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Morpho-molecular identification of alkaline protease producing bacteria from municipal solid wastes in Bangladesh

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Contribution | **Bhuiyan, F. R.,** conceived and designed the experiment, analysed and interpreted the data, review the final manuscript; **T.H. Emon,** analysed the data, and wrote the original manuscript; **M. A. Motaleb** performed the experiments.

Alkaline proteases have become important enzymes that find widespread use in diverse industrial applications. This research was aimed to isolate alkaline protease producing bacterial isolates from protein-based waste. In the current study, proteolytic bacteria were isolated and screened from protein-based wastes. Depending upon the maximum relative diameter of the clear zones, 3 isolates were selected for identification by culturing at alkaline (pH 9) media. The selected isolates were identified as G+ve *Firmicutes* (isolate 1) and G-ve *proteobacteria* (isolate 2 & 3) based on cultural, morphological and biochemical characteristics. The 16S rDNA was amplified through polymerase chain reaction followed by sequencing. Due to insufficient sequence length, first isolate has not been identified at molecular level. Isolate 2 and 3 have been identified as *Stenotrophomonas maltophilia and Pseudomonas hibiscicola* by using different bioinformatics tools. Our study revealed that the bacterial extracellular alkaline protease can be produced from selected bacterial strains which might be potential for industrial and biotechnological applications.

Keywords: Isolation; 16s rDNA; Bacterial enzyme.

INTRODUCTION: Protease [EC 3.4.2124] enzymes are complex molecules responsible for breaking down protein molecules into peptide fragments and amino acids, and they are present in all living organisms (Sharma and Arya, 2019). These enzymes are widely used in industrial processes and are considered a vital component of industrial biotechnology, accounting for 60% of the total enzymes used in various industries worldwide (Ningthoujam *et al.*, 2009; Azad, 2013; Hakim *et al.*, 2018). Enzymes have fascinated the world attention due to their broad range of uses in many fields and are gradually substituting the use of harsh chemicals in various industrial processes (Malathu *et al.*, 2008). More than 3000 different enzymes have been identified and several has been biotechnological and industrial applications (Smita *et al.*, 2012). Most of the proteases used in industrial applications are derived from microorganisms or microbial sources due to their purity, consistency, and cost-effectiveness (Smita *et al.*, 2012). However, most alkaline proteases used for industrial purposes have limitations, including low activity and stability at a wide range of pH levels and around 40% of production cost of industrial enzymes is estimated to be accounting for the cost of growth medium (Giarrizzo *et al.*, 2007). It is well known that the amount of enzyme produced importantly depend on growth conditions and strains. To produce alkaline proteases from an alkalophilic bacterium by using cost-effective growth medium is especially important. Noticeably, proteases are not produced commercially in Bangladesh and tons of proteases are imported every year to use in different industries (Azad, 2013). Thermo-stability is another critical feature of industrial enzymes, yet many microbial proteases have a maximum activity range of 30-40°C, and only a few studies have explored the thermal stability of microbial proteases (Gupta *et al.*, 2002; Shumi, 2004). However, recent research has shown that hot regions, such as soils in such regions, are favourable sites for the growth of thermostable enzyme-producing microorganisms (Fossi *et al.*, 2011). Therefore, a significant need to search for new proteases with novel features for industrial applications from varied bacterial isolates, particularly those that are hyperactive and can produce enzymes that are stable at high temperatures. Recent studies have shown that food and kitchen waste, as well as other protein-based waste such as dairy farm waste, tannery waste, fish market waste, and poultry firm waste, are excellent sources of proteinous items that can be used to isolate high pH-loving bacteria (Hakim *et al.*, 2018; Rahman *et al.*, 2018). These sources of waste are often dumped as a part of municipal solid waste and can be exploited to obtain new microbial strains that produce alkaline proteases. In Bangladesh, the management of Municipal Solid Wastes (MSW) is currently inadequate, resulting in environmental pollution, climate change, and public health hazards. The six divisional cities and other urban areas of Bangladesh produce about 16,000 tons of solid waste every day, a number predicted to increase to 47,000 tons per day by 2025 due to population growth and urbanization (Bahauddin and Uddin, 2012). Rotting MSW emits methane, the second most prevalent greenhouse gas, which has a climate change impact over 20 times greater than carbon dioxide. Organic materials, such as cellulose, protein, and fat, make up almost 70-80% of the MSW (Hasan *et al.*, 2017). This large amount of organic MSW (OMSW) has the potential to be utilized for the production of commercially important enzymes and renewable biomass energy. By doing so, it is possible to mitigate the negative impacts of unmanaged MSW on climate change, public health, and environmental pollution. Studies have shown that sustainable MSW management practices are essential to reduce the harmful impact on the environment and human health in Bangladesh.

