PRODUCTION AND PARTIAL PURIFICATION OF ALKALINE PROTEASE FROM *BACILLUS CIRCULANS* MTCC 7906 USING POTATO PEEL AS SUBSTRATE

G. S Kocher[1,*], Nishu Joshi[2]

[1]Microbiologist, [2]Ph.D. Scholar, Department of Microbiology
Punjab Agricultural University, Ludhiana- 141001

***Corresponding Author:**
E-mail: gskocher@pau.edu

ABSTRACT:

Background: *The present study was carried out to use potato peel which is available in plenty from food processing industries as a substrate for alkaline protease production using B. circulans MTCC 7906.*
Method: *The potential of potato peel as substrate for alkaline protease has been studied for its production and purification was studied by Bacillus circulansMTCC 7906. In order to make the process economically viable the casein was replaced by Potato peel. The results were analyzed using factorial CRD.*
Results: *Alkaline protease activity was increased by2.74fold and was obtained in 96 h of fermentation. Ammonium sulphate precipitation followed dialysis of the fraction 60-90% saturation with maximum specific activity was partially purified using DEAE column chromatography. Purification profile was studied and the fraction with highest specific and enzyme activity were pooled to achieve specific activity of 18.761 U/mg with 6.185 fold purification. The concentration of enzyme was done using PEG had the specific activity of 69.332 U/mg and 22.859 fold purification.*
Conclusion: *The concentrated partially purified enzyme was subjected to SDS PAGE followed by silver staining for both the substrates casein and potato peel showed the protein band for alkaline protease at 40 kDa revealing the purity of the enzyme. Experiments revealed successful utilization of potato peel as an economical substrate for enhanced production of alkaline protease by B. circulans MTCC 7906. Projects which are inclined to reduce global wastage should be encouraged and this is one of the same type.*

Key words: *Alkaline protease, Bacillus circulans, potato peel, purification, production.*

BACKGROUND

India is the second largest producer of fruits and vegetables in the world as it produced 74.877 and 146.554 million metric tonnes fruit and vegetables during 2010-11[1]. Of this, 40% of fresh food is going waste in India[2] which may be mobilized for utilization and byproducts production. In case of potato production alone, India ranked second in world with 45 million tonnes of production in 2012 contributing 12.2 % of the world production [3]. However, about 40% of potatoes are wasted every year, amounting to 10 tonnes per day of residue [4]. Such a huge wastage calls for a look into its alternate uses one of which is production of industrially important enzymes like proteases which are important class of enzymes, constituting>65% of the total industrial enzyme market. Proteases find various industrial applications, such as laundry detergents, leather preparation, protein recovery and meat tenderization [5]. Thus, Potato peel utilization for producing alkaline protease cannot only cut huge amount of global waste, it may reduce the production cost of alkaline proteasesince production cost for the enzyme production medium is 30-40% of the total production cost [6]. The present study was thus carried out to use potato peel (which is available in plenty from food processing industries) as a substrate for alkaline protease production using *B. circulans* MTCC 7906.

METHODS

The microorganism employed in the present study was *Bacillus circulans*MTCC 7906 which is our own isolate from vegetable waste [7]. For inoculum preparation, the cells were grown on Reese medium consisting (gl^{-1}) of Glucose-10.0, Casein-5.0, Yeast Extract-5.0, K_2HPO_4-1.0, $MgSO_4$-0.2 and Na_2CO_3-10.0 and pH was adjusted to 9.5 . A loopful of *Bacillus circulans* MTCC 7906 was inoculated to Reese medium. The

cells were allowed to grow under shaking conditions for 48 h so as to achieve an optical density of more than 1.5 at 580nm. The production of alkaline protease by *Bacillus circulans* MTCC 7906 was studied after replacing casein as substrate from the medium that was statistically optimized using Plackett Burman and subsequently Central composite designs and it was composed of Na_2CO_3 2g, Casein 1.5g, MgSO4 0.05 g, K_2HPO4 0.3 g, yeast extract 1.5g, glucose 3g and agitation 200 rpm per 300ml (unpublished data). Potato peels were analyzed for nitrogen content (2.49% of nitrogen and 14. 7 % of crude protein, [8]) and was accordingly supplemented into the medium to replace casein. Fermentation was carried out in 600 ml glass bottles at scale of 300ml working volume. The alkaline protease production was studied in two different media, one with casein as control and other with potato peel.

The supernatant from the periodic samples was used as crude alkaline protease. The protease activity of these samples was determined in a reaction mixture consisting of 20µl of enzyme, 2 ml of 0.5% casein (in carbonate-bicarbonate buffer, 0.1 M, pH 9.5) and 980µl of distilled water that was incubated at 60°C for 15 minutes. The reaction was stopped thereafter by adding 3 ml of 5% trichloroacetic acid (TCA) and free amino acids released by crude protease from casein hydrolysis were estimated by Lowry method [9]. One unit of enzyme was defined as its amount required to release 1µmole of tyrosine per ml per minute.

