Molecular characterisation and optimisation of isolated extracellular alkaline protease from *Bacillus* sp. from dairy industrial soil

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Abstract:

"Proteases," or proteolytic enzymes, cleave the peptide bonds that join two amino acids. They adhere to the principles of hydrolytic reactions. Both prokaryotic and eukaryotic species manufacture proteolytic enzymes, which have important biological roles. Proteolytic enzymes are highly valuable commercially since the food, dairy, detergent, and leather processing industries all need them. A number of protease-based medicines have been approved, and proteolytic enzymes have also become recognized as therapeutics. Considering their great productivity and ease of enzyme purification, microorganisms constitute a key source of commercial proteolytic enzymes. Engineering new specificities, ensuring their stability, and using proteases as medicines provide significant scientific hurdles in the commercial utilization of proteases. In the current study, a bacterial stain with protease activity has been isolated. The production media has also been optimized using different carbon, nitrogen sources, metal ions, agricultural products, and physical parameters including temperature, pH, and incubation time. The soil samples were taken from the muralya dairy products pvt Ltd and creamline dairy products pvt Ltd Sankarankovil, Tenkasi . The isolated species was discovered to be a Bacillus species, according to the morphological and biochemical examinations. The isolated species' molecular identification was also completed using 16s RNA sequencing, and the results have been submitted to the gene bank under the accession numbers JKSP8 (OP835926.1) and JKSP9 (OP835912.). Using the BLAST similarity index, the two isolated strains JKSP8 and JKSP9 were recognized as *Bacillus sp*. and *Bacillus velezensis*, respectively. Nevertheless, optimizing large-scale protease manufacturing remains a difficult task. Thus, the main focus of this work was on the optimization and characterization of enzyme production with respect to pH, temperature, incubation duration, and various substrates or media for the manufacture of proteases, such as metal iron, carbon, nitrogen sources, and agro-bases. The optimization results reveal that the isolated organism JKSP9

produces enzymes with reasonable physical and resource requirements. Starch and yeast, respectively, were the best sources of carbon and nitrogen where JKSP8 and JKSP9 displayed the highest levels of protease production. When compared to JKSP8, which produced 10.5 U/ml at neutral pH 9, JKSP9 exhibits highest production at this pH. The dehairing of goat skin and the elimination of blood stains were the subjects of an application research for the isolated protease enzyme. Overall, it was determined that JKSP9 strains exhibit effective proteolysis activity and can be used as productive producers of commercially valuable proteases.

Keywords: *Bacteria; Bacillus sp; Bacillus velezensis*; Protease, Dairy waste soil, Optimization

Introduction

Protease enzyme is a group of proteolytic enzymes, which hydrolyse the peptide bonds present in proteins to convert it to shorter of polypeptides and amino acids. Proteases act as biocatalysts; their catabolic nature exhibits the selectivity and specificity the unique peptide bond in the substrate, and the conformational changes of the protein are manipulated by cleaving the peptide bond in the polypeptide chain of the amino acid (Gessesse A., 1997, Panda*et al.,* 2013). Protease cascade controls cellular activity, and the variation in a proteolytic system can cause pathological disorders, which include tumour invasion, neurodegenerative ailment, and inflammatory disorders (Rawlings*et al.,* 2013). Protease actions, adopt their specificity as exopeptidases, which cleave the N and C terminal ends of the free amino acids, and endopeptidases, which are proactively involved in cleaving non-terminal ends (Ward*et al.,* 2011). They are ubiquitous in nature and also an integral component of unicellular and multicellular life on planet Earth, such as animals, plants, and microbes.

The microorganisms are convenient sources of protease production based on their technical and economic advantages (Yadav *et al.,* 2019). It is renowned around 70% of the entire industrial enzyme market and is considered a leading and significant hydrolytic enzyme (Singh*et al.,* 2016). Around 40% of worldwide commercial enzymes are derived from microbial sources

obtained from bacteria, yeast, and filamentous fungi through broth fermentation, Bacillus species are the typical microorganisms in protease production, particularly neutral and alkaline serine proteases (Ward*et al.,* 2011). The industrial protease activities are influenced by the various mediums and parameters such as temperature, incubation period, pH, salt concentration, and other substrate ingredients, There are downsides to using enzymes in healthcare and other industries due to their sensitivity to temperature, pH, and other parameters (Solanki et al., 2021,Choi et al., 2015).

The pursuit of novel characteristic proteases for various industrial implementations increases day by day. The reason behind the use of microbial sources instead of plant and animal sources is that they can be used on a large scale in less time, and their growth conditions can be easily optimised in lab conditions and ease gene modification. As a result, low cost can be consistently achieved. (Rayda*et al.,* 2012). Due to the high demand for proteases in the enzyme industry, there is a need to explore novel strains of specific enzymes, optimise their production, and reconcile the parameters. Hence, the present work is focused on the isolation, characterization, and optimisation of protease from a newly isolated *Bacillus sp.* and *Bacillus velezensis* from muralya dairy products pvt Ltd and creamline dairy products pvt Ltd in sankarankovil, Tenkasi.

