International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Isolation of *Bacillus subtilis* from Dairy Waste and Evaluation of their Protease Kinetics on Fortified Dairy Waste Effluent

Sumit Kumar Dubey[1], Rashmi Parihar[2]

[1]Ph. D. Scholar, Department of Microbiology, Govt. E. Raghavendra Rao Postgraduate Science College, Bilaspur (Chhattisgarh) India

[2]Assistant Professor, Department of Microbiology, Govt. E. Raghavendra Rao Postgraduate Science College, Bilaspur (Chhattisgarh) India
Corresponding Author Email: *rashmiparihar67[at]gmail.com*

Abstract: ***The microbial cells serve asa valuable source of protease due to their diverse metabolic profile and cost - effectiveness. TheBacillus subtilisis widely accepted as a potentalkaline protease producer. These alkaline proteases are widely used as detergent additives, household cleaning agents, partial digestion of proteinaceous food, and waste treatment. The bacterial strains were isolated from dairy waste effluent and among them, the protease - producing B. subtilis was screened. The PPB - 16 (B. subtilis) exhibited the maximum quantitative protease activity of 41 ±0.33 U mL^{-1} on Fortified Dairy Waste Effluent (FDWE). The kinetics study of PPB - 16 (B. subtilis) - derived protease revealed bacterial species has also revealedV_{max} of 8.29 U mg^{-1} proteinand K_m of 2.37mg casein mL^{-1} in FDWE. The prospects include the characterization of protease and their in - silico profiling for the best possible targets that could potentially be used in the food and pharmaceutical sectors. The limitation of the present study is that, as the literature claimed huge diversity in ingredients of dairy waste effluents accordingly protocols for protease production needs to be optimized accordingly.***

Keywords: Alkaline Protease, PPB - 16 (Bacillus subtilis), Fortified Dairy Waste Effluent (FDWE), Kinetics, food and pharmaceutical sector

1. Introduction

Enzymes are active protein molecules that facilitate catalytic reactions in living cells andare ubiquitously distributed in all living cells including plants, animals, and microbes. Cells. The proteases (EC: 3.4) belong to the hydrolase group and participate in proteolytic activity in living cells. It is found in diverse forms including aspartic, cysteine, metalloprotease, serine, and threonine. Additionally, based on the pH, classified into alkaline neutral and acid proteases. The protease has a widespread industrial utility to facilitate chemical reactions (Beg and Gupta, 2003). The proteases have been reported to be utilized in detergent manufacturing, household cleaning additives, and partial digestion of proteinaceous food. Microbial cells nowadays serve asexcellentsources of protease due to diversity, easy genetic manipulation, and cost - effectiveness. However, the proteases are produced by eukaryoticcellsincluding plants and animals, but due to complex cultivation measures, time - consuming and large conditioned area requirements and to follow strict ethical guidelines. Microbial proteases capture 40% of the global enzyme market. In the context of alkaline proteases, the*Bacillus*genus has been recognized asa potent protease producer (Contesini*et al*., 2017; Solanki *et al*., 2021). Among them, *B. subtilis* has been reported as a significant alkaline protease producer (Oda, 2012) andubiquitously distributed in an ecosystem (Harwood *et al*., 2022). This bacterial species has also been revealed for extensive opportunitiesfor stable genetic medications (Hubar *et al*., 2017). Additionally, the proteasesderived from *B. subtilis* have been recognized as GRAS (Rao *et al*., 1998).

As microbial - derived alkaline protease production is expensive (Rao *et al*., 1998) due to the higher cost of substrate or production media. Therefore, the use of inexpensive substrate or waste for protease production might reduce the production cost. The hair - , bakery - , fish - , dairy - , and soya - waste have been earlier studied (Sharma *et al*., 2019) for protease production. The present work was focused on dairy waste because itgeneratesa large quantity of processed wastewaterhavinghigh biological oxygen demand (BOD), protein, carbohydrates, and fats content (Gopinatha Kurup*et al*., 2019) that cause harm to an aquatic ecosystem (Balamane - Zizi and Ait - Amar, 2010) if discharged untreated. The local dairy and bakery waste effluents are often discharged in a municipality or nearby sewerage. Thereby, the work was planned towards the effective waste effluent utilization system for protease production which can further be extended to produce value - added products e. g., organic acids, organic alcohols, biofuels, and so on. The protease kinetics defines how the variables (e. g., substrate concentration) affectthe rate of protease activity. Michaelis–Menten (MM) and Line - weaver Burk (LB) reciprocal plots are well - known for enzyme kinetics studies (Rajendran & Thangavelu, 2008). The LB plot is often used to determine K_m and V_{max} for the enzyme. Hence, the present research work was focusedon theevaluation of the protease kinetics of *B. subtilis*on FDWE.

