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Isolation, Screening, Characterization, and Identification of Alkaline Protease Producing Bacteria from the Dairy Industrial Soil

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ABSTRACT: The purpose of the present study was to isolate, screen and identify protease producing bacteria from two dairy industry soil located in Sankarankoil, Tenkasi district, Tamilnadu, India-627761. The samples are collected and stored based on the microbial standard protocol. Serial dilution and skim agar media are followed for the primary screening to isolate protease producing bacteria. Among twenty isolated microbial cultures two significant organism JKSP1 and MJSP2 are selected based on the highest protease activity zone. The morphological and biochemical tests reveal the isolated species was found to be *Bacillus* species. Molecular identification was also carried out for the isolated species using 16s RNA sequencing and data are submitted to the gene bank with the accession number (OK605054.1) and (ON994262.1). The BLAST similarity shows that isolate JKSP1 is 100% homologous to *Bacillus* sp strain (OQ071600.1) and MJSP2 100% homologous to *Bacillus subtilis* (ON994262.1). Additionally, optimizing protease production on a large scale remains a difficult task. Hence this study mainly focused on enzyme production optimization and characterization with respect to pH, temperature, incubation time and different substrates like metal iron, carbon, nitrogen sources and agro-bases. The optimization results indicate that; the isolated organism MJSP2 shows promising production of enzymes at minimum resources and physical parameters. The overall outcome concluded MJSP2 strains show efficient proteolysis activity and can be considered an effective protease producer for commercial value.

Keywords: *Bacteria*, *Bacillus* sp, *Bacillus subtilis*, Protease, Soil, Optimization.

INTRODUCTION

Proteases are industrial value enzymes with many functional roles in all living organisms in cell growth, amplification of immune system regulation, protein catabolism, cell growth and metastasis etc. (Inácio *et al*., 2015; Solanki *et al*., 2021). They are acknowledged as being economically significant and account for nearly 60% of the market for enzymes used in the production of leather, food, and laundry detergents (Lassoued *et al*., 2015; Liu *et al*., 2017). Proteases are highly active and stable under alkaline conditions (Contesini *et al*., 2018). Based on the mode of action proteases differentiate into several types as exopeptidases and endopeptidases (Razzaq *et al*., 2019). Protease isolation can possibly make use of several sources such as animals, plants and microorganisms. Among those, Microbial proteases have a significant impact on a number of industries, including the pharmaceutical, detergent, photographic, waste-treatment, and tanning sectors etc.

In general, microorganisms process a wide array of protease enzymes in two forms intracellular and extracellular mode. Intracellular proteases are considered important for sporulation and differentiation. Whereas, extracellular proteases function as protein hydrolysis in cell free environments and help cells to absorb and utilize hydrolytic products (Karigar and Rao 2011). Microorganisms based synthesis has several advantages such as they grow rapidly and limited space is only required for growth, so they are the preferred source of extracellular enzymes (Patel *et al*., 2017). The quality of protease production by these organisms is directly influenced by the amount of nitrogen source differ from organism to organism (Souza *et al*., 2015). These extracellular enzymes are produced by different varieties of microorganisms such as fungi, bacteria, yeast, and actinomycetes (Sharma *et al*., 2015). Protease production is dominated by bacterial species, with the genus *Bacillus* being the most prevalent source (Rozanov *et al*., 2021).

Novel protease enzymes must be created in order to use these enzymes for additional purposes. Additionally, industrial waste-produced enzymes from bacteria are a bigger help in demonstrating the importance of turning industrial pollutants into highly useful enzymes, especially proteases (Masi *et al*., 2021). The main purpose of this study is the isolation, screening and identification of morphological and biochemical aspects of protease-producing bacteria from dairy industry soil.

MATERIALS AND METHODS

Sample collection. Soil samples JKSP1 and MJSP2 were collected from two dairy industry Muralya Dairy Products Pvt. Ltd and Creamline Dairy Products Pvt Ltd from the Sankarankoil, Tenkasi district, Tamilnadu, India-627761 respectively. The samples were collected in sterile poly bags, labelled with the date and site of collection and stored in refrigerate at 4˚C till further experimental processing.

