St. Xavier's College
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Department of Life Science and Biochemistry

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SLSC501PR

Internal Assessment Project Report

Isolation and identification of unknown protease producing bacteria from soil

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Introduction

"Proteases are enzymes (biological catalysts) that break down proteins into fragments such as peptides and amino acids"[1]. These are usually found in the digestive tracts of higher organisms, and are also produced by certain bacterial species[2], where the organisms would be dependent on such an ability to gain nourishment and exhibit growth.

Proteases are important in the interconnected industries of biotechnology – to develop compounds, health – as a directed therapy and pharmaceuticals – drug targets. [3]. It is a mainstay in the food industry, especially in the baking and dairy industries.[4]

The present study was undertaken to isolate and identify a bacterial species that produced protease – which would be achieved by screening for protease activity by the hydrolysis of casein, using Skimmed Milk Agar (SMA) plates to grow the sample culture. The latter steps to obtaining a pure isolate, such as DNA Isolation, Biochemical Testing have been discussed in the following section.

Materials and Methods

Sample preparation: Unprocessed cheese was diluted with water to form a suspension. Further serial dilutions were obtained of the factors 10, 100 and 1000, the former of these was used for Gram staining, and the latter 2 dilutions were spread-plated.

Gram Stain: A Gram stain was performed to determine the Gram nature of all of the bacteria present in the sample.

Organisms with thick peptidoglycan layer in the cell wall were Gram +ve/ stained purple; while those which were Gram –ve, contained a thinner peptidoglycan layer, and would stain pink.

Steps performed for Gram Staining: (Modified timing)

1. A loopful of culture was aseptically spread onto a slide, dried and heat fixed.
2. Staining with crystal violet (1 min).
3. Iodine added as mordant (1 min).
4. Decolourisation with acetone-alcohol (20-30 sec). and washing of slide
5. Counter-stained with safranin (4 min), slide was washed and air-dried.
6. Immersion oil put onto slide, and the oil immersion objective lens was used . [5]

Isolation of Protease Producing Species: 0.1ml of the sample was spread-plated on SMA petri dishes to selectively grow protease producers (as demonstrated by casein hydrolysis).

Contents of Skim Milk Agar (SMA)
For 1 L of Distilled Water (D/W): Skim Milk Powder: 28g, Tryptone: 5g, Yeast Extract: 2.5g, Dextrose: 1 g, Agar: 15.0 g. [6]

Colony selection: 6 different bacterial colonies were observed, from which colony '5e' was selected for re-plating onto new plates to obtain a pure culture.

Colony characteristics and Morphology: Colony characteristics of colony 5(e) were studied to aid in identification. Gram staining of the same colony was performed to ensure that only a single species was present, and to simultaneously confirm its Gram nature.

Storage: As the culture was determined to be pure, a loopful of the isolate was inoculated onto an agar slant made of Nutrient Agar and refrigerated for maintenance.

Components of Nutrient Agar (NA): In 1 L of D/W: Peptone (5 g), NaCl (5g), Beef extract (1.5 g), Yeast Extract (1.5 g), Agar (15 g) [7]

Capsule Stain

Principle: Acid fuschin (basic) stains the cell red. Congo red forms a red background that turns blue on addition of acetic acid due to change in pH. Capsule remains unstained.

Steps to perform a Capsule Stain: by Maneval's method:

1. Maneval's A (1% Congo Red) was added to a little culture. & air dried.
2. The slide was flooded with Maneval's B (Acid fuschin, $FeCl_3$, Phenol, Glacial Acetic Acid) and left for 5 minutes.
3. It was washed gently and air dried before observation.[8]

Motility Test:

The hanging drop method was used to determine whether the organism was motile or non-motile. The edge of the drop was observed, since the organisms would be clearly visible, and aerobic bacteria would be easily found at the edge.