2. Materials and Methods

The samples were collected from the 24 sites marked under four sectors inthe Bilaspur Region. The two types of samples viz., Fresh Dairy Effluent (FDE) and Dairy Effluent Soaked in Soil (DESS) were collected from local Dairy units and brought to the laboratory for preliminary experiments as per the research design.

Isolation and Screening of protease - producing*Bacillus* strains

The bacterial strains were isolated by using serial dilution followed by spreading the 10^{-6} titer over the Nutrient Agar Media plates. The protease - producing bacterial strains were qualitativelyscreened on a Skim Milk Agar plate (supplemented with 2% skim milk) using the protocol mentioned by Das and Prasad (2010). Protease - producing bacterial strains were identified as per the key provided by BMDB (Bergey's *et al*., 1974).

Preparation of Fortified Dairy Waste Effluent

The dairy effluent was fortified using glucose (0.5 %), milk powder (1 %), yeast extract (0.5%) and peptone (0.5%). The protein, fat, and sugar content were determined after fortification using Milkotester.

Production of Proteases using *Bacillus* isolates

The protease - producing*Bacillus* isolates weresubjected to submerged fermentation using FDWE at 40^0C, pH of 8.0, and rotation of 150 rpm for 72 h. The fermented FDWE broth was then centrifuged at 8000 rpm to remove bacterial cells and cell - free supernatant was marked as crude protease enzyme. The crude protease was partially purified by ammonium sulfate precipitation (at a gradual saturation from 0 to 65% at 4^0C) method (Kumar *et al*., 1999) followed by dialysisusing 20mM of Tris - HCl buffer at pH of 8.5 for two successive times (Ellaiah and Srinivasulu, 1996) to remove ammonium sulfate fraction. The quantitative protease activity of *Bacillus* species was analyzed by using the method described by McDonald and Chen (1965). The most potent protease - producing bacteria was determined based on the quantitative protease activity and further selected for protease kinetics on FDWE.

Assessment of protease kinetics onFortified Dairy Waste Effluent

The Kinetics of partially purified protease derived from the most potent protease - producing*Bacillus* isolates wasassessedusing LB (Philipp and Bender, 1983). The FDWE was used as a substrate for partially purified protease to determine the kinetics. The substrate concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 mg L^{-1}were taken for kinetic analysis. The temperature of 40^0C and pH of 8.0 were maintained during the assay. The K_m and V_{max} were determined using a plot between 1/V versus 1/S in MS Office Excel 2021 using the equation y= mx + b.

All the experiments were carried out in triplicates to minimize the error. The statistical calculation was done using MS Office Excel 2021.

3. Results and Discussion

The present research work was focused on the evaluation of *Bacillus subtilis* - derived protease kinetics on FDWE. The dairy waste has pH ranges between 6.4 ±0.12 to 8.9 ±0.23, temperature ranges from 23 ±1.0to 31 ±1.0, andmoisture content ranges from 46 ±2.74 to 61 ± 3.53. Likewise, the pH range of 7.2 to 8.8 (Deshannavar*et al*., 2012) and from 7.0 to 8.0 (Arumugam and Sabarethinam, 2008) have been reported earlier. As the *Bacillus* spp. utilize nitrogen sources from media and release ammonium derivatives in fermented broth which tend to increase pH (Parkouda *et al*., 2009; Owusu - Kwarteng *et al*., 2022). The fortified dairy waste effluent (FDWE) has a pH of 7.3, protein content of 2.6 g 100 ml^{-1}, fat content of 1.8 g 100 $ml^{-1,}$ and sugar content of 4.9 g 100 ml^{-1}.

A total of three protease - positive*Bacillus*species were observed from collected samples and were identified as *Bacillus cereus* (PPB - 18), *Bacillus licheniformis* (PPB - 27), and *B. subtilis* (PPB - 16) using the key provided by BMDB (Bergey *et al*., 1974). Similarly, Garcha *et al*. (2016) revealed *B. subtilis* SRS 35, *Lysinibacillussphearicus* B3PO2, *B. cereus* N24, *Bacillus thuringiensis* ODPY, *Brevibacillus brevis* KF152965 strains in unorganized dairy sector wastewater.