OBJECTIVES: The objectives of this study were (i) isolation and screening of alkaline protease producing bacteria from protein based municipal wastes (ii) identification of alkaline protease producing bacteria based on morphological, microscopical and biochemical test (iii) molecular identification of bacterial isolates by 16S rDNA sequencing.

MATERIALS AND METHODS: **Sample collection:** Samples were collected from protein-based waste sources located in different areas of MSW (Municipal Solid Waste) sites. The sources included a milk-based dairy farm waste source in Greenview, Nayabazar, fish market waste from Karnaphuli market dustbin at 2 No Gate, chicken-based waste from Karnaphuli market dustbin, tannery waste from Sagorica, Alangkar, tannery waste from Dewanhat, and pulse waste mixed with soil near Sanmar. The collected samples were from waste that was 3-4 days old. To ensure that only protein-based waste was included in the samples, a preliminary screening was conducted to remove inert materials, plastics, glasses, iron, and other non-protein-based waste components.

Isolation of bacteria from protein waste: To reduce the microbial load, the serial dilution technique was employed. Test tubes (n=9) containing 9 mL of normal saline were prepared, and 1g of waste sample was added to the 1st tube, resulting in a dilution of 10^-1. The test tube was placed on a vortex mixer to mix homogeneously and then the sample was serially diluted up to a dilution of 10^-9. The same procedure was followed for all samples. Nutrient agar medium (0.5% peptone, 0.3% beef extract, 1.8% agar) was prepared with pH9 and sterilized by autoclave and poured onto the petri plates. For the growth of bacterial consortium, spread plating technique was employed by spreading 0.1 mL of the diluted sample evenly using L-rod on nutrient agar medium. The agar plates were then incubated at 30°C for 24 h, and observed for microbial growth. Consortia of bacteria exhibiting various colony morphologies were observed on the plates. Colonies with similar morphologies were considered to be the same bacteria, while different colony morphologies were indicative of different microorganisms.

Screening of alkaline protease producing bacteria: Bacterial isolates from nutrient agar medium were screened using 1% skim milk agar media (1% skim milk, 1% glucose, 1.8% agar, pH 9.0). 0.1M tris buffer pH-9 was used to prepare the media. The media was sterilized and then each distinct colony was placed on the media using toothpick. Following placement, the petri dishes were kept overnight into an incubator at 37°C for 24h to 48 h. Bacterial isolates exhibiting distinct clear zone due to hydrolysis of casein in milk were identified as protease producers. Besides, media containing pH 10 and 11 was also performed but no any colony has been found at

this condition.

Zone ratio was measured using the following formula:

$$\text{Zone ratio} = \frac{Clear\ zone\ diameter\ (mm)}{Colony\ diameter\ (mm)}$$

Depending upon the maximum relative diameter of the clear zones, screening of protease producer was carried out and pure cultures were obtained on SMA media with streak plate method. These colonies were stored for preservation at -20^{0}C with 12% glycerol.

Identification and morphological characterization: Colonies that showed greater clear zone ratio were subjected to bacterial identification based on cultural characteristics, gram staining, motility and various biochemical tests using standard methods and identified using Bergey's manual of systematic bacteriology. The bacterial isolates were subjected to colony character observations on nutrient agar plates, as well as microscopy-based morphological studies such as gram staining, motility test, and endospore staining, following the methods outlined in the study by Masi *et al.* (2014). The selected isolates were inoculated on nutrient agar plates and incubated at 30°C for 24 h and observed for colony characters such as color, shape, surface, and opacity.

Motility test: A small drop of liquid bacterial culture was placed in the middle of a cover glass, attached to a concavity slide, and the slide is then placed under a microscope with a magnification of ×100 using emersion well. A darting, zigzag, tumbling or other organized movement demonstrated positive result whereas no movement or brownian motion only results in negative.