Bacterial strains isolation and Screening. The soil samples were serially diluted, and 0.1 ml of the diluted sample was plated on skim milk agar plates containing Peptone 0.1%, NaCl 0.5%, Agar 2%, and Skim Milk powder 10%. The medium was sterilized at 121 ˚C for 15 minutes at 15lbs pressure. The plates were incubated at 37˚C for 48 hours (hrs). After the incubation period, the plates were observed for colonies showing a zone of clearance. The bacterial colonies were selected based on the highest zone of clearance and a subsequent subculture of JKSP1 and MJSP2 isolate, and maintained in the nutrient agar slant at 4˚C for further study (Battineni *et al*., 2021).

Characterization of bacterial culture. Various biochemical tests were performed to characterize the bacterial culture, including the Fermentation test, Casein hydrolysis, IMViC test, catalase, nitrate test, and urease test. Morphological experiments, such as Gram staining studied out using Bergey's manual of systemic bacteriology (Holt *et al*., 1994).

Bacterial identification using 16S rRNA gene sequencing. The two isolated strain was screened based on the above traits and the efficient isolate was sent to Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India for molecular characterization based on 16S rRNA sequencing. The isolate having the highest biomass, and alkaline protease production in the submerged fermentation study was identified using the molecular technique. The genomic DNA of the isolate was extracted by using the Bacterial Genomic DNA extraction kit according to the manufacturer protocol (Nucleospin® Tissue Kit (Macherey-Nagel)). The isolated DNA was then amplified using the following PCR mix: 0.25µl of bacterial universal 16S rRNA primers forward 16s-RS-F (CAGGCCTAACACATGCAAGTC) and 0.25µl of reverse primer 16s-RS-F(GGGCGGWGTGTACAAGGC), 1µl of genomic DNA and 4µl of PCR grade water were added and the PCR amplification was done. Amplified sequence threads were submitted to the NCBI database and NCBI BLAST (http://www.ncbi.nlm.nih.gov/Blast) was carried out to distinguish the nearest neighbours of the isolates and then a highly similar homologous species were executed (Drancourt *et al*., 2000; Koch and Koch 1995).

Determination of protease activity. The potential protease-producing bacterial strains' protease activity will be evaluated using the universal protease assay with casein as a substrate. 1 ml of potential alkaline protease producing bacterial sample was taken after 48 hrs of incubation with nutrient broth medium at pH 8.5 and centrifuged at 10,000 rpm for 15 min at 4 °C. The tyrosine standard curve will be obtained from the absorbance of the tyrosine concentrations (10, 20, 30, 40 and 50 µg) to determine the protease activity at 620 nm in a UV spectrophotometer (Systronics 117, India) and the enzymatic activity was calculated using the following equation (Rebecca *et al*., 2012).

Unit per/ml =

$$\frac{\text{Micromole of tyrosine equivalent release} \times \text{reaction Volume}}{\text{Sample volume} \times \text{reaction time} \times \text{volume assayed}}$$

Protease enzymatic assay. Casein (Qualigens fin chemicals Pvt Lts, India) 2% solution at 0.5ml along with 2ml of enzyme solution was incubated at 37˚C. The reaction was stopped after 10 minutes by adding 1 mL of 10% trichloroacetic acid. The mixture was centrifuged for 5 minutes at 10,000 rpm. The supernatant was collected. 1 ml of two-fold diluted Folin–Ciocalteu reagent and 5 ml of 0.44M sodium carbonate were added. The blue colour produced after 30 minutes of incubation was measured at 660nm against a reagent blank prepared in the same way but without an enzyme in a UV spectrophotometer (Systronics 117, India). One unit (U) is defined as the amount of enzyme that breakdown the amino acid of 1 micromole of casein per minute (Shafique *et al*., 2021).

