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Alkaline Protease Production by *Bacillus* sp. using Fortified Crude Dairy Effluent

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ABSTRACT: Proteases are widely used in detergent manufacturing, leather processing units, food processing sectors, meat tenderization, and proteinaceous waste treatment. Several attempts have been made consistently to utilize low-cost or waste as a substrate for microbial protease production. Obediently, the purpose of the present research work was focused on the characterization of protease production efficacy of *Bacillus* strains isolated from dairy and bakery waste. A total of 23 bacterial strains were isolated from the waste of local dairy and bakery shops of Bilaspur city situated at 22.09°N 82.15°E geographical location. Among them, the *Bacillus subtilis* PPB3 exhibited maximum protease activity of 42.47 ±0.42 U mL^{-1}. Later, *Bacillus subtilis* PPB3 was optimized in fortified dairy effluent and achieved maximum protease activity of 54.41 ±0.37 U mL^{-1} (relative protease activity - 128.11 ±0.39 %) at pH-8.0, temperature 40°C, and 120 rpm after 72 h of the incubation period. The V_{max} and K_m of PPB-3 (*Bacillus subtilis*-3) derived protease were calculated 66.66 U mg^{-1} protein and 1.73 mg casein mL^{-1} respectively. The optimum utilization of dairy waste effluent (DWE) offers a cost-effective substrate for value-added product synthesis like protease and at the same time could be able to reduce the Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of DWE before discharging it to either for waste effluent treatment unit or other in-house utilities. However, a specialized automatic system needs to be incorporated for the synthesis of proteases from waste dairy effluent using bacteria.

Keywords: Proteases, *Bacillus subtilis*, protease activity, enzyme kinetics, dairy waste effluent.

INTRODUCTION

Proteases i.e., serine endopeptidases (EC. 3.4.21), cysteine endopeptidases (EC. 3.4.22), aspartic endopeptidases (EC. 3.4.23) and metalloendopeptidases are mostly executing the hydrolysis of protein or peptide. Proteases are naturally produced by plants, animals, fungi, and bacteria (Rao *et al*., 1998). The proteases have noteworthy applications in the food industry (e.g., protein hydrolysate, dairy, meat tenderization, etc.), leather dehairing, silk degumming, synthesis of peptide derivates such as nano-supramolecules, detergent, and proteinaceous waste degradation (Razzaq *et al*., 2019; Gupta *et al*, 2002). The extensive demand for proteases is consistently increasing due to their wide range of applications in the industrial sector. Thereby, budget-friendly protease production technologies need to be established. As the commercial supply of plant and animal-mediated proteases is limited due to its expensive production cost and limited scale-up opportunities, requires a large space for cultivation and is associated with ethical considerations (Jisha *et al*., 2013). The microbial fermentation process is often easily doable, efficient, fast, and cost-effective, proffers tremendous genetic modification prospects, and is able to utilize a variety of substrates for enzyme production (Bajaj *et al*., 2013). Hence, microbial communities are generally preferred for the bulk production of proteases by fermentation (Gurumallesh *et al*., 2019). The *Bacillus* genera have been reported as a prominent source of protease due to their higher efficacy in producing extracellular serine protease (Harwood and Cranenburgh 2008; Mienda *et al*., 2014; Cui *et al*., 2018). Serine proteases are categorized into chymotrypsin- and subtilisin-like serine proteases based on their structure (Madala *et al*., 2010). Subtilisin like-Serine protease has numerous industrial applications because it can effectively target a wide range of substrates (Peterle *et al*., 2020). The *B. subtilis* has tremendous commercial applications e.g., as biofertilizer in agricultural (Mahapatra *et al*., 2022), and as probiotics and household cleaners (Lee *et al*., 2019).