Gram and endospore staining: A small colony was picked up from a 24 h old culture with a loop, smeared on a glass slide and fixed by gentle heating. Crystal violet solution was then applied on the smear to stain for 2 min. and washed with running water. Lugol's iodine (5% iodine powder, 10% potassium iodide) was added to act as mordant for 1 min., following washing with running water. Ethanol (95%) was then added, which act as a decolorizer, for 5 sec. The slide was then washed with water and safranin was applied as a counterstain for 45 sec. The slide was then washed with water, blotted and dried in air and then examined under microscope. A loop of organism was taken to a slide and air dried. Slide was covered with a square of blotting paper saturated with 0.5% (w/v) malachite green stain solution. The slide was steamed for 5 min followed by washing in tap water. Safranin (2.5g of safranin in 100mL of 95% ethanol) was added for 30 sec. and washed away with tap water and blot dried. Finally, the slide was examined under the microscope for the presence of endospores (Schaeffer and Fulton, 1933).

Biochemical characterization: Bacterial culture was inoculated in tryptone broth and incubated at 37°C for 48 h. Next, a few drops of Kovac's reagent (5% para-dimethylaminobenzaldehyde in 75mL amyl alcohol & 25mL concentrated HCl) were added to the tubes step by step. The tubes were shaken and were allowed to stand undisturbed for 10 min. to allow a layer to form. The presence of red-colored on the top of the tube is indicative of a positive result (Kumari *et al.*, 2012). Sterile MR-VP broth tubes were prepared and inoculated with the selected isolates and incubated at a temperature of 37°C for 2 days. Post-incubation, the tubes were added with 5 drops of methyl red and were observed for any color change. A positive result is indicated by the color change from yellow to red after the addition of methyl red within 15 sec. (Mazotto *et al.*, 2010). MRVP broth was prepared and 24 h cultures of the selected isolates were inoculated using a sterilized loop followed by incubation at 37°C for 24 h. Following incubation, 1 mL alpha-naphthol was added and shaken then 0.5 mL of 40% KOH was added to the broth and shaken. A positive result was determined by the development of a red color within 1 h after the addition of the reagents (Han, 2012). 3% hydrogen peroxide was used to detect catalase test. An overnight culture of the test organism was obtained and a small amount was mixed with the hydrogen peroxide solution on a sterilized microscopic slide using a sterilized loop. The formation of the bubble during and after mixing was recorded as a positive result (Boominadhan *et al.*, 2009). The oxidase test was done to identify organisms that produce enzyme cytochrome oxidase. A filter paper was taken and soaked with the 1% tetramethyl-p-phenylened-iamine dihydrochloride. The paper was moistened with a sterile distilled water. The colony of each isolate was picked to be tested with wooden or platinum loop and smear in the filter paper. Inoculated area of paper was observed for a color change to deep blue or purple within 30 sec. Colorless means oxidase negative and blue or purple color means oxidase positive (Shields and Cathcart, 2010). The selected isolates were subjected to a carbohydrate fermentation test in an anaerobic environment, utilizing any of carbohydrates such as glucose, sucrose, lactose, mannitol, and galactose. Nutrient broth containing glucose, along with phenol red as a pH indicator was prepared, inoculated with selected isolates and Durham tubes were placed in an inverted position inside the media. The media with Durham tubes were then incubated in an anaerobic environment at 37°C for 24 h to allow for carbohydrate fermentation by isolates. Fermentation of carbohydrates results in formation of organic acids which changes the color of media from pink to yellow and gas production was noted with appearance of gas bubbles in inverted Durham's tubes (Harley and Prescott, 2002). The Simmons citrate agar tubes were prepared and sterilized, followed by inoculation of 24 h old colony through stab inoculation. The tubes were then incubated at 37°C for 24 h. Positive result was demonstrated by a color change from green to a deep Prussian blue color.

Molecular identification: For molecular identification, genomic DNA of the selected isolates was extracted by using Favorgen genomic DNA isolation kit according to the manufacturer protocol. The quality and the quantity of the extracted DNA were analyzed using Nano Drop Spectrophotometer (Model: ND2000, Origin: Thermo Scientific, USA). The PCR amplification of the extracted DNA was carried out using a PCR mix composed of 1µL of forward primer 16S 27F (5- AGA GTT TGA TCC TGG CTC AG -3) and 1 µL of reverse primer 16S 1492R (5- CGG TTA CCT TGT TAC GAC TT -3) (Becker *et al.*, 2002), along with 1 µL of genomic DNA (≤250 ng), 10 µL of Hot Start Green Master Mix (Cat: M7432, Origin: Promega, USA) and 7 µL of PCR grade water. The PCR products were purified by using the SV Gel and PCR Clean up System (Cat: A9281, Origin: Promega, USA) and sequenced (Iqbal *et al.*, 2018). The 16S rDNA sequences obtained were analysed using Chromas 2.6.2 computer program. Sequences were searched using the BLAST search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment with similar sequences was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and a phylogenetic tree was constructed using Molecular Evolution Genetic Analysis (MEGA), version 5.0, as previously described (Azad *et al.*, 2016).