Effect of Incubation time, pH and temperature physical factors on enzyme production. The optimization of various physical parameters on protease production was assessed by growing bacterial culture in the standard growth media with varying incubation time, pH and temperature. Investigation of optimum incubation time for protease production was estimated by different time periods up to 72hrs at 160 rpm with 12 hrs time intervals and 37ºc was maintained for the entire cycle. The medium was prepared with different pH range from 4 to 9 and incubated for 48 hrs respectively. In the case of temperature optimization for growth factor, the medium was maintained with five different temperature ranges from 20°C to 60°C for 48 hrs respectively.

Optimization of carbon, nitrogen and metal ions substrates in protease production. The media used for the production of protease was subjected to optimization with respect to different carbon, nitrogen, metal iron and agro-based substrate concentration. In carbon source optimization the media was supplemented with glucose, galactose, sucrose, starch, lactose, barley, and fructose incubated at 60°C for 48 hours. Similarly to optimize the nitrogen source beef extract, peptone, casein, sodium nitrate, urea, and yeast extract were used. The effect of various metal ions on the production of protease was examined by incubating the production medium at 60°C for 48 hrs in the presence of 10 mM concentrations of metal ions such as cobalt chloride, zinc sulphate, sodium chloride, manganese sulphate, nickel carbonate and magnesium sulphate activities were assayed at standard assay conditions.

Effect of the different agro product as substrates on the protease production. The effect of the appropriate agro-based substrate on protease production, different substrates like groundnut oil cake, coconut oil cake, rice bran, castor, black gram and sesame oil cake was taken under submerged fermentation process and the enzyme

activity was measured as per the standard assay conditions.

RESULTS

Protease isolation from Dairy industry soil bacteria. In order to observe bacterial consortium, bacteria from the dairy industrial pollutant soil were serially diluted and then placed on media using the pour plating method. Each unique morphological characteristic was regarded as a different bacterial species based on colony morphology and was subjected to the streak plate method for pure colony isolation. A total of 20 different protease producing bacterial strains from the two dairy industry soil samples. The isolates were sub-cultured and maintained in media for future tests. Among those, two distinct isolates namely JKSP1 (25 mm), and MJSP2 (33 mm) having the highest zones of inhibition were selected for further studies, and the results are tabulated in and shown in (Fig. 1).

Identification of screened bacterial isolates. The two isolated bacterial strains were grown on a nutrient agar medium to study their morphological characteristics. JKSP1 and MJSP2 isolates showed circular colonies, rough, opaque, and fuzzy white. Gram staining results revealed that the isolates are rod shaped and gram-positive. Morphological characteristics of the isolated bacterial strains and the gram staining results were shown in (Fig. 2). An extensive biochemical characterization of all two isolates were also carried out.

Optimization of Incubation time, pH and temperature physical factors on enzyme production. The production of protease optimization with respect to different physical parameters like pH, temperature and incubation time interval was studied. The result of physical parameters such as incubation on protease production MJSP2 shows maximum production as 6.66 U/ml at 48hrs and minimum production as 3.42 U/ml at 12hrs of incubation when compared to JKSP1. The result also reveals that both strains show a reduction in protease production at 72hrs incubation Table 1 (Fig. 3). In the case of optimization in pH for the protease production, MJSP2 shows maximum production as 6 U/ml in neutral pH 7 when compared to JKSP1 after 48hrs respectively. Whereas, at acidic conditions, less production of protease was observed at 1.3 U/ml and 2.45 U/ml in JKSP1 and MJSP2 at pH 4 respectively Table 2 (Fig. 4). The optimization of temperature shows the promising yield of protease among JKSP1 and MJSP2 as 7.33 U/ml and 7.55 U/ml at 40ºC. Maximum production of protease was observed in MJSP2 media as 9.55 U/ml at 60ºC Table 3 (Fig. 5).