Partial Purification of alkaline protease

The crude enzyme preparation produced under optimized conditions was subjected to precipitation at 0-30%, 30-60% and 60-90% ammonium sulphate saturations[10]. The precipitates were then dissolved in minimum volume of 0.1 M Tris-HCl buffer (pH 9.5) and dialyzed against excess of same buffer for 48 h at 4°C. The activity of alkaline protease was estimated in the protein dialysate and was further purified by DEAE-cellulose column chromatography. For preparing DEAE – cellulose column, excess of 0.5 N HCl was added to about 30 g of DEAE- cellulose, mixed thoroughly, kept for 30 min and supernatant was removed slowly. This procedure was repeated five times followed by washings with distilled water for 3 times. Then sufficient amount of 0.5 N NaOH was added to the above treated DEAE-cellulose in the same way five times and washed with distilled water so that maximum DEAE-cellulose had settled down and its colour became white from creamish. Thereafter, a column of height 49 cm and diameter of 3.0 cm was packed with DEAE cellulose and equilibrated with 0.1M Tris-HCl buffer (pH 9.5) to a length of about 35 cm. The SDS-PAGE of partially purified alkaline protease was performed for the samples after ammonium sulphate precipitation, purification and concentration using analytical gel (12%) and resolving gel (5%). The protein bands were analyzed using silver staining [11]. The SDS page was performed for samples obtained after ammonium sulphate precipitation, purification and concentration.

RESULT AND DISCUSSION

The results analyzed using Factorial CRD are presented in Table 1 which revealed that the use of potato peel as substrate led to a considerable increase in enzyme activity to 10.513 µmol/ml/min. In comparison, control (Casein) produced an enzyme activity of 3.833 µmol/ml/min in 96 h of fermentation period thus leading to 2.74 fold increase in enzyme activity. In most of the reports on alkaline protease production by *Bacillus* sp., an incubation period of 18 to 96 hours has been observed for maximum enzyme production with an alkaline protease activity of 2560 U/ml in *Bacillus* sp., 1070.86 U/ml in *Bacillus* sp. MIG, 589.20 U/ml in *B. circulans* and 0.9×10^4U/ml in *B. alcalophilus* TCCC11004 [12, 13, 7, 14]. The literature also reveals that the use of potato filtrate supported the highest growth and maximum levels of amylase, neutral and alkaline proteases, and polygalacturonate-lyase in comparison to orange filtrate as substrate [8]. The use of potato peel in solid state fermentation for production of cellulolytic enzymes has also been reported elsewhere [4].

Table 1 Extracellular production of alkaline protease by *Bacillus circulans* MTCC 7906

Time (Days)	Control (casein)	Potato Peel Broth
1	0.352	2.568
2	0.554	6.566
3	1.423	8.827
4	3.833	10.513
5	2.627	9.099
CD (5%)	Fermentation Time 0.0654 Treatments 0.0456 Interaction 0.0845	

Partial purification studies on alkaline protease

B. circulans MTCC 7906 grown in 300 ml of enzyme production medium (Modified Reese medium with potato peel) and incubated at 200 rpm produced protease activity of 10.239 µmol/ml/min in 96 h with a corresponding protein of 3.374 mg/ml. Hence a total of 3071.7 U of alkaline protease with a total protein of 1012.5 mg and a specific activity of 3.033 U/mg was available for purification (Table 2).

The extracellular alkaline protease (300ml) was initially subjected to 0-30% ammonium sulphate precipitation for overnight, spun, re-suspended in Tris buffer (pH 9.5) and dialyzed. A purification of 0.624 folds was achieved with a specific activity of 1.895 U/mg of protein (Table 2). The process when repeated by increasing ammonium sulphate concentration to 30-60% and 60-90% sequentially, revealed fold purification level of 0.898 and 2.043 folds, respectively (Table 2). This showed that maximum purification was achieved in 60 – 90 % ammonium sulphate concentration. Hence, this fraction was subjected to DEAE cellulose anion exchange chromatography for further purification.