The local dairy processing units and industries are situated across the world and produce massive nutrient-rich liquid effluent (Briao and Tavares 2007) i.e., the processing of one liter of milk generate about 4 to 5 liters of wastewater (Kolhe and Powar 2011). The literature revealed that about 2 to 3 % of the total processed milk goes into an effluent (Kolhe *et al*., 2009). The Central Pollution Control Board (CPCB) released a manual entitled "Guidelines for Environmental Management of Dairy Farms and Gaushalas" and stated that dairy wastewater should not be percolated to the ground because it might pollute the groundwater and as precautionary the dairy processing

units are directed to prepare paved floors to collect dairy wastewater. Additionally, CPCB suggested proper treatment of collected dairy wastewater before discharge and also should be used for utility purpose only if the wastewater meet standards as prescribed by State Pollution Control Board (SPCB) or CPCB. Dairy effluent has an organic-rich nutrient load such as casein, lactose sugar, and some inorganic salt (Deshannavar *et al*., 2012) that could potentially be served as a good medium for microbial growth and protease synthesis as an alternative scientific management of dairy waste. Thus, the present research work has been focused on the comprehensive evaluation of alkaline protease production by *Bacillus* sp. using fortified crude dairy effluent.

MATERIALS AND METHODS

The waste effluent of a local dairy farm and shops of Bilaspur city were aseptically collected in sterile containers and brought to the laboratory. The bacterial strains were isolated from the collected samples using standard protocol followed by serial dilution (Mazzucotelli *et al*., 2013) and stored as agar slants.

A. Screening of protease-producing bacteria

Each bacterial isolate was qualitatively screened for protease activity. The 10^{-4} dilution of samples (0.5 ml) were aseptically inoculated in Skim-milk Agar Media (SAM) consisting of (w/v) skim milk powder (20.0 gL^{-1}), yeast extract (5.0 gL^{-1}), $MgSO_4$ (0.5 gL^{-1}) and KH_2PO_4 (0.25 gL^{-1}) in double-distilled water at pH 7.0 and incubated at 37 ±2°C for 48 h. The clear zones around the bacterial colonies indicated protease production by bacterial cells. The pure cultures of protease-producing bacterial strains were maintained as Luria-Bertani (LB) agar slant at 4°C until used.

B. Production of protease by submerged fermentation

The LB agar slant-based pure cultures of protease-producing bacterial strains were subjected to seed culture preparation for fermentation. The protease was produced by submerged fermentation as mentioned by Pant *et al.* (2015) with slight modification. The production media (100 mL, w/v) consisted of galactose (0.5 g), casein (1.0 g), peptone (0.5 g), KH_2PO_4 (0.2 g), Na_2CO_3 (1.0 g), and $MgSO_4$ (0.2 g) at pH 8 in double-distilled water. A 4.0 ml of seed culture (5.7×10^6 CFU mL^{-1}) was aseptically inoculated in the production media to initiate the fermentation process. The seed culture was an active 12 h old bacterial culture. The inoculated production media was put inside a rotary shaker incubator at 40°C and 120 rpm for 120 h. The fermented production media (fermented broth) was withdrawn at every 24 h of time intervals until fermentation was completed. The fermented broth was centrifuged at 10000 rpm for 20 min to separate bacterial cells. The clear supernatant was harvested as crude protease.

C. Partial Purification of Crude Protease

The crude protease was partially purified by the standard ammonium sulphate [$(NH_4)_2SO_4$] precipitation method followed by dialysis. Crude protease was precipitated with $(NH_4)_2SO_4$ at an 80% saturation (Junaidi *et al*., 2017) and centrifuged at 10000 rpm for 20 min at 4°C. The pallet was collected and dissolved in a 0.05 mol L^{-1} Tris- HCl buffer (pH 8.0). Dialysis was done with 0.025 M phosphate buffer overnight to remove the ammonium sulfate fraction. Later, this partially purified protease was assigned for protease assay (Meyers and Ahearn 1977) and protein estimation (Lowry *et al*., 1951).