Among them, *B. subtilis* (PPB - 16) was further selected for fermentation as per the experimental design and objective of the present research work. The colony, morphological, and biochemical characteristics of *B. subtilis* (PPB - 16) are shown in Table 1. *B. subtilis* (PPB - 16), *B. cereus* (PPB - 18), and *B. licheniformis* (PPB - 27) exhibited 41 ±0.33 U, 39 ±0.28 U, and 38 ±0.35 U protease activity in FDWE at pH of 7.0, Temperature of 40^oC, RPM of 150 rpm, and time of72h undersubmerged fermentation (Table 2). A similar study was carried out by Mahakhan*et al*. (2022) on alkaline protease production by*Bacillus gibsonii* 6BS15 - 4 using fortified dairy effluent and they revealed 766.60 mg/l of protein and 381.30 mg/l of reducing sugar on effluent. Additionally, they reported 78.85 ± 8.87% of relative protease activity of *B. gibsonii* 6BS15 - 4 on dairy effluent which was fortified with basal media and 0.25% skim milk. El - Gayar*et al*. (2020) revealed that the *Bacillus thuringiensis* that wasisolated from the Mangrove rhizosphere, exhibited 101.22 U/ml of protease activity on dairy industry wastes (50% dairy waste) fortified with 1% skim milk, NaCl, and $CaCl_2$ at pH of 7.0 and 37°C of temperature.

The maximum protease kinetic was found V_{max} (U mg^{-1} protein) of 8.29 K_m (mg casein mL^{-1}) of 2.37with PPB - 16 (*B. subtilis*) - derived protease on FDWE substrate (Fig.1). Similarly, Sumardi *et al*. (2016) claimed that the *Bacillus* sp. derived protease exhibited 0.33 U ml^{-1} of V_{max} and 5.64 U ml^{-1} of K_m at pH 8, and likewise, Abdullah *et al*. (2022) noted 0.307 U g^{-1} of V_{max} and 11.2 mg mL^{-1} of K_m with proteasederivedfrom *B. subtilis.*

Table 1: Colony, Morphological, and Biochemical Characteristics of *B. subtilis*

Characteristics	Colony			Gram stain	Cell Shape	Endospore	Starch hydrolysis	Voges - Proskauer	Citrate utilization	Growth in 6.5% NaCl	Growth at 55^{0}C	Lipase	Gelatine hydrolysis	Catalase
	color	Margin	elevation											
B. subtilis	CW	Irregular	Flat	+	Rod	+	+	+	+	+	-	+	+	+

CW: Creamish White; +: Positive; -: Negative

Table 2: The potent proteasesproducing *Bacillus* species

Bacterial isolates	Identified as per BMDB	Protease activity			
		Qualitative			Quantitative
		Zone diameter (mm)	Colony diameter (mm)	Hydrolysis capacity	
PPB - 16	*B. subtilis*	12	27	2.25	41 ±0.33
PPB - 18	*B. cereus*	10	19	1.90	39 ±0.28
PPB - 27	*B. licheniformis*	14	22	1.57	38 ±0.35

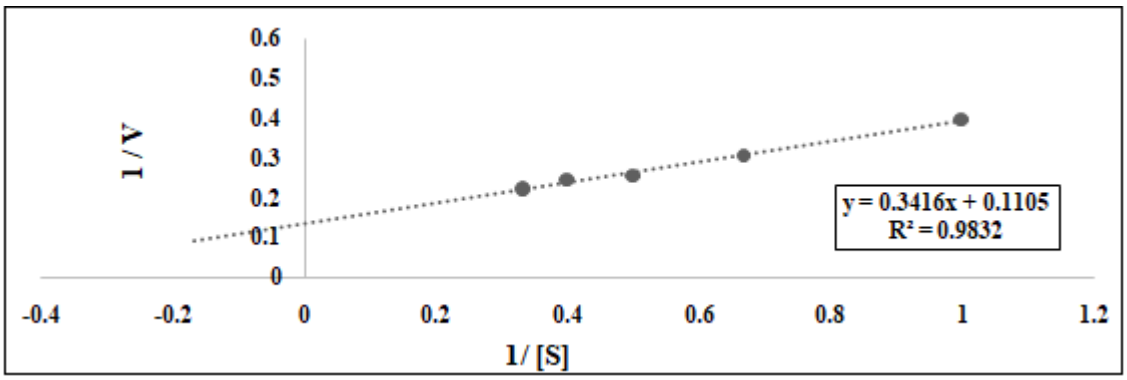

Figure 1: Line weaver - Burk (L - B) double reciprocal plot for Km & Vmax of protease produced by PPB - 16 in FDWE. The graph is plotted between 1/V versus 1/S and y= mx + b was generated by Microsoft Excel 2022.