Steps for the test:

1. A cover slip was taken and Vaseline was applied at the edges, after which a drop of culture was placed in its centre.
2. The cover slip was upturned over a cavity from a cavity slide, such that the drop hung from the centre , and Vaseline sealed any gap between the cavity slide and the cover slip.
3. The slide was viewed under 40 X at the edge of the drop. [9]

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DNA isolation: DNA was isolated from the bacteria and the components were observed by Gel Electrophoresis

Steps to isolate bacterial DNA:
1. Cell lysis followed by centrifugation.
2. Salt added to pellet for precipitate DNA,centrifugated – supernatant taken.
3. Chilled ethanol added and solution chilled – DNA fibres observed – spooled out of the solution. [10]

Gel electrophoresis and PCR:

Bacterial DNA was first observed using Gel electrophoresis, and later amplified by using universal primers of 16S rRNA in a PCR Thermal Cycler.

Gel electrophoresis loading sample:

2 μL loading dye + 7 μL DNA.

PCR Mixture:

gDNA (1 μL), Forward Primer (1 μL), Reverse Primer (1 μL), Taq Master mix (5 μL), Water (2 μL).

Biochemical testing:

Sugar Utilisation Tests - Acid Production: Andrade peptone water was used as the base on which carbohydrates were added for testing acid and gas formation. The carbohydrates used were Galactose, Lactose, Maltose, Mannitol, Sucrose and Xylose.

Andrade peptone water content:

For 1 Litre of D/W, Peptic digest of animal tissue (10 g), NaCl (5 g), and Andrade indicator. (0.1 g)

Procedure: The desired carbohydrates were added to Andrade peptone water, a loopful of culture inoculated, and left overnight to observe acid production (colour change of indicator from colourless to pink-red) and gas formation (bubbles escaping Durham's tube). [11]

IMViC Tests:

1) Degradation of tryptophan to Indole:

The organism was inoculated in Tryptone water (10g Tryptone, 5 g NaCl in 1L D/W) and incubated overnight at 37C, Kovac's Reagent was added and the formation of a brown ring was to be observed. [12,13]

Glucose Phosphate Broth: In 1 L D/W, Peptone (7g), Dextrose (5g), K_2HPO_4 (5g) [14,15]. The broth was inoculated into by the isolate and incubated at 37C overnight, following which aliquots were taken and the following reagents were added to look for colour change as a +ve result: 2) Methyl Red: Methyl Red indicator – immediate reddening would be positive. 3) Voges-Proskauer: Barritt's Reagents A and B (alcoholic α-naphthol, 40% KOH soln) – Pink-red colour within a half-hour would indicate a positive result,

4) Citrate Utilisation Test:

The test was performed to determine whether Citrate could be used by the organism as a singular energy source. Bromothymol blue would show blue colonies of citrate utilising bacteria for a positive result.

Simmon's Citrate Agar Composition: For 1 L of D/W, NaCl (5 g), Sodium Citrate (2 g) $NH_4H_2PO_4$ (1 g), K_2PO_4 (1 g), $MgSO_4.7H_2O$ (0.2 g) Bromothymol Blue (0.08 g), Agar (15 g) [16]

Nitrate Reduction (Nitratase) Test:

Isolate was inoculated onto nitrate broth and incubated overnight, before adding some drops of Sulphanilic Acid, followed by some drops of α-naphthylamine. Red colouration would indicate presence of nitratase to reduce nitrate to nitrite.

Nitrate broth: In 1 L of D/W, Peptone (5g), Meat Extract (3g), Potassium nitrate (1g). [17]

Urease Test:

Isolate was inoculated onto the urea medium, and left overnight – for a few days. Pink colour would indicate a pH increase, and a positive urease result[18]

Urea Broth:In 950 mL D/W, Sterile 40% Urea soln (50mL), Monopotassium phosphate (9.1 g), Dipotassium phosphate (9.5 g), Yeast extract (0.1 g), Phenol red (0.01 g). [19]

Growth in 7% NaCl agar: Isolate was inoculated into an agar slant containing around 7% NaCl and incubated overnight.