Optimization of carbon, nitrogen and metal ions substrates in protease production. In carbon source optimization, the media supplemented with galactose showed a maximum yield of protease production in MJSP2 as 7.22 U/ml when compared to JKSP1 protease production value of 2.1 U/ml respectively Table 4 (Fig. 6). Similarly, the optimization of the nitrogen source casein supplement shows the maximum yield of protease production as 8.56 U/ml by MJSP2 when compared to JKSP1. In this, the media supplement with sodium nitrate and urea also show significant production of protease in MJSP2 after 48hrs Table 5 (Fig. 7). In the case of the effect of various metal ions on the production of protease, the media supplement with zinc sulphate maximum production of protease was observed in both JKSP1 and MJSP2 as 4.5 U/ml and 4.98 U/ml respectively Table 6 (Fig. 8).

Effect of the different agro product as substrates on the protease production. The effect of the different agro-based substrates on protease production results shown maximum production of protease was observed in JKSP1 at ground nut oil cake substrates media as 10.2 U/ml compared to MJSP2 as 4.25 U/ml respectively. Whereas, MJSP2 shows maximum production at coconut oil cake substrates as 8.55 U/ml Table 7 (Fig. 9).

Molecular identification. Genomic DNA of the selected bacterial isolate JKSP1 and MJSP2 was extracted and the same was amplified by polymerase chain reaction (PCR) and visualized using agarose gel electrophoresis. Blast similarity search results revealed that JKSP1 are 100% homologous to *Bacillus* sp strain (OQ071600.1) and MJSP2 100% homologous to *Bacillus subtilis* (ON994262.1). The sequence results were submitted to the gene bank database and the accession number as fallow JKSP1 (OK605054) and MJSP2 (ON994262) respectively.

DISCUSSIONS

The present overall analysis reported 20 different strains from the two dairy industry soil samples out of them two bacterial samples are shown high properties of protease enzyme production as their extracellular products. From our study, it was understood that the amount of yield and success rate depended on the number of isolated samples and site areas. The larger sample size will directly influence the protection rate of the enzyme. The isolated strains underwent initial screening to determine whether they were capable of producing proteases. Following the results of the primary screening of 20 isolates, the highest clearance zone was formed by two potential protease bacteria isolates JKSP1 and MJSP2 isolates (25 and 33 mm) respectively. Bacterial morphological studies based on microscopic analysis and colony morphology of the two isolated cultures revealed that all two were motile rod-shaped bacteria possessing where JKSP1 and MJSP2 isolate is determined to be Gram-positive *Bacillus* species (Økstad *et al*., 2011; Gordon, 2019).

It was reported that Bacillus species are well known and effective for the production of the protease when compare to the other microbial flora (Kuebutornye *et al*., 2019) Submerged fermentation process revealed the higher protease activity of MJSP2 and its commercial potential for larger product recovery. Hence, MJSP2 isolate was considered for further molecular identification, and with the aid neighbourhood method, was found to be *Bacillus subtilis* and also its best protease-production bacteria (Padmapriya and Williams 2012).

In the field of industrial biotechnology significance of protease production becomes commercial impact only after suitable economic growth by means of medium optimization for maximum yields (Chi *et al*., 2007). Hence the present study also focuses on the optimization of the growth media and the

characterization of other physiological factors in protease production. The standard media was optimized initially with different carbon and nitrogen sources as described in Table 4 & 5. In that, the media supplemented with galactoseas a carbon source showed a maximum yield of protease production in MJSP2 as 7.22 U/ml. The results also reveal that lactose supplements have shown less significance in protease production. Similarly, the optimization of the nitrogen source casein supplement shows the maximum yield of protease production as 8.56 U/ml by MJSP2. In this, the media supplement with sodium nitrate and urea no production of protease were observed after 48hrs. Therefore, it was concluded that sodium nitrate and urea are not suitable subtract for protease production. Whereas, In the case of the effect of various metal ions the production of protease was observed a unique impact in both bacterial species the media JKSP1 and MJSP2 as 4.5 U/ml and 4.98 U/ml in media supplement with zinc sulphate respectively.