RESULTS: Alkaline protease producer bacteria isolation from MSW: About six different MSW source samples were serial diluted and plated on nutrient agar medium to observe the growth of bacterial consortium. Out of 6 samples, bacterial isolates have been found in 5 sources when cultured in nutrient agar medium (figure 1). A total of 5 different protease-producing bacterial isolates were isolated from MSW sample. The isolates were sub-cultured and maintained in NA media for future tests.

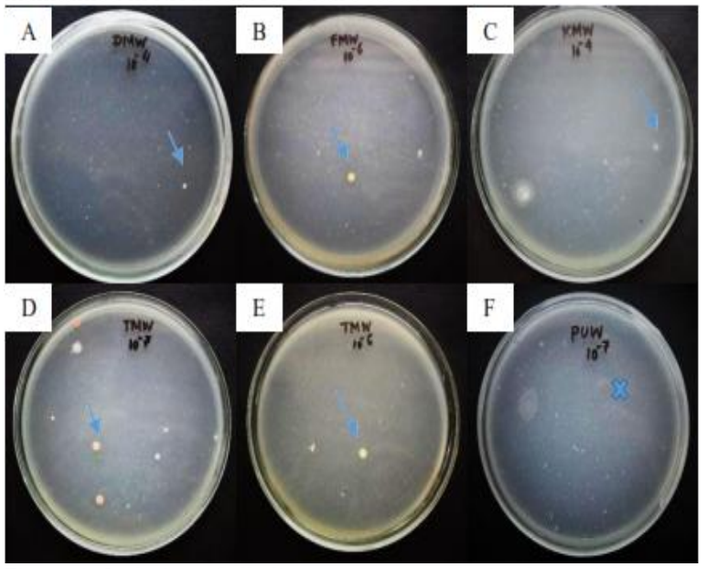

Figure 1: Isolation of alkaline protease producing bacterial isolates using SMA from different sources of MSW.

Screening of potential alkaline protease-producing bacterial isolates: Out of 5, three isolates exhibited significant clear zone and pure culture of the isolates was obtained in SMA media shown in figure 2, respectively. The efficiency of zone clearance of the isolates is summarized in table 1.

Identification of screened bacterial isolates: About 3 pure bacterial isolates namely isolate 1, 2, and 3 were subject to biochemical tests. Isolate 1, 2 and 3 exhibited organish, yellowish and greenish white colonies, respectively. All 3 isolates had raised, smooth, and opaque colonies. Morphological examination revealed that all three isolates were motile rods having no endospore. Isolate

1 was identified as G+ve whereas isolate 2 and 3 were classified as G-ve based on the results of the gram staining test. Morphological characteristics of these isolates are summarized in table 2 and gram staining results are presented in figure 3.

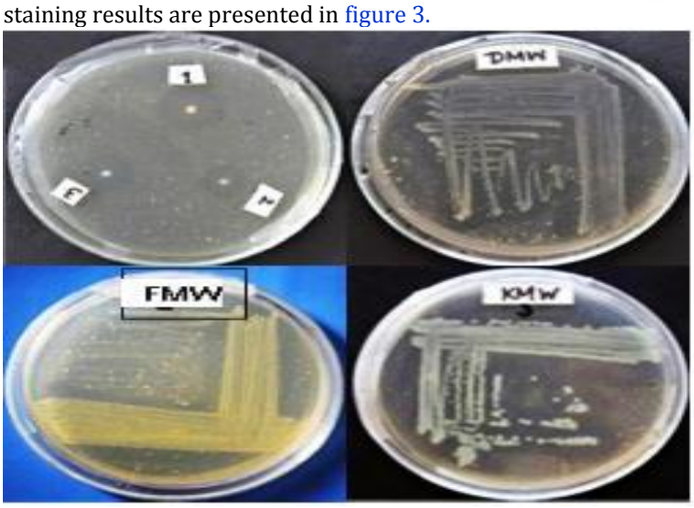

Figure 2: Screening of three proteolytic bacterial isolates and their efficiency test on SMA media and pure cultures of three distinguished isolates. DMW, FMW and KMW represent isolates from dairy, fish and chicken-based waste source accordingly.