Table 2: Purification Profile of Alkaline protease produced by *B. circulans* MTCC 7906

Purification Step	Volume (ml)	Enzyme Units (µmole/ml)	Total Enzyme Units (µmol/ml)	Protein (mg/ml)	Total protein (mg)	Specific Activity (µmol/mg)	Fold Purification
Crude enzyme extract	300	10.239	3071.7	3.374	1012.5	3.033	1
Ammonium Sulphate Saturation (%)							
0-30	2.0	4.264	8.528	2.250	4.50	1.895	0.624
30-60	1.5	7.495	11.242	2.750	4.125	2.725	0.898
60-90	10.0	12.653	126.53	2.0414	20.413	6.198	2.043
DEAE-Cellulose anion exchange column chromatography	25.0	5.190	129.75	0.276	6.915	18.761	6.185
PEG (Mol. Wt. 8000) Concentrated Enzyme	1.5	11.838	17.756	0.256	3.921	69.332	22.859

Different workers have reported variable specific activities and fold purification of purified alkaline proteases. In most of the reports on ammonium sulphate fractionation of enzyme solution, maximum yield of precipitated alkaline protease has been observed between 60-90% [15,16,17,18]. Whereas Rao *et al*[19] observed a2.2 fold increase of specific activity (1679 U/mg) with 60% ammonium sulphate, Ahmed *et al* [20] precipitated the crude enzyme at 70% saturation with specific activity of 55.71 U/mg and 1.11 fold purification.

DEAE-cellulose anion exchange chromatography

The dialyzed protein fraction recovered with ammonium sulphate saturation between 60-90% was loaded in DEAE cellulose column. A total of 32fractions of 5mleach were collected at a flow rate of 1 ml per minute and analyzed for alkaline protease activity and proteins. Elution patterns of proteins along with enzyme activity showed a single peak (Figure1a). A comparison of specific protease activity with NaCl concentration gradient also revealed a single sharp peak between 0.6-0.8 M NaCl concentrations (Figure1b), thus revealing the purity obtained with column chromatography. Hence, the protein fractions corresponding to the peak (fractions 16 to 24) were pooled that had a combined specific activity of 18.761 U/mg with 6.185 fold purification (Table 2). After DEAE column chromatography, the pooled fractions were subjected to concentration using PEG molecular weight 8000 that led to increase in specific activity to 69.332 U/mg with 22.859 fold purification. These findings are in accordance with several earlier reports showing 40.38 fold purification with specific activity of 34171.46 U/mg of protein by using Gel-filtration [21]. In another report, 50 fold purification was reported with specific activity of 143550 APU/mg in *Bacillus* sp. 2-5 [22]. Rao *et al* [19] observed 11.9% fold purification using Sephadex G-100 with 9000 U/mg specific activity. Elsewhere, purification of alkaline protease from *B. alcalophilus* TCCC11004 to 4.8 and 6.8 fold using Sephadex G - 75 and SP - Sepharose HP, respectively and 1.49 fold purification with 74.66 U/mg specific activity in *B .subtilis* have been reported[14, 20].

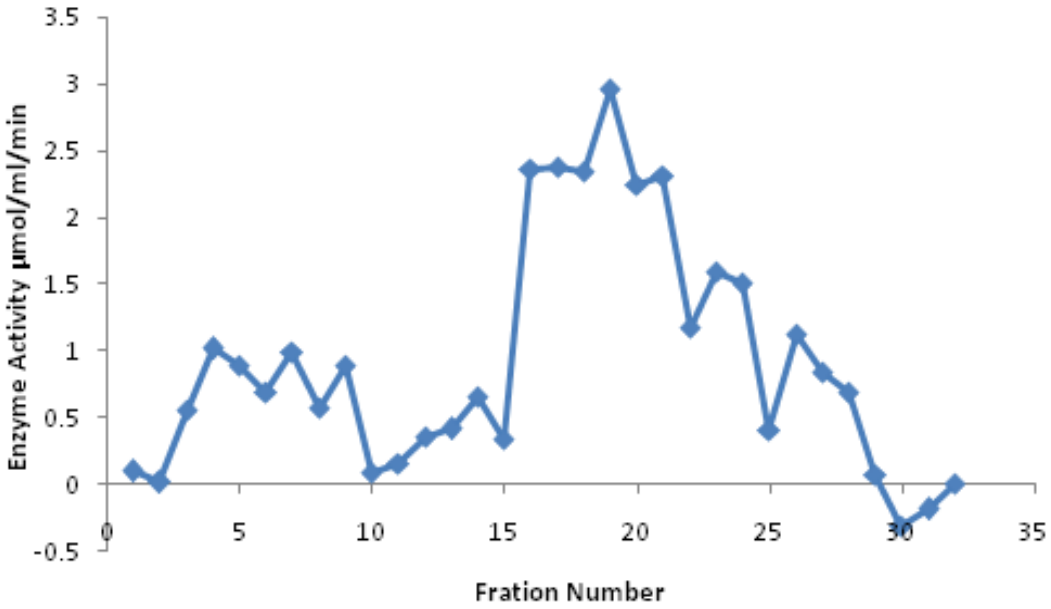

Figure: 1(a)