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MATERIALS AND METHODS

Sample collection

Soil samples were collected from two dairy industries, Muralya Dairy Products Pvt. Ltd and Creamline Dairy Products Pvt Ltd, in Sankarankoil, Tenkasi district, Tamilnadu, India-627761. The samples were collected in sterile plastic bags that were labelled with the time and

location of collection, and they were kept in the refrigerator at 4 °C until 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 sterilised at 121 ˚C for 15 minutes at 15 lbs of pressure.For 48 hours, the plates were incubated at 37°C hrs. Following the incubation time, the colonies on the plates were checked for a zone of clearing.The bacterial colonies were selected based on the highest zone of clearance and a subsequent subculture of JKSP8 and MJSP9 isolates, and maintained in the nutrient agar slant at 4˚C for further study (Battineni*et al.*, 2021).

Characterization of bacterial culture

Fermentation, Casein hydrolysis, IMViC, Catalase, Nitrate, and Urease assays were among the biochemical procedures used to evaluate the bacterial culture. Using Bergey's manual of systemic bacteriology, morphological investigations, such as Gram staining, were carried out(Holt*et al.*, 1994).

Bacterial identification using 16S rRNA gene sequencing

Based on the biochemical characteristics, the two isolated strains were tested, and the effective isolate was forwarded to the Rajiv Gandhi Centre for Biotechnology in Thiruvananthapuram, India for molecular characterization using 16S rRNA sequencing. Using a molecular method, the isolate with the highest biomass and alkaline protease synthesis in the submerged fermentation investigation was found.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: 0.25 µl of bacterial universal 16S rRNA primers forward 16s-RS-F (CAGGCCTAACACATGCAAGTC)and 0.25 µl of reverse primer 16s-RS-F(GGGCGGWGTGTACAAGGC), 1 µl of genomic DNA and 4 µl of PCR grade water were added and the PCR amplification was done. Amplified sequence were 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 and Koch*et al*., 1995).

Inoculum preparation

JKSP8 and JKSP 9 strains **were** inoculated on nutrient broth (37ºC) for 24 hours.

Enzyme production

Production of protease from JKSP8 and JKSP 9 was carried out in a medium containing the following (g/l): glucose 1.0, peptone 10.o, yeast extract 0.2, $Cacl_2$ 0.1, K_2HPo_4 0.5 and $MgSo_4$ 0.1 and maintained at 37ºC for 48 h. The pH of the medium was adjusted at 7.0 before sterilization. Sterile broth medium (900 ml) was inoculated with 100 ml inoculum and incubated at 35ºC for 48 h, then centrifuged at 15000rpm for 10 min at 0ºC and the clear crude enzyme supernatant was stored at - 20ºC for further studies.

Determination of protease activity

The prospective protease-producing bacterial strains 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).

$$\textbf{Unit per/ml} = \frac{\text{Micromole of tyrosine equivalent release X reaction Volume}}{\text{Sample volume X reaction time X volume assayed}}$$

Protease enzymatic assay

Casein (Qualigens fin chemicals Pvt Lts, India)2% solution at 0.5mlalong 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 and the mixture was centrifuged at 10,000 rpm for 5 minutes in order to collect the supernatant. 1 ml of two-fold diluted Folin– Ciocalteureagent 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 aminoacid of 1 micromole of casein per minute (Shafique*et al*., 2021).

The effect of Incubation time and pH on the production of protease

The optimization of physical parameters such as incubation and pH on protease production was assessed by varying incubation time, and pH. Investigation of optimum incubation time for protease production was estimated by different time periods as 12, 24, 36, 48, and 72hrs up to at 160 rpm with 37ºc was maintained for the entire cycle. In case of pH optimization, the medium

was prepared with six different pH range as 4, 5, 6, 7, 8 and 9 and incubated for 48 hrs respectively.

The effect of temperature on the production of protease

Temperature optimization for protease production was carried out with five different temperature elevated ranges from 20°C to 60°C in the order of 20°C, 30°C, 40°C, 50°C and 60°C for 48 hrs respectively.

Optimization of carbon and nitrogen substrates in protease production

The media that was used to produce protease was optimised with regard to various carbon and nitrogen substrate concentrations.The media was supplemented with glucose, galactose, sucrose, starch, lactose, barley, and fructose and incubated at 60°C for 48 hours in the carbon source optimisation process.Similarly beef extract, peptone, casein, sodium nitrate, urea, and yeast extract were used to optimisation the nitrogen source in protease production.