D. Quantification of protease activity

The qualitatively screened bacterial colonies with significant protease activity were then acquired for quantitative protease assay. Protease assay was carried out as per suggested by Meyers and Ahearn (1977) with minor modifications. The 2.0 mL of substrate (2.0% casein in 0.1 M Tris HCl buffer at pH 7.0) was incubated with 0.5 mL of partially purified protease and incubated for 10 min at 40˚C. Then, the enzyme-substrate reaction was stopped using 4.0 ml of 5% Trichloroacetic acid (TCA) and filtered by Whatman Filter Paper No. 1 to remove solid partials which may interrupt spectrophotometric analysis. The 3.0 mL of sodium hydroxide (0.1N) and, 2.0 mL Folin-Ciocalteau's (FC) reagent in double distilled water (at a ratio of 1:1) were added. Sodium hydroxide was added to neutralize TCA. The reaction mixture was then incubated in a dark place for 30 min to develop color which is directly propositional to the concentration of tyrosine released in the reaction mixture. Wavelength was selected based on spectrogram A (λ). Maximum absorption was observed at 643 nm. Thereby, the tyrosine released was measured by a spectrophotometer at 643 nm. Tyrosine standard curve was prepared between a range of 10-100 mg mL^{-1} and was used to calculate µ mole of tyrosine released. One unit (U) enzyme is defined as the amount of protease require to hydrolyze casein to produce colour equivalent to 1.0 µmole of tyrosine per minute at 37ºC in presence of FC reagent. The protease activity was calculated by the formula (Pant *et al*., 2015; Sigma-Aldrich, 2022) given below:

$$\text{Enzyme U mL}^{-1}\text{:}\ \frac{(\mu\text{ mole Tyrosine equivalents released})\ (11.5)}{(1)(10)(2)}$$

Where 11.5: total volume of an assay in mL
2: volume (mL) used in Colorimetric Determination
1: volume of enzyme used for assay
10: time (min) of assay

Enzyme units per mg of protein was calculated using following formula:

$$\text{Enzyme Units/mg protein:}\ \frac{\text{Units/mL enzyme}}{\text{mg protein/ml enzyme}}$$

E. Identification of Bacterial Stains

The potential protease-producing bacterial strain was identified based on morphological, physiological, and biochemical characteristics as per described in Bergey's Manual of Determinative Bacteriology (BMDB) (Holt *et al*., 2004).

F. Optimization of protease production on dairy effluent

The nutrient content of sterilized crude dairy effluent was analysed (APHA-AWWA-WPCF, 1980; APHA, 1995; ICMR Manual of Standards of Quality for Drinking Water Supplies, 1975) and allowed the potent protease-producing bacterial strain to grow on it. The effect of pH (6.0 to 9.0), temperature (30 to 60°C), time of incubation (24 to 120 h), and metal ions (viz., Ca, Mg, and Zn) on protease activity were analyzed. Acetate buffer (pH 4.0 to 5.0), Phosphate buffer (pH 6.0 to 8.0), and Glycine-NaOH buffer (pH 9.0) were used to maintain pH. All the experiments were carried out in 100 mL Erlenmeyer flasks filled with 50 mL of the reaction mixture at 120 rpm. A one-factor-at-a-time approach was used for the optimization.

G. Protease kinetics

Protease kinetics was assessed by changing the substrate concentration (0.5, 1.0, 1.5, 2.0, and 2.5 %) at constant protease concentration. V_{max} and K_m calculated by Lineweaver-Burk double reciprocal plot. Enzyme kinetics was analysed by changing the substrate concentration from 0.5 to 2.5 % at a constant enzyme concentration. V_{max} and K_m calculated by Line weaver-Burk double reciprocal plot followed by the following equation:

$y = mx + c.$

where, m - a slope of the line, c - intercept, x - vertical value, and y - horizontal value.

RESULTS AND DISCUSSION

The present research investigation aimed to optimize protease production by *Bacillus* sp. using dairy waste effluent. The major results are briefly mentioned below:

1. Screening of protease-producing bacteria: A total of 23 bacterial strains were isolated from the waste of local dairy and bakery shops in Bilaspur City. Among them, 5 bacterial strains PPB-1 (Protease Producing Bacteria-1), PPB-2, PPB-3, PPB-4, and PPB-5 were qualitatively screened based on the clear zone (mm) 10.38 ±0.14, 17.47 ±0.27, 21.03 ±0.36, 13.79 ±0.84 and 12.20 ±0.65 around the bacterial colonies respectively (Fig. 1 and 2).

