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Advances in Bioresearch

ORIGINAL ARTICLE

Production, purification and characterization of protease from *Bacillus subtilis*

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ABSTRACT
This study was conducted in the laboratories of Biology Department, faculty of Science, which deal with isolation and purification of protease by Bacillus subtilis which carried out for enhanced production of protease using casein(10%) as the substrate of enzyme , the production was carried out by submerged fermentation , the best conditions were the isolated of protease in synthetic medium , it gave high titer of protease activity , the ammonium sulfate as nitrogen source , incubation period 48 h , the casein as carbon source, incubation temperature 37℃ and pH= 7, the protease was purified using precipitation by ammonium sulphate (60%), dialysis against sucrose and ultra filtration, analyses of the protease for molecular weight was carried out by SDS-PAGE electrophoresis which revealed 32 KDa, the refined protease had a maximum activity at pH = 9 and in temperature 30 °C.
Keywords: *Protease, Bacillus subtilis, Purification*

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INTRODUCTION

B. subtilis is the best studied model organism of the Gram-positive lineage , it is naturally transformable and has an extremely powerful genetic toolbox , it is fast growing and easy to cultivate and produces several commercially important products, most notably proteases and amylases [1]. hydrolytic enzymes are ubiquitous in nature and have been found in all living forms encompassing the eukaryotes like plants, animals, fungi, protists as well as the prokaryotic domains of bacteria and archaea. Even several viruses are also known to encode their own proteases [2].The microbial proteases came into the focus and quickly became quite popular owing to several advantages associated with them , microorganisms as source of proteases, offer several inherent advantages like rapid production rates, lower investment in terms of land and time requirements, not being subjected to climatic influences [3].

MATERIAL AND METHODS

Bacterial isolates

Bacterial isolates (B.s1, B.s2 and B.s3) were obtained from the Al-Amin center in Najaf city , and bacterial isolates identification were based on vitek-2 system .

B.subtilis inoculum Preparation :

It was ready according to [4].

Table 1: Compositions of growth (synthetic) medium of *B.subtilis*

S/N	Ingredients Quantity	(g/L)
1	Lactose	10 g
2	Casein	10 g
3	KH2 PO4	2 g
4	K2 HPO4	2 g
5	MgSO4 .7H2 O	1 g

Protease isolation

The selected strains of isolates were transmitted casein broth medium and incubated for 48 h on a rotary shaker (200 rpm) at room temperature [4]. After incubation, the culture media were centrifuged at 5000 rpm for 20 min. at 4° C and the cell free supernatants were collected and used as the crude enzyme for protease assay.

Protease assay

Protease activity was assayed according to [5]. The enzyme unit is defined as the amount of enzyme capable of changing the optical absorbance by 0.001 at a wavelength of 280 nm of the reaction solution per minute under reaction conditions, and enzyme activity was estimated by applying the following equation:

$$\text{Enzyme activity} = \frac{\text{Absorption at a wavelength of 280 nm}}{\text{Volume of the enzyme solution} \times \text{reaction time} \times 0.001}$$

Where :

Reaction time: 20 minutes

Volume of the enzymatic solution: 0.2 ml

Amount of increase in absorption per enzymatic unit: 0.001

Protein determination

Protein content was calculated according to [6].

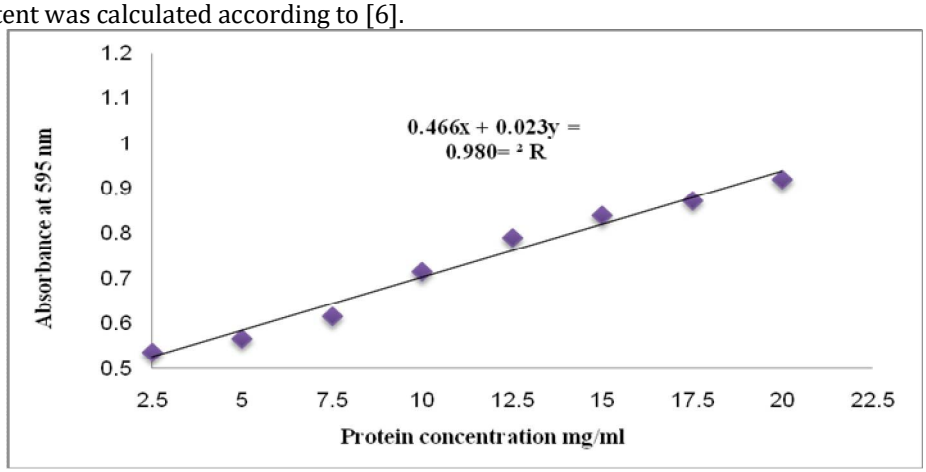


Figure 1: Standard curve for the various concentration of bovine serum albumin (BSA) at 595 nm

