability of enzyme:

The optimum pH of enzyme activity was determined to be 8 and 8.5 Table 7. The enzyme activity decreased slowly at acidic pH values, pH 6.5 the activity decreased 2.8% while at pH 5 the activity decreased by 77% of its maximal activity. Tunga *et al* (2003) reported that the maximum activity of Alklaine protease from *Aspergillus parasiticus* at pH 8, and the activity decreased under acidic conditions till 80% of the maximum at pH5. The enzyme was stable at pH 8 for 50 min. at 50 °C, while at pH 10 the activity decreased 30% after 1hr at 50°C (table 8). These results are nearly similar to those obtained by lee et al (1991) and Rhaman et al (1994) on *Bacillus* sp and B. *Stearothermophilus* respectively.

Table 7. Effect of pH on the Activity of the Purified proteolytic enzyme

pH	Sp. Activity u/mg protein	PH	Sp activity u/mg protein
5	322	7.5	1390
5.5	799	8.0	1400
6.0	1185	8.5	1400
6.5	1360	9.0	1390
7.0	1369	-	-

Table 8: The relative activity of different pH at different periods.

	Relative activity %					
Time (min) pH	10	20	30	40	50	60
5	100	100	92	88	72	60
6	100	100	100	100	100	100
7	100	100	100	100	100	100
8	100	100	100	100	100	98
9	100	100	100	95	90	89
10	11	1	93	82	75	70

Temperature optimization studies at pH 8.5, showed that 50°C and 55°C to be optimum temperature (Table 9). The stability against temperature at pH 8.5 (Table 10) showed that the relative activity did not change after 1hr between 30°- 50°C, the relative activity at 70°C after 1hr decreased by 35% from maximal activity. J.K. young *et al* (2000) reported that the optimal temperature for *Bacillus subtilis* protease enzyme was 50°C. They reported also that the protease retained its initial activity from 25°-50°C but was rapidly inactivated at higher temperature and the protease activity completely inactivated at 60°C.

Table 9: Effete of Temperature on the activity of purified proteolytic enzyme.

Temp. °C	Sp activity u/mg protein	Temp. °C	Sp activity u/mg protein
25	320	50	1400
30	997	55	1400
35	1003	60	1390
40	1165	95	1372
45	1375	70	1300

Table 10: Thermal stability of the purified enzyme at different periods.

	Relative activity %					
Time (min) Temp °C	10	20	30	40	50	60
30	100	100	100	100	100	100
35	100	100	100	100	100	100
40	100	100	100	100	100	100
45	100	100	100	100	100	100
50	100	100	100	100	100	/00
55	100	100	100	100	100	98
60	100	100	100	100	98	95
65	100	100	100	100	90	80
70	100	98	93	85	79	65

4- Effect of different cations on the purified enzyme:

The activating or inhibiting effect of metal ions on alkaline protease enzyme were investigated at a final concentration 0.1M Results illustrated in Table 11 show that Zn^{2+}, Mg^{2+} and Cu^{2+} increased, the activity by 64.0, 20.13 and 17.33% respectively. However Co^{2+}, K^{+} and Fe^{2+} had inhibitory effect on enzyme activity by 13.07, 20 and 17.34% against the control. El.Shafei and Rezkallah (1998) reported that Mn^{2+} and Zn^{2+} had inhibitory effects on enzyme activity of alkaline protease from *Bacillus thruingiensis* by 6.6 and 26.6% respectively. Hamed *et al* (1997) proved that Zn^{2+} and Mn^{2+} increased the activity of alkaline protease form *pseudomonas* sp by 339.4% and 119.7% respectively while Fe^{2+}, K^{+} and Co^{2+} at 0.1M decreased the activity. The activation or inhibition of the enzyme activates by metal ions might be due to the effect of metals on the active sites of proteases enzyme.

Table 11 : Effect of metal ion on purified protease activity.

Activators			Inhibitors		
Metal ions	Sp. Activity	Relative activity%	Metal ions	Sp. Activity	Relative activity%
Control	7500	100	Control	7500	100
Zn^{2+}	12300	164	CO^{2+}	6520	86.93
Mn^{2+}	9010	120.13	Fe^{2+}	6200	82.66
Cu^{2+}	8800	117.33	K^{+}	6000	80.0

5- Effect of detergent on the enzyme activity:

The data in Table (12) indicated that the enzyme was stable in the liquid detergent formula. The relative activities when 1% enzyme used was 99% of the activity. When 2% of enzyme was used, the relative activity decreased to 97%. This data are shown similar to those obtained by Tunga *et al* (2003) where they found that the relative activities of protease from solid state culture of *Asp Parasiticies* decreased at the enzyme concentration of 2% than 1%.

Table 12: Effect of detergent on enzyme activity.

Enzyme concentrate	Relative activity %
1%	99
2%	97

REFERENCES

Agrawal, D, Potidar, p; Banerjee, R and patil, S. (2004) Purification of alkaline protease by *Penicillin* sp. Under SSF conditions and its application to say protein hydrolysis. Process Biochemistry 39 (2004) 977-981.