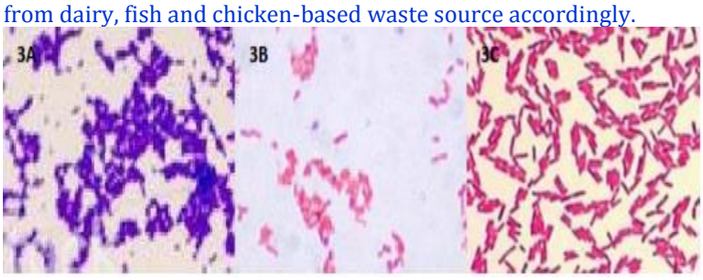

Figure 3: Gram staining of three isolates. 3A, B, C indicates isolates 1, 2 and 3, respectively.

Sample source	Isolates	Zone diameter (mm)	Colony diameter (mm)	Efficiency
DMW	1	16	2	8
FMW	2	14	2	7
KMW	3	12	2	6
TMW	4	-	-	-

Table 1: Screening of efficient alkaline protease producing isolates on the basis of zone of clearance.

Morphological Characteristics	Bacterial Isolates		
	Isolate 1	Isolate 2	Isolate 3
Colony surface	Smooth	Smooth	Smooth
Colony color	Organish	Yellowish	Greenish white
Colony size and shape	Small, circular	Medium, circular	Big, circular
Visual quality	Opaque	Opaque	Opaque
Colony height	Raised	Raised	Raised
Biochemical Characteristics			
Gram test	Non sticky	Sticky	Sticky
Gram's staining	Positive	-	-
Shape	Rod	Rod	Rod
Motility	Positive	Positive	Positive
Spore	Negative	Negative	Negative
Ability to grow in the air	Positive	Positive	Positive
Citrate test	Negative	Positive	Positive
Catalase test	Positive	Positive	Positive
Indole test	Negative	Negative	Negative
Glucose fermentation	Positive	Negative	Positive
Oxidase production	Negative	Negative	Negative
Mac conkey Agar	Negative	Positive	Positive
MR test	Positive	Negative	Positive
VP test	Positive	Negative	Positive

Table 2: Morphological and biochemical characteristics of bacterial isolates.

A thorough biochemical analysis was conducted for each of the three isolates and is tabulated in table 2. It was observed that Isolate 1 and 3 exhibited positive results for glucose fermentation, Voges-Proskauer test, and methyl-red test, whereas isolate 2 showed negative results for all of these tests. All 3 isolates showed negative results for indole and oxidase production tests. On the other hand, all isolates demonstrated positive results for citrate and catalase tests, except for isolate 1 which showed a negative result in the citrate test. Based on the results of cultural, morphological characteristics and biochemical tests isolate 1 has been identified as G+ve Firmicutes (*Bacillus sp),* and 2, 3 were identified as G-ve proteobacteria.

Molecular identification: Genomic DNA of the selected bacterial isolates was extracted and subsequently amplified by polymerase chain reaction (PCR) for molecular identification by 16S rDNA sequencing. The PCR amplicon from the genomic DNA of Isolate 2 and 3 were approximately 1433 and 1380 base pair, respectively (figure 4). Due to insufficient base pair of isolate 1 (397 bp) in sequencing, it was omitted in further identification process. Blast result revealed that isolate 2 DNA sequence was 98.2% homologous to the nucleotide of *Stenotrophomonas maltophilia* strain IAM 12423 (NR_041577) and isolate 3 showed 99.24% sequence similarity with *Pseudomonas hibiscicola* strain ATCC 19867 (NR_024709). The sequences are deposited in NCBI database and can be retrieved through project accession ID PRJNA1022797.

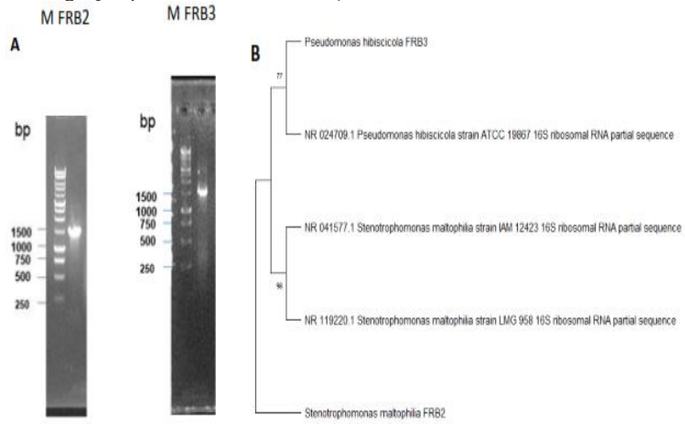

Figure 4: (A) Purified amplicons from both isolates in agarose gel electrophoresis M: denotes 1 kb DNA ladder (Marker) and (B) Phylogenetic tree showing the relationships of FRB2 and FRB3 with other related species.