Enzyme purification

Ammonium sulfate fractionation

The ammonium sulfate was added in different saturation ratio (20,40,60 and 80%) to reach the optimum ratio of ammonium sulfate by adding gradually the amount of salt to each 70 ml of the crude protease enzyme in ice bath and magnetic stirrer, centrifuge the solution for 25min at 6000 rpm. Dropped the supernatant and take the precipitate and dissolved it in 5 ml distilled water and both protein content and the activity of enzyme were determined for each separate fraction [7].

Dialysis against sucrose

The gained precipitation (in solution) was introduced into dialysis bag with a size of (14.3mm) and the obtained protease enzyme preparation was saved in the refrigerator at 4ºC for additional purification[8].

Ultrafiltration of Protease

As modified step to protein purification Concentrator with molecular weight cut-off (10 KDa) [9].

Molecular weight Determination of protease

The molecular weight of purified protease was determined by SDS- PAGE according to [9].

Characterization of amylase

Optimal pH for enzyme activity

The casein (substrate) was prepared with dissimilar pH ranges (4, 5,6, 7 ,8 , 9,10).

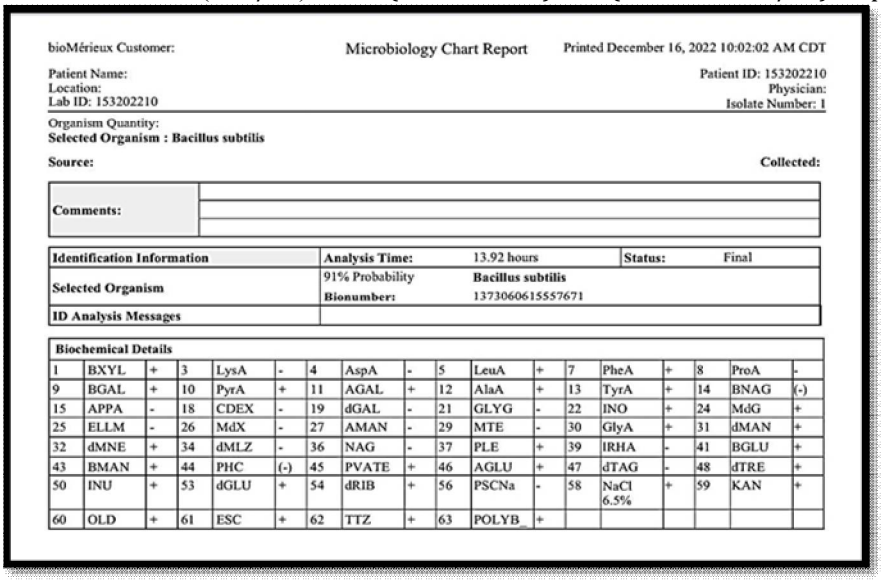
Optimal temperature for enzyme activity

The casein (substrate) was prepared in tubes ,the tubes were incubated in dissimilar temperatures (10, 20,30 ,40 , 50) °C for 24h .

RESULTS AND DISCUSSION

Identification of bacterial isolates

The results of the vitek-2 system showed that the probability of (B.s1, B.s2, B.s3) (95,98,91)% respectively is *Bacillus subtilis* figure (2).The enzymatic activity of the isolates was calculated, B.s3 was the most efficient with a rate of (73U/ml), while (B.s1and B.s2) was (65 and 59,5 U/ml) respectively .



bioMérieux Customer: Microbiology Chart Report Printed December 16, 2022 10:02:02 AM CDT

Patient Name: Patient ID: 153202210
Location: Physician:
Lab ID: 153202210 Isolate Number: 1

Organism Quantity:
Selected Organism : Bacillus subtilis

Source: Collected:

Comments:	

Identification Information	Analysis Time:	13.92 hours	Status:	Final
Selected Organism	91% Probability	Bacillus subtilis		
	Bionumber:	1373060615557671		
ID Analysis Messages				