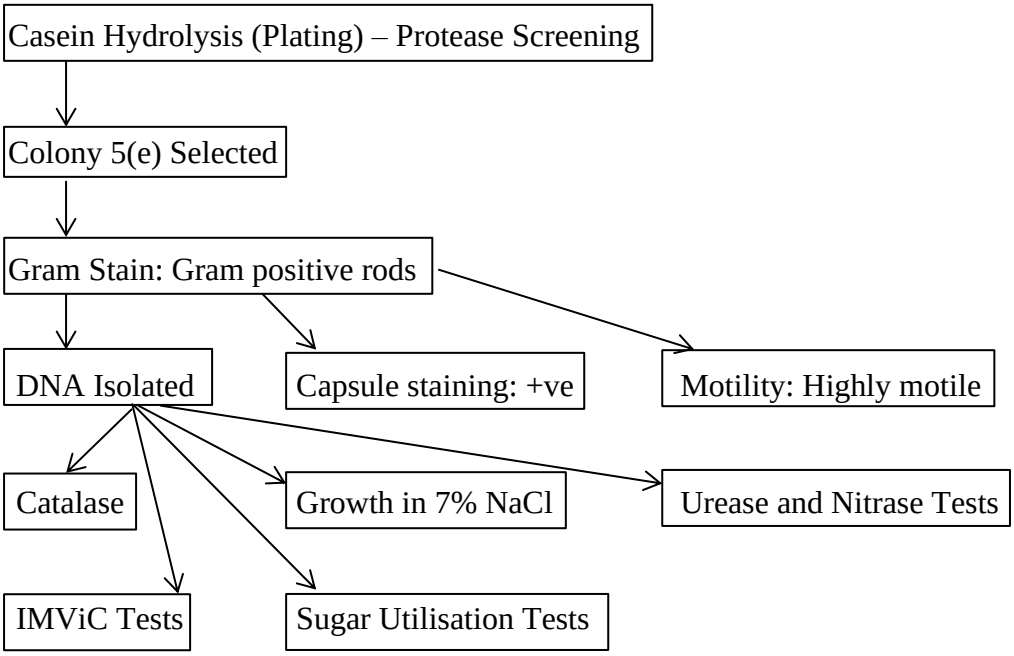
Identification of species:

The results from these biochemical tests, along with colony and other characteristics held the means to identify the protease producing organism.

Bergey's manual of Determinative Bacteriology, and Bergey's manual of Systematic Bacteriology were used to identify the isolate. [20,21]

Result

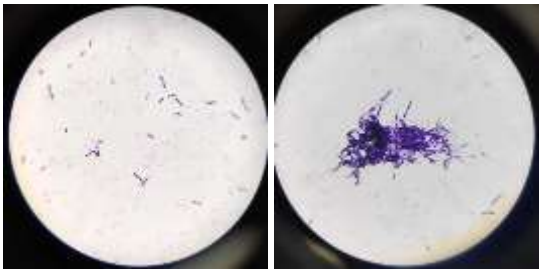
3.1: Path of Action for the unknown isolate



3.2: Gram Stain Characteristics

Characteristic	Observation
Colour	Purple
Shape	Rods
Arrangement	Clusters
Gram nature	Positive

Gram Staining (100 X Visual Field)



3.3: Colony characteristics

Characteristic	Observation
Colour	Buff/ light yellow
Opacity	Opaque
Edge/Margin	Undulate/ Wavy
Shape/ Form	Circular to Irregular
Size	~1-2 mm
Elevation/ Height	Umbonate
Texture	Matte, Rough

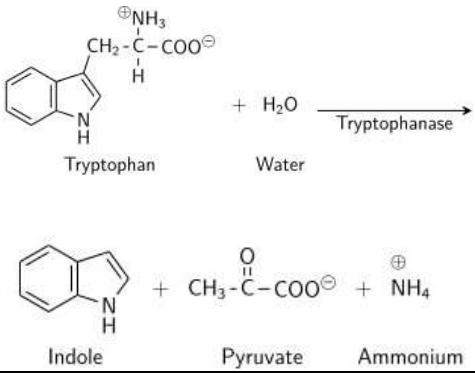
Colonies of the isolate 5(e)

3.4 Biochemical Testing:

Tests 1-6 refer to sugar utilization for acid production, 7-10 are the IMViC group of tests, 11 looked at growth on 7% NaCl (slight halotolerance), 12 and 13 tested for the presence of the enzymes nitrase and urease, respectively.