The effect of metal ions substrates on the production of protease

By incubating the production medium at 60°C for 48 hours in the presence of 10 mm concentrations of metal ions, including cobalt chloride, zinc sulphate, sodium chloride, manganese sulphate, nickel carbonate, and magnesium sulphate activities, it was possible to examine the impact of different metal ions on the production of protease.

Effect of the different agro product as substrates on the protease production

Different substrates, including rice bran, wheat bran, sesame oil cake, groundnut oil cake, and coconut oil cake, were taken under submerged fermentation conditions and their effects on the

production of proteases were studied. The enzyme activity was then measured in accordance with the requirements of the standard assay.

Application studies

Blood stain removal

Washing performance analysis of the *Bacillus sp.* and *Bacillus velezensis* protease enzyme preparation is done.

Animal blood was used to stain the cotton fabric and it was air dried. The stained cloth pieces were taken in separate flasks. In 3 flasks, the treatment was given in following combinations.

1. 100 ml detergent solution (7mg/ ml) + stained cloth piece.
2. 100 ml detergent solution (7mg/ ml) +enzyme solution + stained cloth piece.
3. 100 ml tap water + stained cloth piece.

They were incubated at 60ºC for 30 minutes. Later the cotton fabric was taken out, mixed in tap water , dried and examined.

Dehairing of goat skin

Small pieces of goat skin which are freshly obtained from a local slaughter house and rinsed to remove excess blood, were placed into 20ml of enzyme solution. After 8 hours of inoculation at 37ºC , the skins were taken out and the hair was gently hand pulled to test wheather it had parted from the skin.

Goat skin was treated with distilled water and also with purified protease enzyme.

RESULTS

Protease isolation from Dairy industry soil bacteria

Bacteria from the dairy industry pollution soil were serially diluted and then applied to media using the pour plating method in order to observe bacterial consortia. Based on colony morphology, each distinct morphological trait was considered to be a different bacterial species and was submitted to the streak plate method for pure colony isolation. From the two dairy sector soil samples, a total of 20 distinct bacterial strains that produce proteases were initially screened. The isolates were sub-cultured and maintained in media for future tests. Among those, two distinct isolates namely JKSP8(28 mm), and JKSP9 (25 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. JKSP8 and JKSP9 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 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 time on protease production JKSP9 shows maximum production as 8 U/ml at 72hrs

and minimum production as 2.36. U/ml at 12hrs of incubation when compared to JKSP8 as 1.08 U/ml at 12 hrsTable 1 (Fig. 3). In the case of optimization in pH for the protease production, JKSP9 shows maximum production as 10.5 U/ml in neutral pH 9 when compared to JKSP8 as 7.5 U/ml after 48hrs respectively. Minimumproduction was observed at acidic pH 4 for both strains JKSP8 and JKSP9 as 1.8 and 2.56 U/ml (Fig. 4).

Optimization of temperature physical factors on enzyme production

The optimization of temperature shows the promising yield of protease was observed in JKSP9 as 7.6 U/ml at 50 °c. Whereas, both strains show more or less equal production rate at 40°c as 4.1 and 5.2 U/ml respectively (Fig. 5).

Optimization of carbon, and nitrogen substrates in protease production

In carbon source optimization, the media supplemented with sucrose showed a maximum yield of protease production in JKSP8 as 5.12 U/ml andJKSP9show protease production signifance in media supplement with starchas5.58 U/ml respectively. The media supplement with lactose show equal production rate in both strains JKSP8 and JKSP9 as 5.02 and 4.95 U/ml respectively Table 4 (Fig. 6). Similarly,the optimization of the nitrogen source yeast supplement shows the maximum yield of protease production in both strains JKSP8 and JPSP9 as 6.95 and 7.24 U/ml respectively.In this, the strain JKSP9show significant protease production in media supplement with all substrate used as nitrogen source after 48hrs Table 5 (Fig. 7).

Optimization of metal ions substrates in protease production

In the case ofthe effect of various metal ions on the production of protease,the media supplement with sodium chloride maximum production of protease was observed in JKSP9 as 5.2 U/ml and

the media supplement with magnesium sulphate in JKSP8 show maximum production as 4.9 U/ml 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 JKSP8 at castor substrates media as 6.95 U/ml compared to JKSP9 as 4.85 U/ml respectively. Whereas, JKSP8 and JKSP9 shows similar production at sesame oil cake substrates as 6.28 and 6.25 U/mlrespectively Table 7 (Fig. 9).