Biochemical Details

1	BXYL	+	3	LysA	-	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	+	10	PyrA	+	11	AGAL	+	12	AlaA	+	13	TyrA	+	14	BNAG	(-)
15	APPA	-	18	CDEX	-	19	dGAL	-	21	GLYG	-	22	INO	+	24	MdG	+
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	-	30	GlyA	+	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	-	37	PLE	+	39	IRHA	-	41	BGLU	+
43	BMAN	+	44	PHC	(-)	45	PVATE	+	46	AGLU	+	47	dTAG	-	48	dTRE	+
50	INU	+	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_	+						

Figure 2: Vitek-2 system diagnosis of *Bacillus subtilis*

Protease purification

Precipitation with ammonium sulfate

The ammonium sulfate used in different saturation ratios (20 ,40 ,60 , and 80)% , then the 60% ratio was chosen as finest ratio for precipitate the crude extract of enzyme , when the specific activity reached to (1.910 U/mg) , with a purification fold (1.209) and yield (0.757) as shown in table (2) , while the additional saturation ratios (20 ,40 ,80) gave low down titer of specific activity (0.425, 0.832, 1.320 U/mg) respectively. The saturation rate is (60%), which is the best, and this percentage was used by a number of researchers in purifying the enzyme from the bacterium *B.subtilis*, which was used to precipitate the enzyme from the filter of the culture of this bacterium. The ammonium sulfate was used in precipitation of the enzyme because it elevated soluble and inexpensive compared with the further salts, unchanged in pH and enzyme stability, the concentration by ammonium sulfate depending on equilibrate the charges found in protein surface and disrupt of the water layer surrounding it, that leads to precipitate it, this research was agreed with [10] when they purified protease from *B.subtilis* used ammonium sulfate with 60 % saturation ratio.

Table 2: The protease purification steps from *B.subtilis*

Purification steps	Volume (ml)	Activity (U/ml)	Total activity (U)	Protein con. (mg/ml)	Specific activity (U/mg)	Fold	Yield %
Crude enzyme	1000	73	73000	46.220	1.579	1	100
Ammonium sulfate precipitation (60)%	700	79	55300	41.347	1.910	1.209	0.757
Dialysis	30	179.25	5377.5	32.449	5.524	3.497	0.073
Ultrafiltration	8	97.25	778	11.771	8.261	5.230	0.010

Dialysis against sucrose

The acquired ammonium sulfate precipitate was introduced into dialysis bag against sucrose overnight in a refrigerated room, then the specific activity reached to (5.524U/mg) with a purification fold (3.497) and yield (0.073) as shown in table (2) .The additional results of [11] they purified protease from *B. subtilis* used dialysis bag against sucrose, the specific activity was (4196.4 U/mg) with purification fold (5.18).

Ultrafiltration of Protease

The enzyme solution produced from dialysis was placed in a concentrator tube with molecular weight cut-off (10 KDa).Supernatant was collected and calculated at 280 nm absorbency. the specific activity

reached (8.261U/mg) with purification fold (5.230), shown in table (2) .The other results recorded by [12] when they get specific activity (11.5 U/mg) with purification fold (2.74) when they purified protease from *B. subtilis* by using ultrafiltration method .

Determination of molecular weight

In order to examine the purity of the protease, which was purified from *B.subtilis*, polyacrylamide gel electrophoresis under denaturing and with concentration 12.5 %? Electropherogram of Protease revealed a single band, which corresponded to the molecular mass of approximately 32kD as shown in figure (3).According to this analysis result, this result agree withwith the researchers' study[10] and [13] using electrophoresis that the molecular weight of the alkaline protease enzyme is between (16-32 KDa) from *Bacillus sp.*