In the case of optimization in pH for the protease production, MJSP2 show maximum production as 6 U/ml in neutral pH 7 when compare to JKSP1 after 48hrs respectively. Whereas, at acidic pH 4 conditions, low production of protease was observed in both bacterial species. There are several reports point out that, pH 6-7 is the optimum pH for protease production (Ahmed *et al*., 2011). It also reports that alteration in the ionic bond pattern in protein will reduce the catalytic functions (Siddiqui and Thomas 2008). Hence the protease production was observed maximum at higher pH and lower in acidic pH. Temperature optimization also play important role in the bio-product of microorganism. It is directly proportional to the energy requirement and consummation in the fermentation industry to achieve economic growth (Chen and Jiang 2018). The optimization of temperature result shows the promising yield of protease among JKSP1 and MJSP2 as 7.33 U/ml and 7.55 U/ml at a minimum temperature 40ºC. At the same time when increasing the temperature the production rate also significantly increased to 9.55 U/ml at 60ºC in MJSP2. The effect of the different agro-based substrates on protease production results indicates maximum production of protease in JKSP1 at ground nut oil cake substrates media as 10.2 U/ml compared to MJSP2 as 4.25 U/ml respectively.

Advanced development in genomics and proteomics technology make a significant role in bacterial identification and characterization. Genomic DNA of the selected bacterial isolate JKSP1 and MJSP2 were identified as *Bacillus sp* strain and *Bacillus subtilis* species. The result of the sequence of the isolated species is deposited in Genbank for future molecular identification of the species as JKSP1 (OK605054.1) and MJSP2 (ON994262.1) respectively. Bacterial molecular characterization data help in expose the mechanisms that microbial functions at the molecular level, which leads to the invention of new proteins and enzymes for the development of valuable bio-product in agriculture and industrial biotechnology. It also may lead pathways to develop new vaccines and other therapeutics products.

Table 1: Optimization of the Incubation Time Interval.

Sr. No.	Incubation In Hrs	Enzyme Activity (Unit/ml)	
		JKSP1	MJSP2
1.	12	3.06	3.42
2.	24	3.45	2.55
3.	36	4.3	4.82
4.	48	5.23	6.66
5.	72	4.1	4.32

Table 2: Optimization of the pH.

Sr. No.	pH	Enzyme Activity (Unit/ml)	
		JKSP1	MJSP2
1.	pH4	1.3	2.45
2.	pH5	2.2	1.69
3.	pH6	3.02	3.02
4.	pH7	4.2	6
5.	pH8	5.84	4.2
6.	pH9	4.79	4.79

Table 3: Optimization of the Incubation Temperature Interval.

Sr. No.	Temperature	Enzyme Activity (Unit/ml)	
		JKSP1	MJSP2
1.	20 ºC	2.55	4.1
2.	30 ºC	4.6	4.22
3.	40 ºC	7.33	7.55
4.	50 ºC	6.54	8.43
5.	60 ºC	6.45	9.55

Table 4: Optimization of the Carbon source substrate.

Sr. No.	Carbon Source	Enzyme Activity (Unit/ml)	
		JKSP1	MJSP2
1.	Glucose	1.06	2.42
2.	Galactose	2.1	7.22
3.	Sucrose	2.35	2.62
4.	Starch	5.02	5.62
5.	Lactose	3.45	2.25
6.	Bareley	6.59	4.5
7.	Fructose	4.2	5.23

Table 5: Optimization of the Nitrogen source substrate.

