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Department of Life Science and Biochemistry

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Internal Assessment Project Report

Isolation and identification of unknown protease producing bacteria from cheese and whey protein

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Isolation and Identification of unknown bacteria from cheese and whey protein

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Introduction

Enzymes are the biocatalyst used for enhancing metabolic rate of reaction. A large number of enzymes are produced in vivo having great importance in industries. Protease is the most important enzyme produced industrially. Protease enzyme is naturally present in all organisms and it corresponds to 1-5% of the total protein content. It is the third largest group of industrial enzymes and has a wide sale of 60%. It can hydrolyse peptide bonds in proteins and they are also called peptidase or proteinase or proteolytic enzymes. Protease re the oldest and the most common family of enzymes that are involved in almost every process or organism's physiology. Due to their wide range substrate specificity, proteases are used in many industrial applications such as leather processing, detergent formulations, baking, brewing, peptide synthesis, cheese manufacture, waste treatment, silk industry, organic synthesis etc.

Proteases are classified into three groups based on their acid base behaviour that is, acid, neutral, and alkaline proteases. Acid proteases have a pH range of about 2.0-5.0 and they are produced only by fungi. Neutral pH of protease ranges from 7.0-8.0 and they are mainly of plant origin and finally proteases with pH above 8 are said to be alkaline proteases.

Proteolytic enzymes are ubiquitous in nature and they are found in all living organisms such as plants, animals and microbes. The microbial production of proteases is preferred more than the other sources because microbes can be grown and cultivated in a very small space. They grow faster and cab be genetically modified easily.

The current study aims to isolate protease producing bacterial stains from the given protein sample.

Materials And Methods

Sample Selection

The sample of cheese and whey protein was mixed together thoroughly with warm water and was collected in a plastic container. The mixture obtained was used for bacteriological analysis.

Isolation of microorganisms

One gram of sample was weighed aseptically and diluted with 9ml of saline. The solution was diluted serially to obtain 10^{-1}, 10^{-2}, 10^{-3} dilutions. 0.1 sample of all the three dilutions were spread on the nutrient agar plates in duplicates and they were incubated at 37˚c for 24hrs. Skim milk agar is commonly used to demonstrate proteolysis of organisms capable of hydrolysing casein. Proteolytic bacteria use the enzyme caseinase to hydrolyze casein and form soluble nitrogenous compounds displayed as a clear zone around the colonies. Therefore, the isolates were streaked on the skim milk agar and was incubated at 37˚c for 24-48hrs. The bacterial colonies with zone of

clearance around their colonies were selected and maintained on nutrient agar plate.

A gram stain was also performed with the isolate and was observed under 10X

Isolation of pure culture from the isolate

The inoculated plates were incubated in an incubator at 37˚C for 24-48 h. After 24–48h at 37°C, colonies which exhibited the largest cleared zones were selected and was further incubated in culture media for further 48 hrs and checked for protease production. The isolates were screened on the basis of larger zone on milk agar medium and further confirmed.

Next, a second gram stain was performed and observed.

Bacterial DNA Isolation

The DNA is organized in rings or circular plasmids, which are in the cytoplasm. The DNA extraction process frees DNA from the cell and then separates it from cellular fluid and proteins so you are left with pure DNA.

The three basic steps of DNA extraction are

1) lysis
2) precipitation
3) purification.

Gel Electrophoresis

The extracted genomic DNA was made to run in agarose gel electrophoresis with bromophenol. This technique separates the DNA fragments according to their size. Bromophenol is usually used as a tracking dye during agarose or polyacrylamide gel electrophoresis. It has a slight negative charge and will migrate the same direction as the DNA, allowing to monitor the progress of DNA moving onto the gel. 2 microliter of bromophenol and 7 microliter of genomic DNA was used for loading. The given steps are involved in gel electrophoresis

Pour a standard 1% agarose gel using 1g of agarose in 100mlTAE into the gel tray

Place the agarose gel into the electrophoresis unit and keep it for solidifying

Fill TAE until the gel is submerged

Add bromophenol to the DNA sample and with the help of a micropipette carefully load the DNA ladder into a lane of the gel. Should not spill

Run the gel at 80-150V until the dye reaches 70-80% of the way down the gel

Disconnect the electrophoresis until and remove the gel. Visualise the DNA bands using UV as the light source.

Polymerase chain reaction

The genomic DNA culture of 5E was used for polymerase chain reaction (PCR) test of 16s RNA analysis. This test is used for exponentially amplifying the DNA sequences and generate much more copies of that particular sequence. After amplification the DNA sample was again allowed to run on an agarose electrophoresis gel and the DNA bands were observed under UV

For Polymerase chain reaction

1 microliter genomic DNA + 1 microliter forward primer + 1 microliter reverse primer + 5 microliter Taq Master +2 microliter water for injection. This mixture

in an Eppendorf tube was introduced in thermal cycler of amplification.

Amplification in PCR is don by three main steps:

Denaturation at 94-95˚C causes the double stranded template DNA to get separated into two single strands.