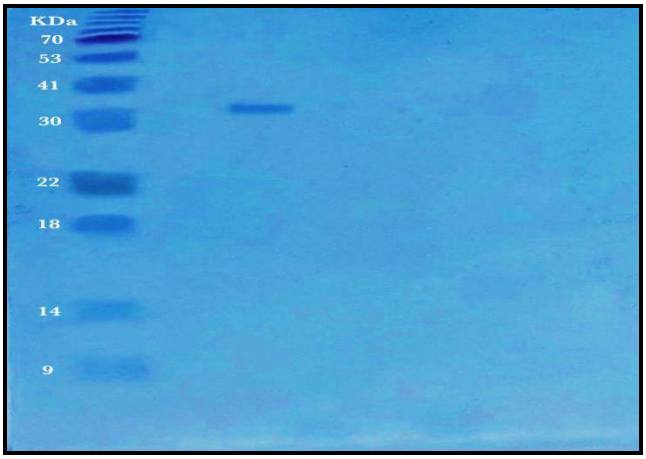


Figure 3: The polyacrylamide gel electrophoresis of the protease from *B.subtilis* under denaturing conditions. Lane (1) standard molecular weight markers, lane(2) purified protease produced from the ultrafiltration.

Protease characterization

The finest pH for protease activity

The figure (4) shown the rising the activity of protease purified from *B.subtilis*with rising the pH until reach to greatest activity (65.5U/ml) in pH = 9 then it began to decreased in higher pH values (52.5U/ml) in pH= 10 and this agree with [14]. The change in the pH of the reaction mixture results in the change in the ionic nature of the amino and carboxylic acid components of the enzyme;this in turn affects both the conformational status of the enzyme and catalytic site thus altering its activity [15].

The finest temperature for protease activity

To establish the best temperature of protease activity purified from *B.subtilis*,the enzyme reaction was done in dissimilar range of temperature (10 - 50) °C , and the results shown in figure (5) rising the activity of enzyme with increasing the temperature until reached to highest activity of protease (50.75U/ml) in 30°C then it began to decreased in elevated temperature values (46.25,43 U/ml) in 40 ,50°C respectively . Previous also protease purified has been described for the influence of temperature by (11).

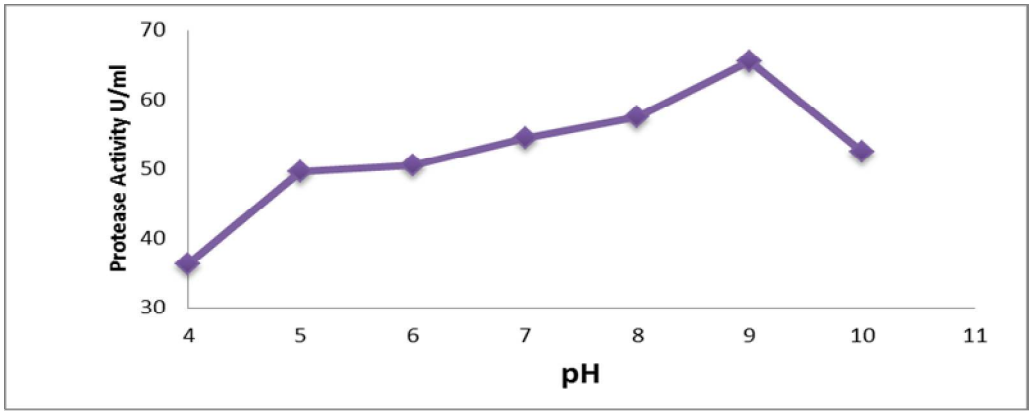


Figure 4: Effect of diverse pH on the activity of purified protease from *B. subtilis*