DISCUSSION: Municipal solid waste is a useless, unwanted, discarded materials arising from domestic, agricultural, industrial, trade and commercial sources as well as public services (Alamgir and Ahsan, 2007). Rapid urbanization has led to high annual production of MSW and made solid waste management a serious problem today. In Chittagong city corporation area, around 1258.61 (Ton/Day) waste has been produced during dry season and 1837.57 (Ton/Day) during wet season (Alamgir and Ahsan, 2007; YPSA, 2024). Therefore, the unprocessed MSW is the main cause of environmental pollution and public health hazards in many cities, especially in developing countries like Bangladesh. To overcome these problems, biotechnology is an interesting tool to add value to the processes involved in MSW disposal/reuse. Therefore, the alternate or suitable solution is utilization of MSW by an eco-friendly approach. With these views, our project study was conducted for isolation, screening and identification of alkaline protease producing bacterial isolates from protein based municipal wastes. Among six samples protease producing bacteria have been found from 5 waste sources. Bacteria from tannery sources did not show protease activity though protease produced by Halophilic bacteria was reported (Mellado *et al.*, 2005). Potential proteolytic bacteria grown on SMA media (pH 9) indicates that the colonies are alkaliphiles (organism grows well in alkaline pH) and they might produce highest amount of protease enzyme at alkaline pH. Morphological test suggests that, all 3 isolates were smooth and opaque colonies with distinct colony color. Biochemical identification of the 3 isolates revealed that isolate 1 and 3 could ferment glucose and showed positive result for VP and MR test. All isolates have citrate and catalase activity except isolate 1 which doesn't possess the citrate activity. All 3 isolates were incapable of producing indole and oxidase when tested. Based on the comparison of biochemical analysis, isolate 1 was found to be G+ve Firmicutes (*Bacillus sp),* and 2, 3 G-ve proteobacteria. Hakim *et al.* (2018) isolated and identified *Bacillus subtilis* from MSW as alkaline protease producers. Jessika *et. al.*, produced alkaline protease from *Bacillus licheniformis (*Dos Santos Aguilar *et al.,* 2019*).* Saggu and Mishra (2017) also characterized thermostable alkaline proteases from *Bacillus infantis SKS1* isolated from garden soil. For accurate confirmation of these isolates further molecular approach was commenced by using 16s rDNA sequencing. Since the sequencing quality was not good and the sequence length is very small it was not possible to identify the isolate 1 at molecular level. Based on the

sequencing result, BLAST search revealed the isolate 2 and 3 as *Stenotrophomonas maltophilius* strain and *Pseudomonas hibiscicola,* respectively. *S. maltophilia* has been recovered from soils and plant roots, animals invertebrates, water treatment and distribution systems, wastewater plants, sinkholes, lakes , rivers (Brooke, 2012). (Miyaji *et al.* (2005) isolated a novel extracellular alkaline serine protease from *S. maltophilia* strain S-1. A novel psychro-tolerant bacterium *Stenotrophomonas maltophilia* (MTCC 7528) with an ability to produce extracellular, cold-active, alkaline, and detergent-stable protease was isolated from soil samples obtained from Gangotri glacier, Western Himalaya, India was also reported by Kuddus and Ramteke (Kuddus and Ramteke, 2009).

CONCLUSION: The extracellular alkaline protease producers were isolated from protein based MSW and screened on SMA medium. Three proteolytic bacterial isolates were identified initially as G+ve Firmicutes (isolate 1), and G-ve proteobacteria (isolate 2 and 3) based on cultural, microscopical and biochemical test. Molecular identification through 16S rDNA sequencing and bioinformatics analysis revealed the Isolate 2 as *Stenotrophomonas maltophilia*, and isolate 3 as *Pseudomonas hibiscicola*. Due to short sequence length isolate 1 could not be identified but all cultural, microscopic and biochemical results align this isolate with *Bacillus* sp.

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INFORMED CONSENT: The author(s) have reviewed the entire manuscript and approved the final version before submission.

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