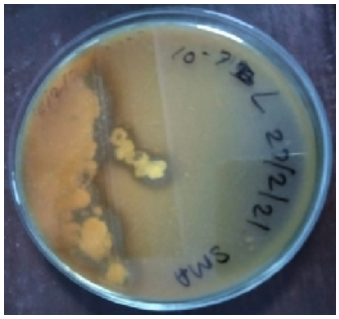
Sr. No.	Nitrogen Source	Enzyme Activity (Unit/ml)	
		JKSP1	MJSP2
1.	Beef extract	5.42	5.36
2.	Peptone	6.89	6.56
3.	Casein	5.41	8.56
4.	Sodim nitrate	4.99	7.45
5.	Urea	5.2	6.5
6.	Yeast extract	3.35	5.23

Table 6: Optimization of the Metal Ions substrate.

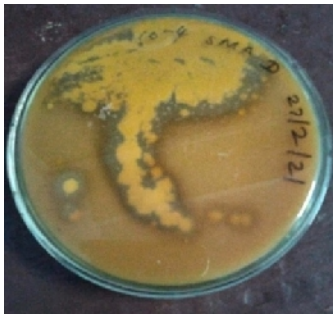
Sr. No.	Metal Ions	Enzyme Activity (Unit/ml)	
		JKSP1	MJSP2
1.	Cobalt chloride	2.5	3.85
2.	Zinc sulphate	4.5	4.98
3.	Sodium chloride	3.89	3.5
4.	Mangonoussulphate	4.21	3.45
5.	Nickel carbonate	4.2	4.56
6.	Megnesiumsulpahte	3.6	3.5

Table 7: Optimization of the Agro-based substrate.

Sr. No.	Agro-based substrate	Enzyme Activity (Unit/ml)	
		JKSP1	MJSP2
1.	Ground nut oil cake	10.2	4.25
2.	Rice bran	7.5	5.66
3.	Castor	4.36	4.25
4.	Black gram	4.9	5.12
5.	Sesame oil cake	8.65	8.45
6.	Coconut oil cake	7.62	8.55

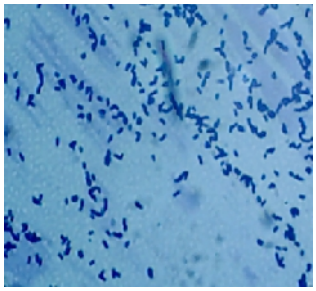


(a) JKSP1

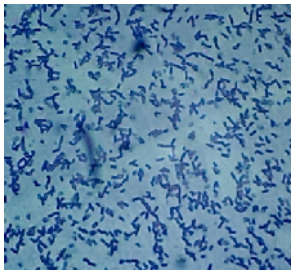


(b) MJSP2

Fig. 1. Screening of protease producing bacteria isolates.



(a) JKSP1



(b) MJSP2

Fig. 2. Gram staining of bacteria isolates.

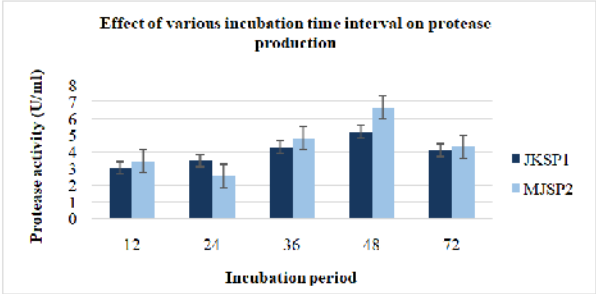


Fig. 3. Effect of various incubation time interval on protease production.

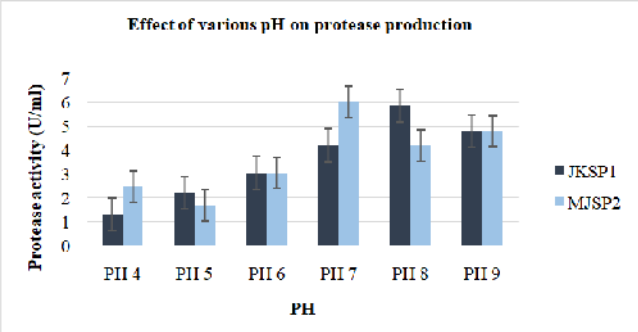


Fig. 4. Effect of various pH on protease production.