Baliey JE. And Ollis, D.F. (1977). In: Clark BJ, Takay B, Bradley JW. Editors. Biochemical Engineering Fundamentals. New York: McGraw Hill 1977:196.

El. Shafei, H.A. and Rezkallah. L. A. (1998). Production and properties of Alkaline protease by *Bacillus thuringiensis* var alesti. Egypt. J. Microbiol. 33 (4). 541-556.

Gomeri, G. (1959). Preparation of buffers for use in enzyme studies Method Enzymol. 1: 138-146.

Greenberg, D.M. (1957). Phenol color method. Method Enzymol 2:54.

Hamed, A.A.; Saad, M.M. and Saad, M.A. (1997). The influence of certain physicochemical properties on production and purification of alkaline protease from *pseudomonas* sp. Egypt J. Appl. Sci; 12 (2) 369-388 (1997).

Hindazlotink, Y., Hironhi, I.; Shigeki, M. and Shinichi, I. (1983). "Purification and flourometric assay of protenase A from yeast Anal. Biochem., 134 (1): 210.

Kaufman, S. (1971). Fractionation of protein mixture with organic solvents. Metod Enzymol., 21: 233-267.

Lee, W.J; CHO, Y.J. and Sonand. G.M. (1991). Characteristic and actin pattern of alkaline protease produced from *Bacillus* sp CW-1121. Korean Biochem. 24 (6): 537.

Lowery, O.H., Roserbrough, N.J.; Fair, A. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 255-275.

Malacthi, S. and Charkraborty, R. (1991). Production of alkaline protease by a new *Aspergillus flarus* isolated under solid-substrate fermentation conditions for use as a depilation agent" Appl. Environ. Microbiol. 57, pp. 712-716.

Manonmani, H.K and Joseph, R. (1993). Purification and properties of an extracellular proteinase of *Trichoderma koning*" Enzyme Microb. Techniol. 15. pp. 624-628.

Nasuno, S. and Ohara, T. (1972). Purification of alkaline proteinase from *Aspergillus condidus*. Agric. Boil. Chem. 36, pp. 1791-1796.

Rohman R.N.Z.A.; C.N. Ampon, K., Basri, M; Zin, W.W; Yunus; W and Salleh, A.B. (1994). Purification and characterization of heat-stabel alkaline proteafrom *Bacillus stearothermopili*; Applied Microbiol and Biotechnol., 40(60): 822.

Saad, M.M. (1995). Alkaline protease from *Streptomyces Venezuela* DM 4027 Egypt. J. Microbiol, 30 (3) pp. 355-368.

Saad, M.M. and Hamed, H.A. (1997). Purification, Crystallization and some Enzymatic Properties of alkaline protease of *streptomyces venezulae* DSM 4027" Bull. NRC, Egypt. 22, (3). pp 311-321.

Tunga, R; Shrivastave, B and Banerjee, R. (2003). Purification and characterization of protease from solid state cultures of *Aspergillums parasiticus* Process Biochemistry 38 (2003) 1553-1558

Young, J; Shih, I; Tzeng, Y. and Wang. S. (2000). Production and purification of protease from *Bacillus subtilis* that can eproetinize crustacean wastes. Enzyme and Microbial Technotogy. 26: 406-413 (2000).

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إنتاج وتنقية وخواص إنزيم البروتيز القلوى من فطر ترايكودرمافيردى

مجدى على جاد الله
قسم التكنولوجيا الحيوية الميكروبية – المركز القومى للبحوث – القاهرة – مصر

تمت الدراسة على الظروف المناسبة لإنتاج وتنقية إنزيم البروتيز القلوى من فطر الترايكودرما فيردى والمعزولة من التربة المصرية. وجد أن الانزيم المحلل للكازين ينتج بنسبة كبيرة عند نمو الفطر لمدة ستة أيام فى بيئة مزودة بـ ١,٥% (و/ح) من النشا كمصدر للكربون مع دقيق فول الصويا (٠,١٤% نيتروجين) كمصدر للنيتروجين، وكانت درجة حموضة البيئة فى البداية ٦,٥ على درجة حرارة ٢٨°م. وقد تم الحصول على الإنزيم النقى بدرجة ٣٠,٠٦ باستخدام الترسيب بالاسيتون والفصل بواسطة السفادكس (ج-١٠٠). وجد أن الإنزيم النقى ذو نشاط عالى عند درجة حموضة ٨,٥ ودرجة حرارة ٥٠°م، كذلك درجة ثبات حتى ٥٠ دقيقة على درجة الأس الهيدروجينى ٨ ودرجة حرارة ٥٠°م. كذلك وجد أن الإنزيم النقى يزداد نشاطه باستخدام مركبات الزنك والمنجنيز و النحاس بينما مركبات كل من الكوبلت والحديد والبوتاسيوم مركبات تعمل على تثبيط النشاط الإنزيمى بدرجات مختلفة.وقد وجد أن النشاط الإنزيمى ثابت فى حالة استخدام المنظفات السائلة المنتجة محليا.