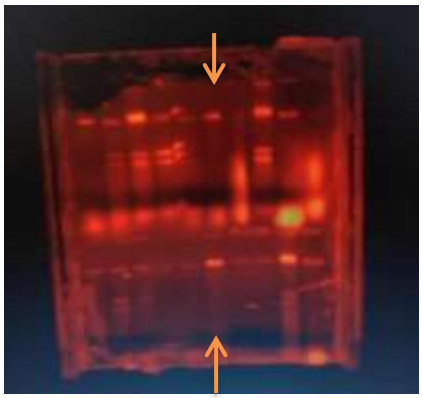
Observations:

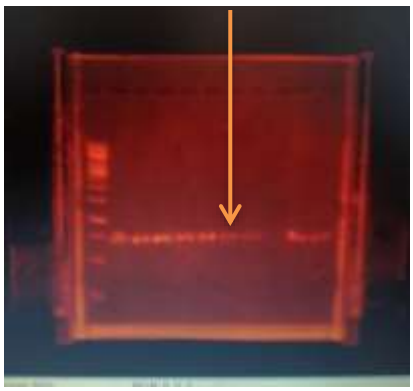
Test	Reaction	Result	Inference
1. Galactose	Andrade indicator – acidic medium Colourless to pink		+ve. Acid is formed from galactose.
2. Lactose	Andrade indicator – acidic medium Colourless to pink		-ve. Acid is not formed with lactose.
3. Maltose	Andrade indicator – acidic medium Colourless to pink		+ve. Acid is formed from maltose.
4. Mannitol	Andrade indicator – acidic medium Colourless to pink		-ve. Acid is not formed from mannitol.

5. Sucrose	Andrade indicator – acidic medium Colourless to pink		+ve. Acid is formed from sucrose.
6. Xylose	Andrade indicator – acidic medium Colourless to pink		-ve. Acid is not formed from xylose.
7. Indole [12]			-ve. Tryptophan does not get degraded into Indole by the given organism.
8. Methyl Red [14]	$Pyruvic\ acid$ $\rightarrow Mixed\ acid\ (pH\ less\ than\ 6)$		+ve. Acidic end-products are formed by metabolism of the species.
9. Voges-Proskauer [15]	a)$2(Pyruvate) = Acetoin + 2CO_2$ b)$Acetoin + NADH + H^+ \rightarrow$ $2,3 - Butanediol$ $+ NAD^+\ (ph\ 6\ and\ above)$		-ve. Acetoin is not formed as an end-product of metabolism of the species.

10. Citrate utilisation [16]	Citric acid $\xrightarrow{citrase}$ Mixed acid and/or Acetoin		-ve. Species is unable to use citrate as the sole Carbon source, i.e., it lacks the enzyme citrase.
11. Growth in 7% NaCl	No reaction, only growth to be observed.		+ve. The species was found to be at least slightly halotolerant.
12. Nitratase [17]	$NO_3 \xrightarrow{nitrate\ reductase} NO_2$		+ve. Nitrate reduction to nitrite took place with the species.
13. Urease [18]	$(NH_2)_2CO + 2H_2O \xrightarrow{urease} CO_2 + H_2O + 2NH_3$		-ve. Urea degradation did not take place with the species.

3.5: Genomic DNA Electrophoresis and PCR of 16 S rRNA. {Orange arrows mark the wells loaded with the isolate 5(e).}





From the gels, it could be summarised that the isolation of DNA and PCR amplification were successful – the amplified region appeared to be between 750 and 1000 base pairs long, when compared to a 1 Kb DNA Ladder.

3.6: Identification

The organism obtained from the unprocessed cheese sample was shortlisted to be one of *Bacillus pantothenticus, Bacillus soli, or Bacillus galactosidilyticus*.

Discussion

Identification was carried out by following the key at the back of Bergey's Manual of Determinative Bacteriology, to identify the genus. Once the genus was determined to be *Bacillus*, the internal chapter key for the genus was followed and characteristics (biochemical – sugar utilisation, hydrolysis tests; colony characteristics, motility, temperature, pH, salt concentrations, etc.) compared to get to the closest estimate of *Bacillus pantothenticus*, however – Bergey's Manual of Systematic Bacteriology (Vol 3) was simultaneously consulted, and comparisons of biochemical tests, colony characteristics and general characteristics led to two more possibilities: *Bacillus soli* and *Bacillus galactosidilyticus.* [20,21]

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