4. Conclusion

Protease enzymes havetremendousapplications in the industrial sector includingdetergent manufacturing, food processing, tannery, pharmaceutical preparations, agricultural practices, bioenergy generation, and waste treatment methods. The microbial proteases attract scientific communities due to easy cultivation, diverse substrate utilization, diverse metabolic profile, effective genetic manipulation opportunities, and less associated ethical concern as compared to other related living organisms producing the enzyme. The genus *Bacillus*is a potent producer of alkaline proteases and can utilize a broad array of substratesfor protease production. Dairy waste effluent has a high nutrient content. Thereby, the protease production by *B. subtilis* using FDWE and protease kinetics has been assessed in the present study. The PPB - 16 (*B. subtilis*) exhibited maximum quantitative protease activity and significant K_m and V_{max}in FDWE. The prospects of the present research work include the molecular characterization of protease enzymes and their *in - silico* profiling for the best possible targets in the food and pharmaceutical sectors.

Acknowledgment
The authors would like to extend their warm gratitude to **Dr. D. K. Shrivastava** (Head, Department of Microbiology, Govt. E. R. R. P. G. Science College) for the lab facilities. The present work was carried out in the PG lab, Department of Microbiology, Govt. E. R. R. P. G. Science College, Bilaspur (C. G.).

References

[1] Abdullah, R., Asim, M., Nadeem, M., Nisar, K., Kaleem, A., &Iqtedar, M. (2022). Production, optimization, purification, kinetic analysis, and applications of alkaline proteases produced from *Bacillus subtilis* through solid - state fermentation of agricultural by - products. *Kuwait J. Sci.*, 49 (4), 1 - 15.
[2] Arumugam, A. &Sabarethinam, P. L. (2008). Performance of a three - phase fluidized bed reactor with different support particles in the treatment of dairy wastewater. *ARPN Journal of Engineering and Applied Sciences*, 3 (5).
[3] Beg, K. B. & Gupta, R. (2003). Purification and characterization of an oxidation - stable, thiol - dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme Microbiol. Technol*, 32, 294 - 304.
[4] Bergey, D. H., Buchanan, R. E., & Gibbons, N. E. (1974). Bergey's Manual of Determinative Bacteriology. (8th ed.), USA, *The Williams and Wilkins Co., Baltimore*, 15 - 36.

[5] Contesini, F. J., Melo de, R. R., & Sato, H. H. (2017). An overview of Bacillus proteases: from production to application. *Critical Reviews in Biotechnology*, 38 (3), 321–334. https: //doi. org/10.1080/07388551.2017.1354354

[6] Das, G. & Prasad, M. P. (2010). Isolation, purification & mass production of protease enzyme from *Bacillus subtilis*. *International Research Journals of Microbiology*, 1, 26 - 31.

[7] Deshannavar, U. B., Basavaraj, R. K., & Nandini, M. N. (2012). High rate digestion of dairy industry effluent by upflow anaerobic fixed - bed reactor, *Journal of Chemical and Pharmaceutical Research*, 4 (6), 2895 - 2899.

[8] Ellaiah, P. & Srinivasulu, B. (1996). Production of extracellular protease by *Streptomyces fradiae*. *Brazilian Journal of Microbiology*, 38, 41 - 47.

[9] El - Gayar, K., Essa, A., & Abada, E. (2020). Whey Fermentation for Protease Production Using *Bacillus thuringiensis*; Isolated from Mangrove Rhizosphere Soil in Jazan, Saudi Arabia. *Polish Journal of Environmental Studies*, 29 (3), 2167–2176. https: //doi. org/10.15244/pjoes/110583

[10] Garcha, S., Verma, N., & Brar, S. K. (2016). Isolation, characterization and identification of microorganisms from unorganized dairy sector wastewater and sludge samples and evaluation of their biodegradability. *Water Resources and Industry*, 16, 19–28. https: //doi. org/10.1016/j. wri.2016.10.002

[11] Gopinatha Kurup, G., Adhikari, B., &Zisu, B. (2019). Recovery of proteins and lipids from dairy wastewater using food grade sodium lignosulphonate. *Water Resources and Industry*, 22, 100114. https: //doi. org/10.1016/j. wri.2019.100114

[12] Harwood, C. R. & Kikuchi, Y. (2022). The ins and outs of Bacillus proteases: activities, functions and commercial significance. *FEMS Microbiol Rev*., 46 (1), fuab046. https: //doi. org/10.1093/femsre/fuab046

[13] Hubar, C. H., Shen M. C., Lin J. S., Yang N. C. (2017). Nattokinase decreases plasma levels of fibrinogen, factor VII, and factor VIII in human subjects. *Nut. Res.*, 29, 190–196.