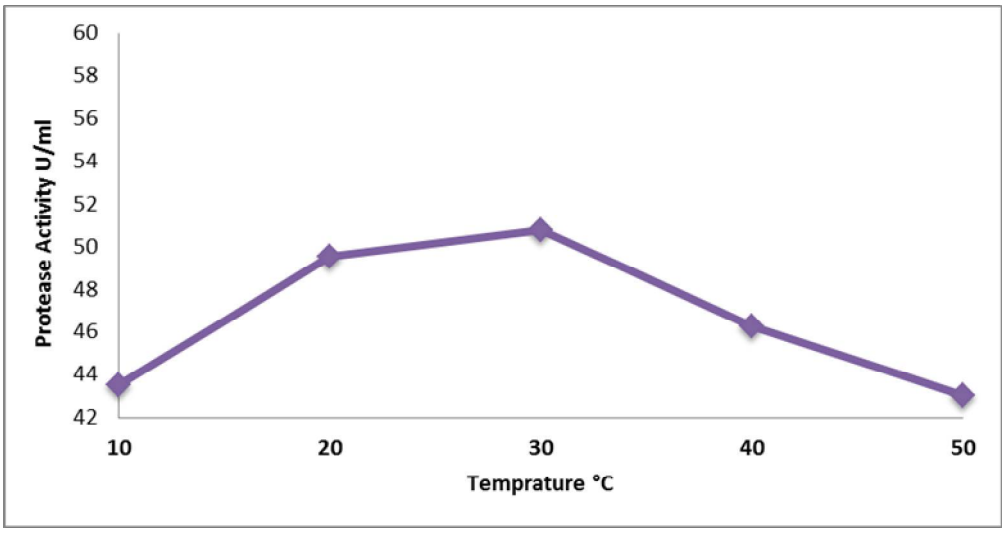


Figure 5: Effect of diverse temperature on the activity of purified protease from *B.subtilis*

REFERENCES

1. Jeffery Erringtonand Lizah T van der Aart . (2020). Microbe Profile: ***Bacillus subtilis***: model organism for cellular development, and industrial workhorse. Errigton and Aart, Microbiology.166:425-427.
2. Bernardo R, Hongying S, Fabio P, Antonio GJ (2018) Plant viral proteases: beyond the role of peptide cutters. Front Plant Sci 9:666. https://doi.org/10.3389/fpls.2018.00666.
3. Preeti Solanki, Chayanika Putatunda, Anil Kumar, Ravi Bhatia and Abhishek Walia .(2021). Microbial proteases: ubiquitous enzymes with innumerable uses. 3 Biotech 11:428. https://doi.org/10.1007/s13205-021-02928-z
4. Saraswathy N, Yeole GS, Parikh AJ, Meena C. Production and optimization of protease from Bacillus licheniformis NCIM 2044. Asian J Pharma Life Sci, 2013; 3:9-15.
5. Senior, B.W.(1999). Investigation of the types and characteristics of the proteolytic enzymes formed by diverse strain of *Proteus* sp. J. Med. Microbiol. 48:623-628.
6. Bradford, M.; (1976), a rapid and sensitive method for the determination of microgram amounts of protein using the principle of protein dye binding. Analytical Biochemistry: 72, 248-254.
7. Dennison C. (2002). A guide to protein isolation. Kluwer Academic Publishers. New York .
8. Adeola ,S. , Adesegun, Folorunso O. , Samuel, Okedeyi O., Olumuyiwa, Ogungbe B Folasade and Gbeliho O. Grace . (2013) . Partial Purification and Characterization of Extracellular Protease of Proteus vulgaris and its Inhibition by the Volatile Oil of *Syzygium samarangense* . Asian Journal of Biomedical and Pharmaceutical Sciences; 3(21) , 30-35.
9. Bollag, D.;Rozycki , M. D. and Edelstein, S. J.(1996). Protein Methods . 2nd edition. Wiley-Liss . New York.107-129.
10. Adinarayana, K. ;Ellaiah, P. and Prasad,D.S. (2003).Purification and Pratial Characterization of Thermostable Serine Alkaline protease from a newly isolated *Bacillus subtilis* PE11.AAPS.pharm.sc.Tech.;4(4)Article 56:530.
11. E. M. El-Safey and U. M. Abdul-Raouf .(2004). Production, Purification And Charactrization Of Protease Enzyme From Bacillus Subtilis. International Conferences For Development And The Environment In The Arab World, Assiut Univ., March 23-25. p 14.
12. K. Shanti Naidu.(2011). Characterization and Purification of Protease enzyme . Journal of Applied Pharmaceutical Science 01 (03); 107-112.
13. Kaur, M.; Dhillon, S. ; Chaudhary, K. and Singh, R. (1998). Production, purification and characterization of a thermostable alkaline protease from *Bacillus polymyxa*. Indian J. Microbiol., 38: 63-67.
14. Si, J.-B., Jang, E.-J., Charalampopoulos, D. and Wee, Y.-J. (2018). Purification and characterization of microbial protease produced extracellularly from Bacillus subtilis FBL-1. Biotechnology and Bioprocess Engineering, 23 (2). pp. 176- 182. ISSN 1226-8372 .
15. Prakash B, Vidyasagar M, Madhukumar MS, Muralikrishna G, Sreeramulu K. (2009) .Production, purification and characterization of two extremely halotolerant, thermostable, and alkali-stable α amylases from Chromohalobacter sp. TVSP 101. Process Biochem. 44:210–215.