Molecular identification

Genomic DNA of the selected bacterial isolate JKSP8 and JKSP9 was extracted and the same was amplified by polymerase chain reaction (PCR) and visualized using agarose gel electrophoresis. Blast similarity searchresults revealed that JKSP8 are 100% homologous to Priestiaflexa strain (MF143520.1) and JKSP9 100% homologous to Bacillus velezensis (MT516333.1). The sequence results were submitted to the gene bank database and the accession number as fallow JKSP8 (OP835926.1) and JKSP9 (OP835912.1) respectively.

Application studies

Blood stain removal

Blood stain were destained **by** JKSP8 and JKSP9 within 25 minutes and 30 minutes respectively and no stain removal when washed with distilled water.

Dehairing of goat hair

Goat skins were dehaired by JKSP8 and JKSP 9 within 8 hours and 12 hours respectively.

JKSP9 showed increased dehairing efficiency.

DISCUSSIONS

The two soil samples from the dairy sector shown 20 different strains in present analysis. Among twenty samples were examined, in that two bacterial strains produced maximum external proteases. The maximum zone was absorbed inJKSP8 and JKSP9 isolates as (28and 25 mm) respectively. The morphological follow by microscopic analysis and colony morphology of the two isolated cultures revealed that the isolated bacteria are motile rod-shaped and determined to be Gram-positive *Bacillus species* (Økstad et al., 2011 and Gordon*et al*., 2019).According to a report, when compared to other microbial flora, Bacillus species are well recognised and efficient for the production of protease (Kuebutornye*et al*., 2019) Submerged fermentationprocess revealed the JKSP9 higher protease activity and its shows the capability in commercial potential for larger production.

Advanced development in genomics and proteomics technology make a significant role in bacterial identification and characterization (Yue et al., 2023). Genomic DNA of the selected bacterial isolate JKSP8 and JKSP9 were identified as *Bacillus subtilis* species. The result of the sequence of the isolated species is deposited in Genbank for future molecular identification of the species as JKSP8 (OP835926.1) and JKSP9 (OP835912.1) respectively. A recent genetic analysis of Bacillus velezensis highlights the species' uniform dispersion in the environment along with its numerous fungi and bacteria-inhibiting abilities. In addition to these, it has a rich metabolite pool. The gene function of Bacillus velezensis will be better known with the advancement of molecular research technologies, which may enhance the sustainability of the application (Ye et al., 2018). Bacterial molecular characterization data reveal the molecular

processes by which microbes function, leading to the discovery of novel proteins and enzymes for the synthesis of valuable bio-products in agriculture and industrial biotechnology.

The major aim of the study is to focuses on the optimization of the growth media and the characterization of other physiological factors in protease production. Hence it was report that, significance of protease production becomes commercial impact only after suitable economic growth by means of medium optimization for maximum yields (Chi*et al*., 2007). The standard media was optimized initially with different pH, in this JKSP9 show high production as 10.5 U/ml in basic pH 9 when compare to JKSP8 after 48hrs respectively. Whereas, at acidic pH 4 conditions, reduction of protease production was observed in both JKSP8 and JKSP9.It reflect the outcome of report by Ahmed *et al.,* 2011, production of protease was significantly in pH 6-7. It also reports that alteration in the ionic bond pattern in protein will reduce the catalytic functions (Siddiqui*et al*., 2008). Hence the protease production was observed maximum at higher pH and lower in acidic pH. In case of incubation time optimization in protease production, different time periods was followed as 12, 24, 36, 48, and 72hrs. In this, JKSP9 shows maximum production as 8 U/ml in 72hrs of incubation. Low production rate was observed in JKSP8 as 1.08 U/ml at 12 hrs. In addition, increasing the incubation time show increase in protease production rate in both JKSP8 and JKSP9 respectively.

The bioproduct that microorganisms produce is greatly influenced by temperature optimisation. To accomplish economic expansion, the energy consumption and requirements in the fermentation sector are directly correlated (Chen *et al*., 2018). The result reveals that, the

high yield of protease was observedin JKSP8 as 7.6 U/ml and 7.1 U/ml in 50ºc and 60ºc. Additionally, it was concluded that, when increasing the temperature the production rate also significantly increased in both JKSP8 and JKSP9.

Some specific nutrients are necessary for the growth and maintenance of metabolic processes in microorganisms (Garcia-Lopez *et al.,* 2021). Depending on the microorganism, different amounts and types of nutrients are needed such as water, an energy source, nitrogen, vitamins, and minerals (Saeed*et al.,* 2013).Optimization of carbon and nitrogen sources supplement was as described in table 5. In that, the sucrose supplemented media for carbon source showed a high yield of protease production in JKSP9 as 5.58 U/ml. In the nitrogen source optimization, media supplement with casein shows the high yield of protease production in both JKSP8 and JPSP9 as 6.95 and 7.24 U/ml respectively. In this, the all media supplementsfor nitrogen source show promising significant productions in both JKSP8 and JKSP9 after 48hrs.Whereas, the effect of various metal ions substrate optimizationsodium chloride supplemented media show high protease productionin JKSP9as 5.2 U/ml. whereas, the media supplement with cobalt chloride shows less protease production in JKSP8 and JKSP9 as 2.4 U/ml and 2.45 U/ml when compare to other supplement used for metal ion source. The results reflect the general statement as, sources of carbon and nitrogen are crucial components in the development of protease in culture medium (Pant *et al.,* 2015).