2. Production of protease by submerged fermentation: Crude protease was produced using submerged fermentation at 40°C and 120 rpm. The notable crude protease activity was observed between 48 to 96 h of incubation.

3. Partial Purification of Crude Protease: The purification fold was recorded 4.37, 3.71, 4.42, 4.37, and 3.61 for PPB 1, PPB 2, PPB 3, PPB 4, and PPB 5 correspondingly upon ammonium sulphate precipitation (Table 1).

4. Quantification of protease activity: The protease activity of PPB-1, PPB-2, PPB-3, PPB-4, and PPB-5 were observed (U mL^{-1}) 36.92 ±0.31, 17.24 ±0.89, 42.47 ±0.42, 21.65 ±0.57, and 19.49 ±0.27 (Fig. 3).

5. Identification of Bacterial Stains: The significant protease-producing bacterial strains i.e., PPB-1, PPB-2, PPB-3, PPB-4, and PPB-5 were identified as *Pseudomonas aeruginosa*-1, *Staphylococcus aureus*-2, *Bacillus subtilis*-3, *Bacillus megaterium*-4, and *Bacillus cereus*-5 correspondingly as per their morphological, physiological and biochemical characteristics as described in BMDB. The maximum protease activity 42.47 ±0.42 U mL^{-1} was observed with PPB-3 (*Bacillus subtilis*-3).

6. Optimization of protease activity on dairy effluent: The bacterial proteases were partially purified using the ammonium sulfate precipitation method and the purification table along with Total microbial protease activity (U), Total protein content (mg), Specific activity (U mg^{-1}), Protease recovery (%), and Fold Purification are mentioned in Table 1. The crude dairy effluents consisted of biomolecules viz., Protein (59.73 mg/L), Carbohydrate (0.19 mg/L) and fat (0.048 mg/L) and inorganic nutrients viz., nitrogen (62.39 mg/L), organic phosphate (9.51 mg/L), magnesium (16.72 mg/L), sulfate (159.47 mg/L), potassium (14.09 ppm). The fortified dairy effluent (0.5 gram/L peptones and 0.5 gram/L yeast extract) was found to be suitable for Bacterial growth. Maximum protease activity of 54.41 ±0.37 U mL^{-1} (relative activity -128.11 ±0.39 %) at pH-8.0, temperature 40^0C, and 120 rpm after 72 h of the incubation period under optimized conditions was observed with PPB-3 (*Bacillus subtilis*-3) (Table 2).

7. Protease kinetics: A graph was plotted between 1/V versus 1/S for the partially purified protease of PPB-3 (*Bacillus subtilis*-3) at a varied concentration of substrate. The equation value; y= 0.026x + 0.015 was generated by Microsoft Excel 7.0. The plot was found to be linear with a slope of line = 0.026 and Intercept = 0.015. The V_{max} and K_m have calculated 66.66 U mg^{-1} protein and 1.73 mg casein mL^{-1} (Fig 4).

The nutrient content of dairy effluent is treated and discharged into the waterbodies or soaked in nearby lands (Tikariha and Sahu 2014; Custodio *et al*., 2022). The present research rationale was to produce value-added products from waste effluent before treatment. A total of 23 bacteria were isolated from the waste of local dairy and bakery shops in Bilaspur City. PPB-3 (*Bacillus subtilis*-3) exhibited maximum protease production efficiency of 42.47 ±0.42 U mL^{-1} activity at 40°C (pH-7.0). Works of literature have also reflected the temperature range between 37 to 42°C, pH range between 8.5 to 9.0, and agitation rate 150 rpm for alkaline protease from *Bacillus* spp (Rao *et al*., 2017; Agrawal *et al.*, 2012; Sharmin *et al.*, 2005; Sarker *et al.*, 2013). However, an agitation rate of 120 rpm was optimized for the present study. Dutta *et al.* (2005) isolated *Pseudomonas* sp. from soil and observed protease activity of 2000 U in 50 mL (40 U/mL) of crude extract at pH 8.0 and 45°C. Similarly, PPB-1 (*Pseudomonas aeruginosa* 1) was found to be positive for protease production but *P. aeruginosa* is often associated with community-acquired infections (Vincent, 2003). Likewise, PPB-2 (*Staphylococcus aureus* -2) was observed for proteases production but *Staphylococcus aureus* has been reported to produce cysteine, serine, and metalloproteinase for executing the pathogenesis in host cells (Pietrocola *et al*., 2017). Hence, it was assumed that *P. aeruginosa* and *S.*