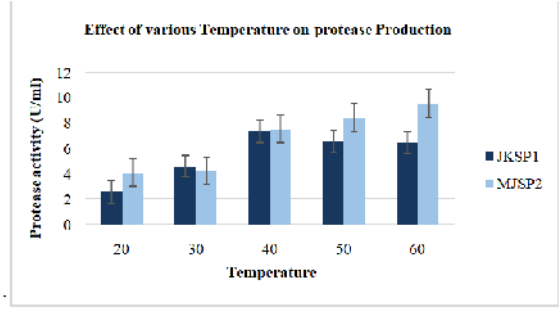


Fig. 5. Effect of various Temperature on protease Production.

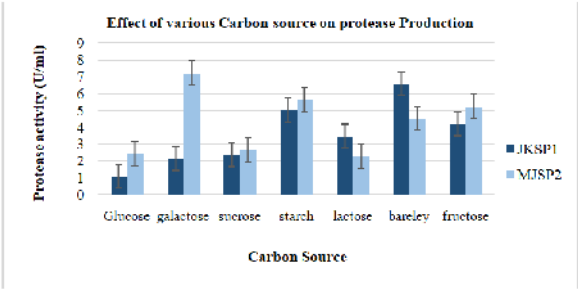


Fig. 6. Effect of various Carbon source on protease Production.

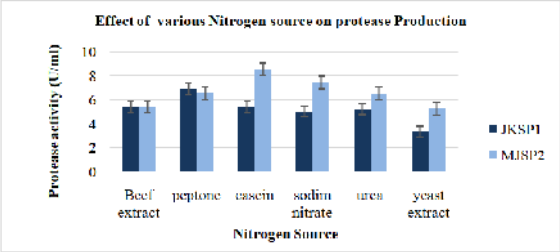


Fig. 7. Effect of various Nitrogen source on protease Production.

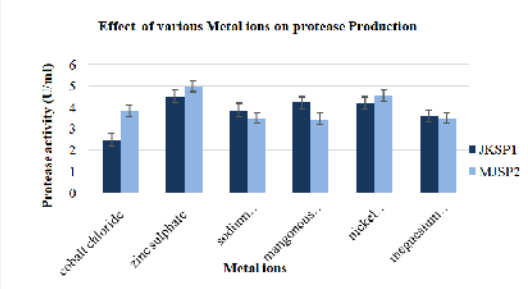


Fig. 8. Effect of various Metal ions on protease Production.

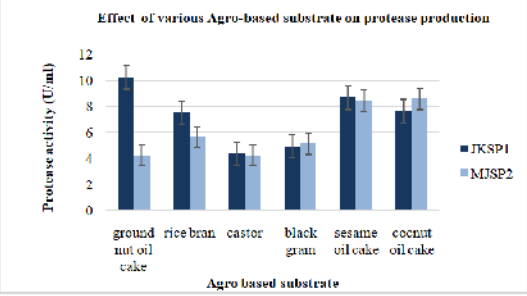


Fig. 9. Effect of various Agro-based substrate on protease production.

CONCLUSIONS

The present study was focused on isolating, screening, and identification of protease producing bacteria from dairy industry soil samples. Among 20 isolated samples, two bacterial isolates showed potent protease activity on skim milk agar plate assay. The bacterial isolates were then further screened for alkaline protease production using the submerged fermentation method and the isolated MJSP2 was observed to have a maximum protease enzyme. Based on morphological and biochemical characteristics the strain was identified as Bacillus species which was later identified as *Bacillus sp* strain and *Bacillus subtilis* species using 16S rRNA sequencing techniques.

FUTURE SCOPE

Future applications and uses of enzymes made from bacterial sources have been recognized for their significant use in waste management and enzyme engineering for biotechnological applications. The genetic characterization of bacteria's protease-producing genes has a promising effect on the large-scale production of enzymes to meet market demand.

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Conflict of Interest. None.

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