The effect of the different agro-based substrates on protease production, JKSP8 show high production rate in JKSP8 as 6.95 U/ml at castoradded substrates media. Whereas, low production rate was observed in JKSP9 as 3.82 U/ml in rice bran added substrates media. Additionally, JKSP8 show significant production rate in all agro-based substrates (Table

7).Overall, the outcomes corroborate that the production of protease significantly varies with temperature and pH. The other media substrate component also influences the product and growth of the bacterial species (Maghsoodi *et al*., 2013, Kumar Bajaj *et al.,* 2013).

Reports by Venugopal and Saramma (2007) proved the efficiency of protease in the removal of blood stains. Here in the present study, JKSP8 showed higher and speedy destainig by 25 minutes rather than JKSP9 within 30 minutes. From this study, we conclude that protease enzyme from JKSP8 and JKSP9 could be used as detergent and protease enzyme from JKSP8 could act as good detergent.

The dehairing protease from *Bacillus* sp. SB12 can be profitably employed as powerful alternative of polluting lime-sulphide method for reducing pollution load in effluent substantially without affecting the quality of the leather produced (Briki *et al*., 2016). Here, in this present study, JKSP8 and JKSP9 showed speedy dehairing within 8 hours and 12 hours respectively. So we can conclude that, this enzyme could be used in leather industry. Application of microbial enzyme will be eco-friendly.

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  nzymes in leather industry help develop eco-friendly process much useful for the environment.

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Figures

Fig. 1: Effect of incubation time interval on the rate protease production.