aureus derived-proteases are often responsible for pathogenic activities. The dairy effluent consisted of organic matters at high levels e.g., 810.33 mg/L of chemical oxygen demand, 797.91 mg/L of biochemical oxygen demand, and 47.3 mg/L of total suspended solids (TSS), and often discharged into the water reservoir or open land (Nabbou *et al*., 2020). The dairy effluent was fortified to ensure significant bacterial growth. The first level of optimization exhibited 52.01 ±0.15 U mL^{-1} (RPA-122.46 ±0.19 %) at pH 8.0, 40°C, 120 rpm, and 72 h of the incubation period (Table 2).

Later, the second level of optimization relied on the effect of cofactors. The protease activity was increased by 54.41 ±0.37 U mL^{-1} (RPA-128.11 ±0.39 %), 54.07 ±0.65 (RPA-127.31 ±0.28), and 53.90 ±0.37 (RPA-126.91 ±0.38) respectively upon the addition of $CaCl_2$, $MnSO_4$, and $ZnSO_4$ at the concentration of 5.0 mM (Table 2). Fedatto *et al.* (2006) conducted a study on bacteria and they found cofactors i.e., Mg^{2+}, Ca^{2+}, Zn^{2+}, and Na^{+} (1.0 mmol l^{-1}) to be effective in inducing protease activity. Ba^{2+}, Ca^{2+}, Fe^{3+}, Mn^{2+}, and Mg^{2+} have been reported to enhance protease activity (Wen *et al*., 2022). Mahmoud *et al.* (2021) isolated alkaline protease-producing *B. subtilis* and *B. cereus* from soil and revealed that *B. subtilis* D9 isolates exhibit maximum proteolytic activity at 50°C under pH 9.5. The Fe^{3+} ion encouraged protease activity. The proteolytic bacteria *Bacillus subtilis* AKAL7 was screened from poultry wastes which exhibited 8335.34 U/mg protein with 45.67-fold purified protease at 40°C under pH 9.0 and also stimulated by Mg^{2+}, K^{+}, and Ca^{2+} (Al Hakim, *et al*., 2018). Sumardi *et al*. (2018) extracted protease enzymes from *Bacillus* sp. and upon addition of Mn^{2+} activator, the 0.28 U/ml V_{max} and K_m 4.60 U/ml at 30°C under pH 8.0. While we found 66.66 U mg^{-1} protein V_{max} and 1.73 mg casein mL^{-1} K_m. But similar results were observed as reported by Iqbal *et al.* (2018), they documented Km of 0.03064 µM and Vmax 69.76 U/mL (under pH of 8.0, temperature 50°C, and incubation period of 72 hours).

The aeration, pH, temperature, incubation period (Hameed *et al*., 1999; Puri *et al*., 2002), and metal ions concentration (Varela *et al*., 1996) significantly influenced extracellular alkaline protease production in bacterial cells. The present experimental cross-check revealed the same and PPB-3 (*Bacillus subtilis*-3) was found to be significant for protease production using DWE. Furthermore, recently, Rosazza *et al*. (2023) claimed that *B. subtilis* has extraordinary adaptability to sustain a wide range of soil environments and they produce extracellular proteases to extract their food from a variety of simple and polymeric food sources which makes them suitable for food waste digestion.