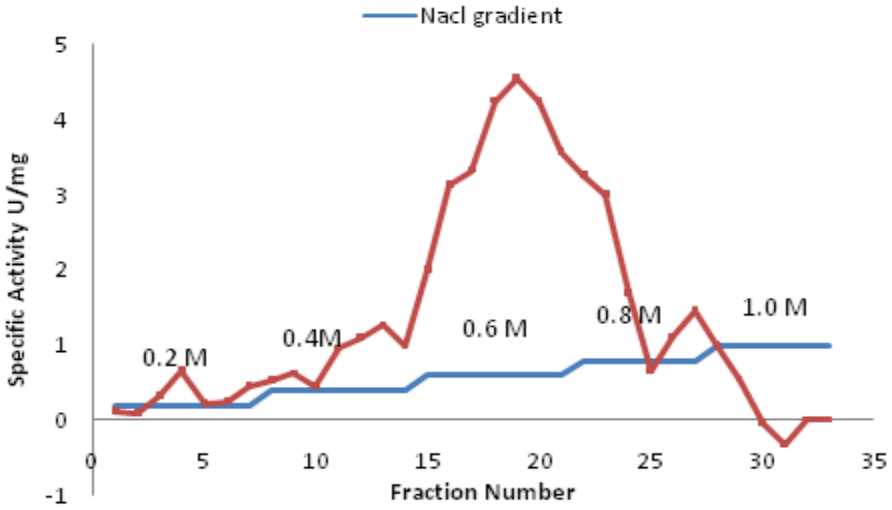

Figure: 1(b)

Figure1: Elution Profile **a)** Enzyme activity **b)** Specific activity and NaCl concentration (0.2-1.0 M) of the precipitate obtained at 60-90% saturation during ammonium sulphate fractionation, in DEAE cellulose anion exchange chromatography

SDS-PAGE of alkaline protease

The partially purified alkaline protease (pooled 16-24 fractions) resolved on a SDS-PAGE (5% stacking and 12% running gel) was found to be a homogenous monomeric protein as evident by a single band corresponding to 40kDa on SDS-PAGE relative to the standard molecular weight marker of 20-60 kDa (Fig. 2). In literature, the alkaline protease from *Bacillus* sp. is reported as a monomeric band with a molecular weight ranging from 15 to 35 kDa[23, 24, 25] with few reports of higher molecular weights of 42 kDa from *Bacillus* sp. PS719 [26] and a very high (90 kDa) from *B. subtilis*[27]. Gimenez *et al* [28] and Studdert *et al* [29] also reported halophilic alkaline proteases with molecular weight in range from 40 to 130 kDa. Hence, a wide variation in the molecular weight of alkaline protease from different *Bacillus* sp. has been observed.

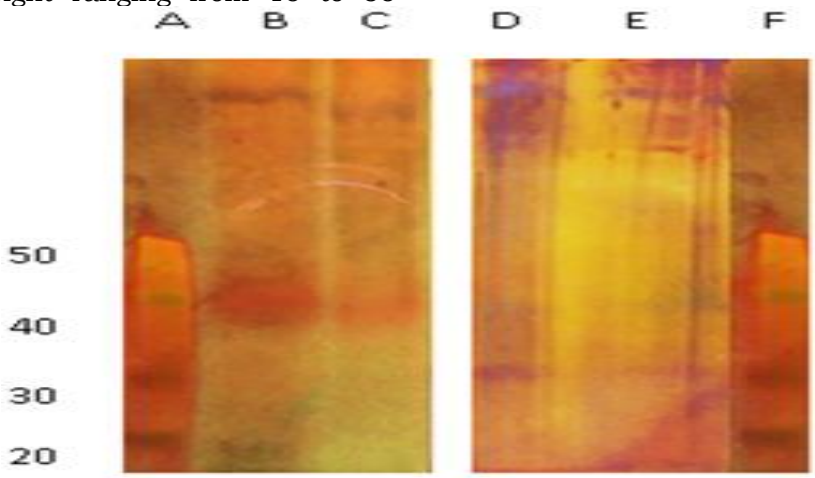

Figure 2: Enzyme profile of alkaline protease of *Bacillus circulans*MTCC 7906: lane A & F: the molecular marker (20-60 kDa), B & C lane: partially purified enzyme of alkaline protease on substrates Casein and potato peel, respectively. Lane D & E: crude alkaline protease on substrates casein and potato peel respectively.

CONCLUSION

Hence**,** our experiments revealed successful utilization of potato peel as an economical substrate for enhanced production of alkaline protease by *B. circulans* MTCC 7906 and also for its partial purification as well. Further studies on scale up of the enzyme production and its immobilization are in the progress in our laboratory so that commercial level technology may be developed.

Competing Interests: There are no competing interests.

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