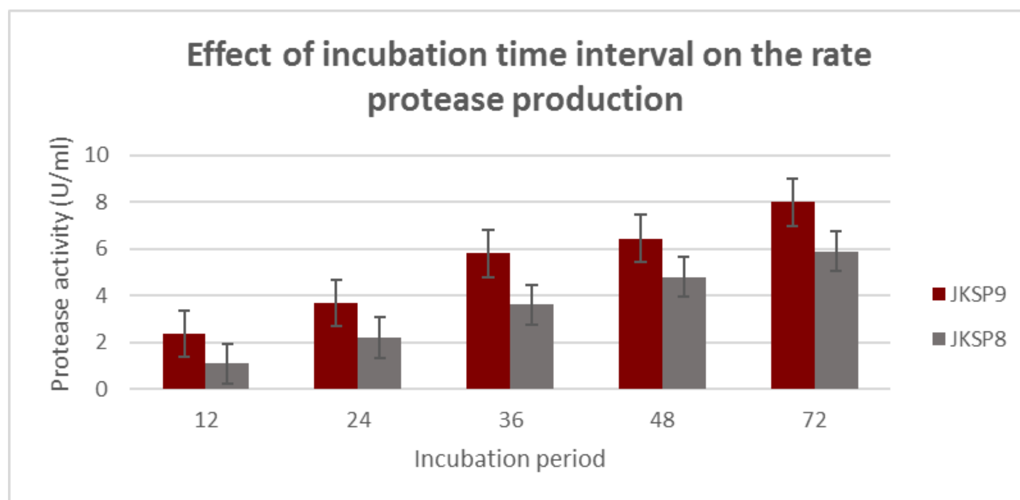

Fig. 2: Effect of pH on the rate of protease production

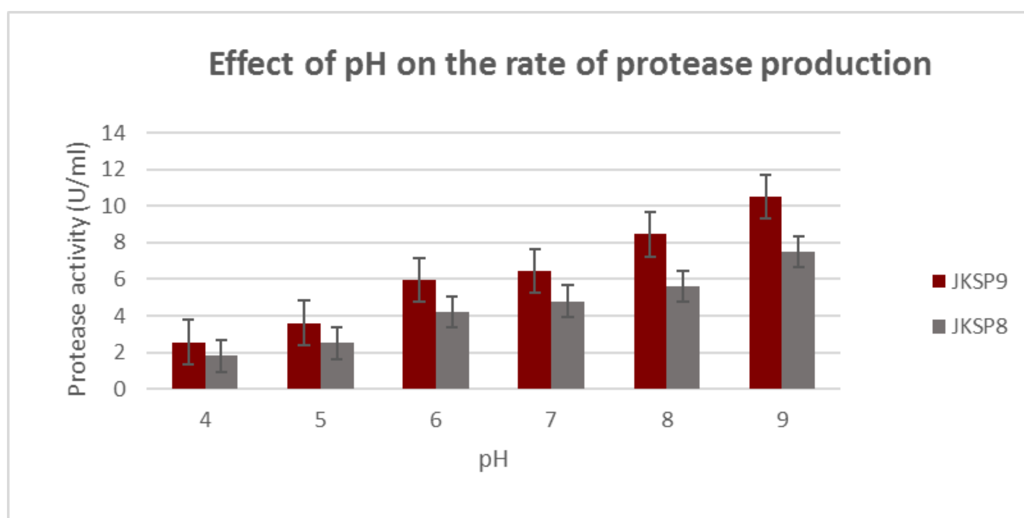

Fig. 3 : Effect of temperature on the rate of protease Production

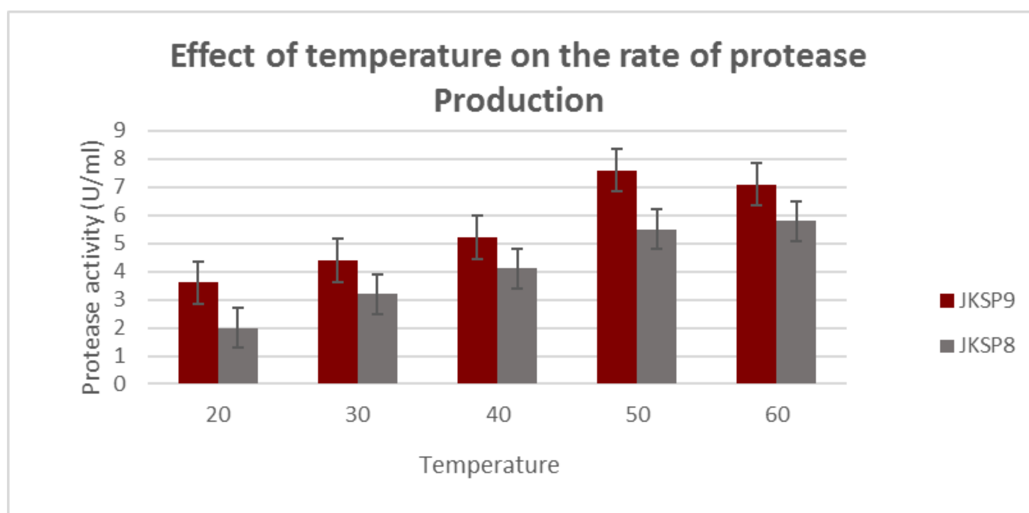

Table 1:Protease activity rate at different Incubation Time Intervals.

S.No	INCUBATION (Hrs)	PROTEASE ACTIVITY (U/ml)	
		JKSP8	JKSP9
1	**12**	1.08	2.36

2	**24**	2.2	3.68
3	**36**	3.6	5.8
4	**48**	4.8	6.45
5	**72**	5.9	8

Table 2:Protease activity rate at different pH.

S.No	pH	PROTEASE ACTIVITY (U/ml)	
		JKSP8	JKSP9
1	pH4	1.8	2.56
2	pH5	2.5	3.6
3	pH6	4.2	5.96
4	pH7	4.8	6.45
5	pH8	5.6	8.45
6	pH9	7.5	10.5

Table 3:Protease activity rate at different Incubation Temperature.

S.No	TEMPERATURE	PROTEASE ACTIVITY (U/ml)	
		JKSP8	JKSP9
1	20 ºC	3.6	2
2	30 ºC	4.4	3.2
3	40 ºC	5.2	4.1
4	50 ºC	7.6	5.5
5	60 ºC	7.1	5.8

Table 4: Protease activity rate at different Carbon source substrate.

S.No	CARBON SOURCE	PROTEASE ACTIVITY (U/ml)	
		JKSP8	JKSP9
1	Glucose	2.85	3.45
2	Galactose	3.45	2.60
3	Sucrose	5.12	3.45
4	Starch	4.85	5.58
5	Lactose	5.02	4.95
6	Bareley	5.1	4.51

7	Fructose	4.89	4.52

Table 5:Protease activity rate at different Nitrogen source substrate.

S.NO	NITROGEN SOURCE	PROTEASE ACTIVITY (U/ml)	
		JKSP8	JKSP9
1	Beef extract	6.25	5.62
2	Peptone	5.28	6.23
3	Casein	5.65	6.25
4	Sodium nitrate	4.85	7.12
5	Urea	4.9	6.5
6	Yeast extract	6.95	7.24

Table 6: Protease activity rate at different Metal Ions substrate.