[14] Kumar, C. G., Tiwari, M. P., & Jany, K. D. (1999). Novel alkaline serine proteases from alkalophilic *Bacillus* sp purification and some properties. *Process Biochemistry*, 34, 441 - 449.

[15] Mahakhan, P., Apiso, P., Srisunthorn, K., Vichitphan, K., Vichitphan, S., Punyauppa - path, S., &Sawaengkaew, J. (2022). Alkaline Protease Production from *Bacillus gibsonii* 6BS15 - 4 using Dairy Effluent and Its Characterization as a Laundry Detergent Additive. *Journal of Microbiology and Biotechnology*, 33 (2), 195–202. https: //doi. org/10.4014/jmb.2210.10007

[16] Oda, K. (2012). New families of carboxyl peptidases: serine - carboxyl peptidases and glutamic peptidases. *Journal of Biochemistry*, 151 (1), 13–25.

[17] Owusu - Kwarteng, J., Agyei, D., Akabanda, F., Atuna, R. A., &Amagloh, F. K. (2022). Plant - Based Alkaline Fermented Foods as Sustainable Sources of Nutrients and Health - Promoting Bioactive Compounds. *Frontiers in Sustainable Food Systems*, 6. https: //doi. org/10.3389/fsufs.2022.885328

[18] Parkouda, C., Nielsen, D. S., Azokpota, P., Ivette Irène Ouoba, L., Amoa - Awua, W. K., Thorsen, L., Hounhouigan, J. D., Jensen, J. S., Tano - Debrah, K., Diawara, B., & Jakobsen, M. (2009). The microbiology of alkaline - fermentation of indigenous seeds used as food condiments in Africa and Asia. *Critical Reviews in Microbiology*, 35 (2), 139–156 https: //doi. org/10.1080/10408410902793056

[19] Parkouda, C., Thorsen, L., Compaoré, CS., Nielsen, D. S., Tano - Debrah, K., Jensen, J. S., Diawara, B., & Jakobsen, M. (2010). Microorganisms associated with Maari, a Baobab seed fermented product. Int. *J. Food Microbiol*., 142, 292–301. https: //doi. org/10.1016/j. ijfoodmicro.2010.07.004

[20] Philipp, M. & Bender, M. L. (1983). Kinetics of subtilisin and thiolsubtilisin, Mol. Cell. Biochem.51 (1), 5–32. https: //doi. org/10.1007/bf00215583

[21] Rajendran. A. & Thangavelu, V. (2008). Evaluation of various unstructured kinetic models for the production of protease by *Bacillus sphaericus* - MTTC511. *Engineering in Life Sciences*, 8, 179 - 185.

[22] Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and Biotechnological Aspects of Microbial Proteases. *Microbiology and Molecular Biology Reviews*, 62 (3), 597–635. https: //doi. org/10.1128/mmbr.62.3.597 - 635

[23] Sharma, M., Gat, Y., Arya, S., Kumar, V., Panghal, A., & Kumar, A. (2019). A review on microbial alkaline protease: an essential tool for various industrial approaches. *Mary Ann Liebert, Inc., Industrial Biotechnology*, 15 (2), 69 - 78, https: //doi. org/10.1089/ind.2018.0032

[24] Solanki, P., Putatunda, C., Kumar, A., Bhatia, R., & Walia, A. (2021). Microbial proteases: ubiquitous enzymes with innumerable uses.3 *Biotech*, 11 (10), 428. https: //doi. org/10.1007/s13205 - 021 - 02928 - z

[25] Sumardi, Agustrina, R., Ekowati, C. N., & Pasaribu, Y. S. (2018). Characterization of protease from Bacillus sp. on medium containing FeCl3 exposed to magnetic field 0.2 mt. *IOP Conference Series: Earth and Environmental Science*, 130, 012046. https: //doi. org/10.1088/1755 - 1315/130/1/012046