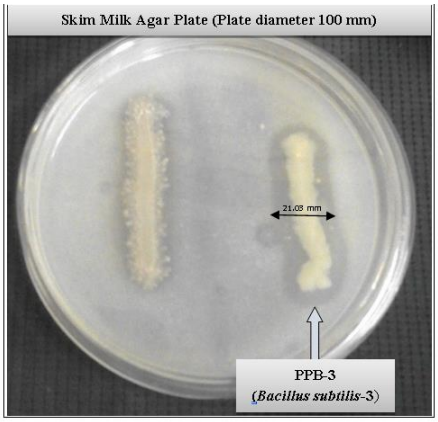

Fig. 1. Qualitative screening of PPB-3 (*Bacillus subtilis*-3).

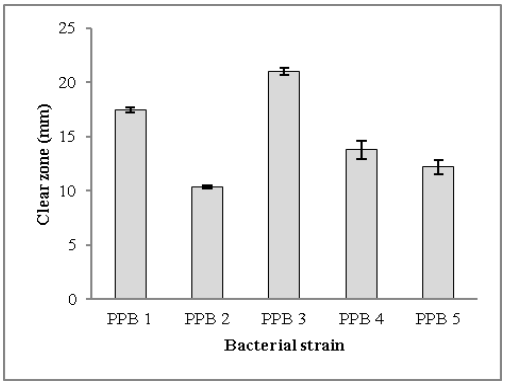

Fig. 2. Qualitative screening of protease-producing bacteria.

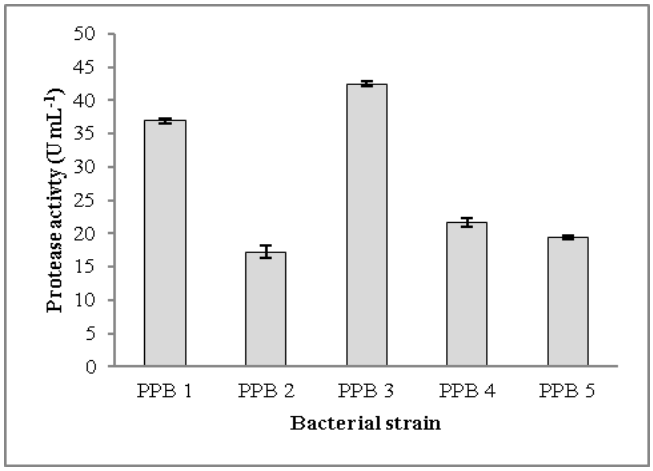

Fig. 3. Quantitative screening of protease-producing bacteria.

Table 1: Partial purification table for crude proteases derived from PPB-3 (*Bacillus subtilis*-3).

Test strains	Purification	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Protease recovery (%)	Fold Purification
PPB 1	Crude extract	50	435.27	124.86	3.48	100	1.0
	$(NH_4)_2SO_4$ precipitation	5	184.60	11.99	15.39	42.41	4.42
PPB 2	Crude extract	50	206.94	60.62	3.41	100	1.0
	$(NH_4)_2SO_4$ precipitation	5	86.20	5.78	14.91	41.65	4.37
PPB 3	Crude extract	50	534.21	148.39	3.60	100	1.0
	$(NH_4)_2SO_4$ precipitation	5	212.35	15.87	13.38	39.75	3.71
PPB 4	Crude extract	50	247.62	72.82	3.40	100	1.0
	$(NH_4)_2SO_4$ precipitation	5	108.25	6.72	16.10	43.71	4.37
PPB 5	Crude extract	50	229.22	63.67	3.60	100	1.0
	$(NH_4)_2SO_4$ precipitation	5	97.45	7.48	13.02	42.51	3.61

Table 2: Optimization of protease activity of PPB-3 (*Bacillus subtilis*-3).