S.NO	METAL IONS	PROTEASE ACTIVITY (U/ml)	
		JKSP8	JKSP9
1	Cobalt chloride	2.4	2.45
2	Zinc sulphate	3.54	3.2
3	Sodium chloride	3.89	5.2
4	Mangonous sulphate	4.35	4.5
5	Nickel carbonate	4.85	4.44
6	Magnesium sulpahte	4.9	2.5

Table 7: Protease activity rate at different Agro-based substrate.

S.No	AGRO-BASED SUBSTRATE	PROTEASE ACTIVITY (U/ml)	
		JKSP8	JKSP9
1	Ground nut oil cake	4.22	6
2	Rice bran	5.82	3.82
3	Castor	6.95	4.85
4	Black gram	6.42	5.2

5	**Sesame oil cake**	6.28	6.25
6	**Coconut oil cake**	6.21	4.25

Fig. 4: Effect of Carbon source on the rate of protease Production

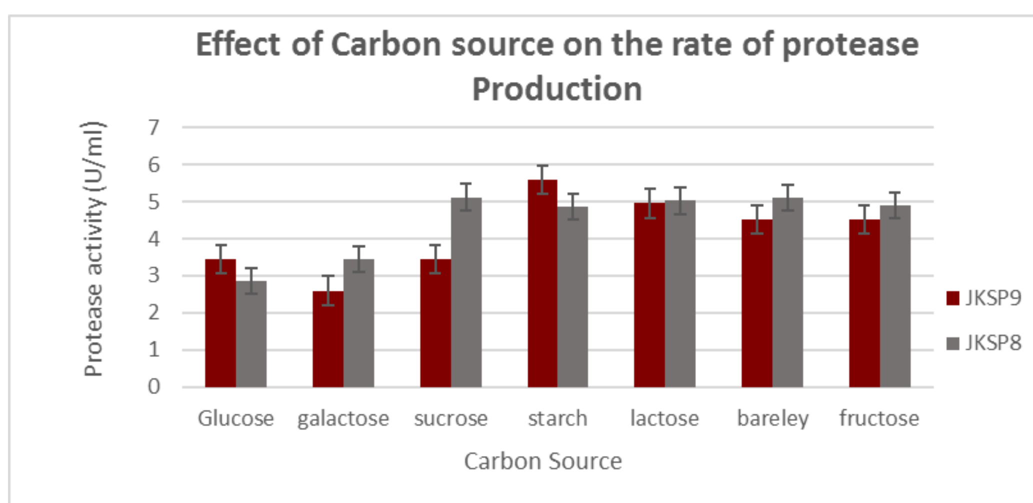

Fig. 5: Effect of Nitrogen sources on the rate of protease Production

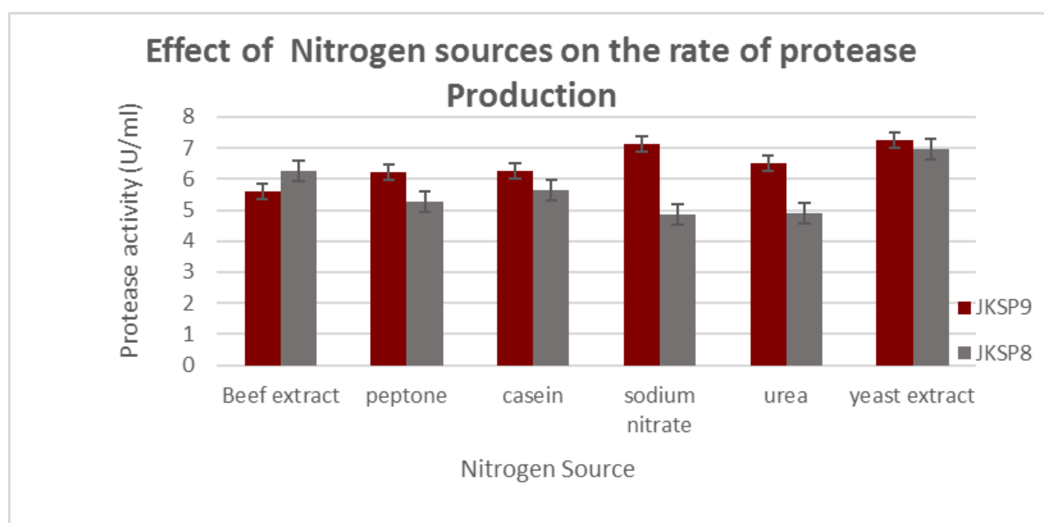

Fig. 6: Effect of Metal ions on the rateof protease Production

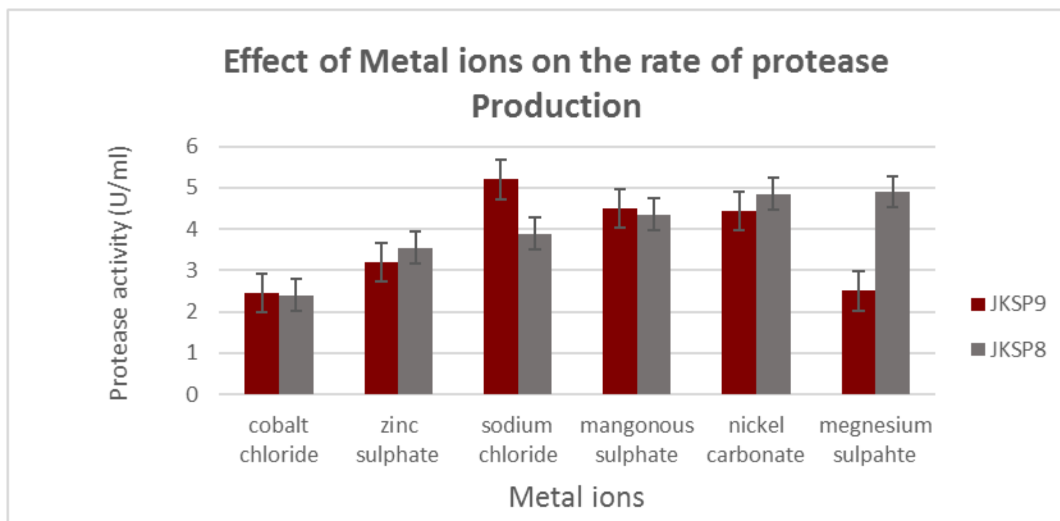

Fig. 7: Effect of Agro-based substrate on the rate of protease production

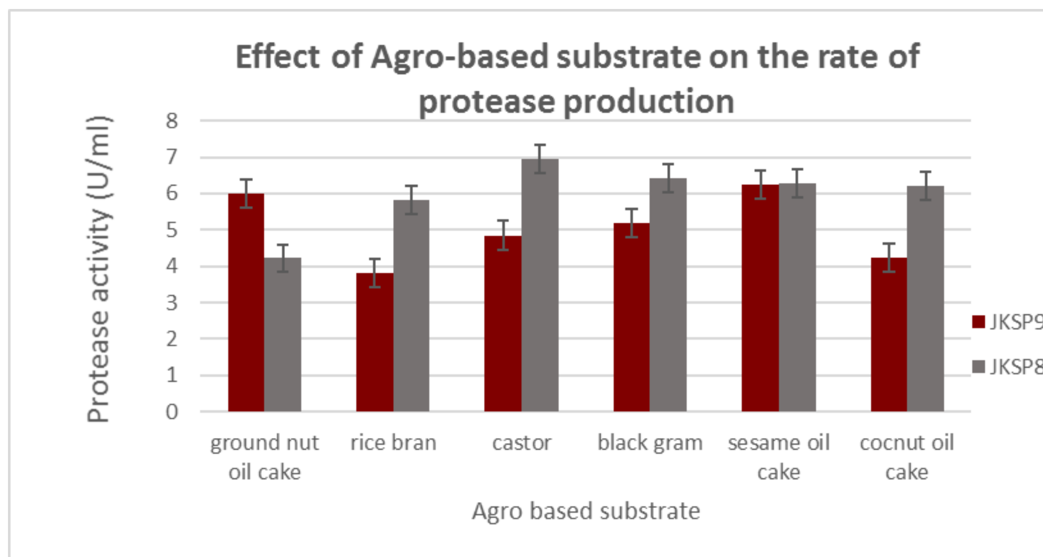

Fig 8: Destaining – Washing performance analysis of the Bacillus sp. and Bacillus velezensis enzyme preparation in the presence of the commercial detergent surf Excel (a)Cloth stained with blood washed with tap water (b)blood stained cloth washed with detergent (c) blood stained cloth

washed with protease enzyme of the Bacillus sp.(d) blood stained cloth washed with detergent and purified protease enzyme Bacillus sp.

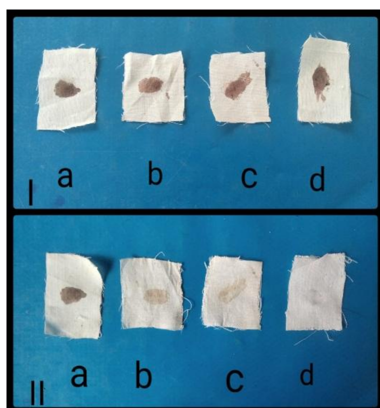

Fig 9: Dehairing :
A- Goat skin treated with distilled water
B -Enzymatically treated with purified Bacillus sp and Bacillus velizensis protease