Constant Parameters	Variable unit	Variable unit value	Protease activity (U mL^{-1} ±SD)	PPB-3 (*Bacillus subtilis*-3) Relative protease activity (% ±SD [a])
pH- 7.0, Temperature- 37°C, Time- 72 h, and Rotation- 120 rpm	Nil	Nil	42.47 ±0.42	100
Temperature- 37°C, Time- 72 h, and Rotation- 120 rpm	pH	4.0	33.23 ±0.36	78.25 ±0.59
		5.0	35.81 ±0.75	84.31 ±0.17
		6.0	37.91 ±0.93	89.27 ±0.31
		7.0	42.06 ±0.14	99.03 ±0.49
		8.0	**50.45 ±0.23**	**118.78 ±0.62**
		9.0	41.76 ±0.71	98.33 ±0.20
pH- 8.0, Time- 72 h, and Rotation- 120 rpm	Temperature (^{0}C)	20	26.87 ±0.50	63.27 ±0.34
		30	38.12 ±0.31	89.76 ±0.42
		40	**51.49 ±0.62**	**121.24 ±0.37**
		50	46.59 ±0.86	109.71 ±0.61
		60	42.23 ±0.13	99.43 ±0.42
Temperature- 40°C, pH- 8.0, and Rotation- 120 rpm	Time (h)	24	37.89 ±0.79	89.21 ±0.37
		48	41.01 ±0.28	96.57 ±0.66
		72	**52.01 ±0.15**	**122.46 ±0.19**
		96	43.38 ±0.44	102.14 ±0.78
Temperature- 40°C, pH-8.0, Time- 72, and Rotation- 120 rpm	Zinc (mM)	1.0	51.75 ±0.12	121.85 ±0.45
		5.0	**53.90 ±0.37**	**126.91 ±0.38**
		10.0	52.64 ±0.42	123.95 ±0.16
		15.0	41.30 ±0.17	97.25 ±0.45
	Manganese (mM)	1.0	52.98 ±0.73	124.75 ±0.48
		5.0	**54.07 ±0.65**	**127.31 ±0.28**
		10.0	51.60 ±0.86	121.50 ±0.88
		15.0	46.31 ±0.33	109.04±0.65
	Calcium (mM)	1.0	52.32 ±0.21	123.19 ±0.74
		5.0	**54.41 ±0.92**	**128.11 ±0.39**
		10.0	53.61 ±0.63	126.23 ±0.61
		15.0	49.76 ±0.40	117.17 ±0.44

[a] Relative protease activity was calculated against 42.47 ±0.42 (100%) for PPB-3 (*Bacillus subtilis*-3) as per Table 1.

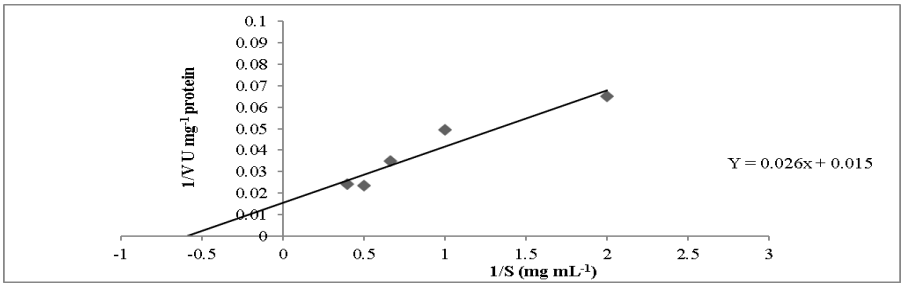

Fig. 4. Line weaver-Burk double reciprocal plot for the determination of Km & Vmax of partially purified protease of PPB-3 (*Bacillus subtilis*-3).

CONCLUSIONS

Lots of emphasis have been made to minimize the cost of protease production in earlier literature. Hence, the present work was focused on protease production by fortified dairy effluent. The PPB-3 (*Bacillus subtilis*-3) was found to be significant for protease production and further, its protease kinetics optimized in fortified dairy effluent. The production of protease from dairy effluent

reflects the significant utilization of dairy waste effluent. Similar industrial wastes i.e., bakery, food processing unit, and so forth, were further evaluated for a variety of enzyme production to meet the demand of the global enzyme market in the near future.

FUTURE SCOPE

PPB-3 (*Bacillus subtilis*-3) could further be optimized for large scale protease production from fortified dairy effluent or similar waste effluents in near future.

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Conflict of interest. None.

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