Annealing at 50-56˚C cause the primers to attach to the template DNA

Extending at 72˚C causes the Taq Polymerase enzyme to bind the new strand of DNA is made

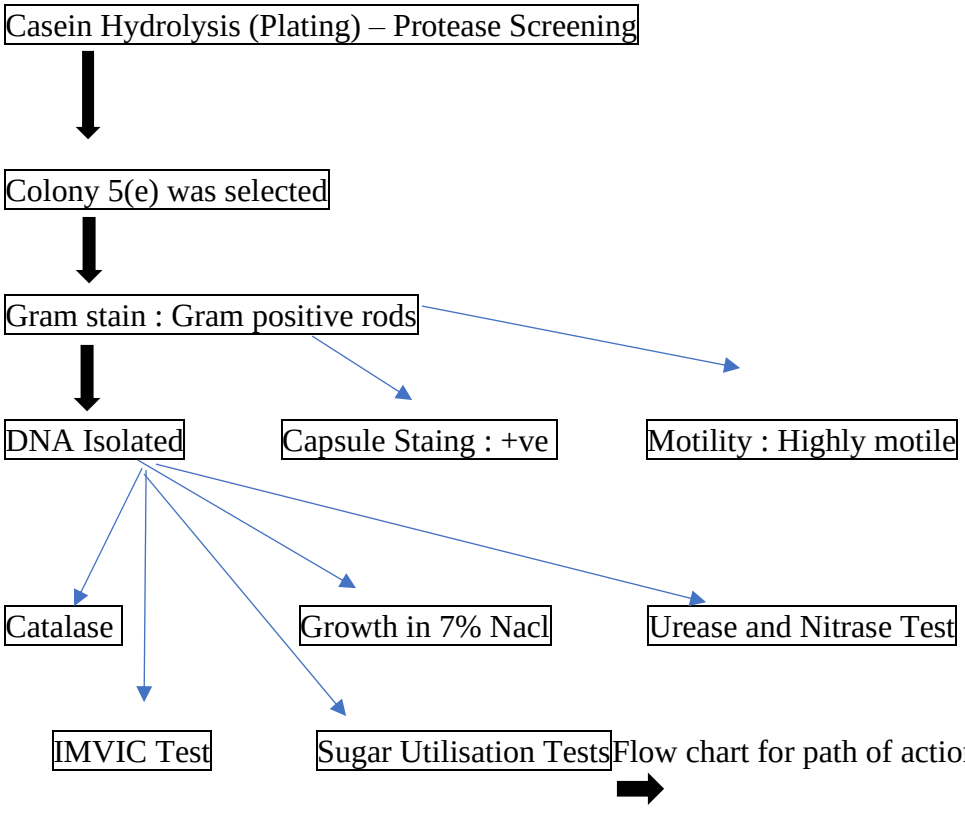
Motility Test

The motility test of the obtained bacterium from the culture was performed in a cavity slide. A drop of the culture is introduced in the cavity slide which is then covered by sticking a cover slip with petroleum jelly. The slide is then inverted and is observed under the microscope using the oil immersion objective.

Biochemical Testing

After the motility test, the biochemical tests were performed. Biochemical tests are the tests used for the identification of bacteria species based on the differences in the biochemical activities of different bacteria. Bacterial physiology differs from one species to the other. identification based on structural differences is not possible in case of bacteria, because structural differences, which may differentiate one species of bacteria from the other, are not discernible even under a microscope. The structural differences with respect to shape, size and arrangement of bacteria only help in the process of identification, because there are many species of bacteria having similar shape, size and arrangement. Therefore, ultimately, the identification of bacteria is mostly based on the differences in their biochemical activities.

Result And Discussion



Flow chart for path of action of unknown

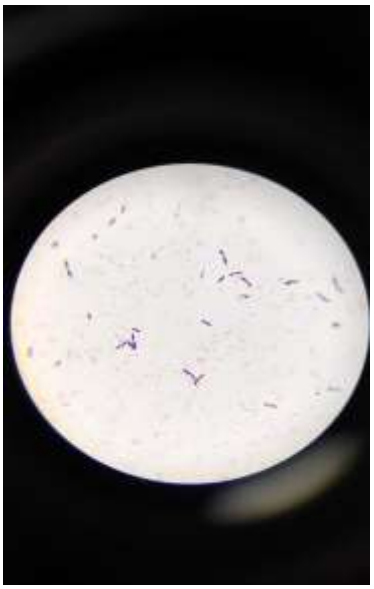
Size	Shape	Edge	Elevation	Opacity	Texture	Pigmentation
Big	Irregular	Lobate	Flat	Opaque	Rough	White

Table 1 Colony characteristic on nutrient agar plate

Isolated colonies on nutrient agar plates

Table 2 Results of staining and motility

Sr. No.	Staining	Result
1.	Gram staining 1	Positive
2.	Gram staining 2	Positive
3.	Motility	Motile

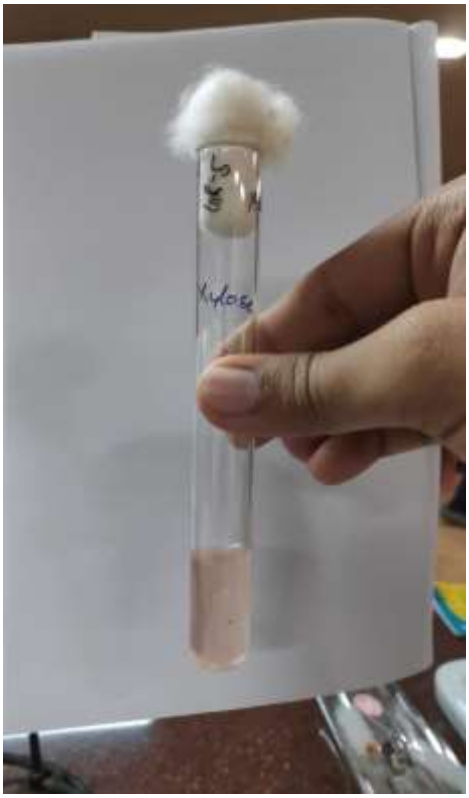


Result for electrophoresis and PCR

The first gel electrophoresis performed and it was observed that the DNA was properly isolated and the bands were also visible

The second gel electrophoresis was done to know if the PCR was worked properly or not and if one single band in the gel was observed. From this we concluded that the 16s RNA Gene sequence has been

Tryptophan



Xylose

Mannose

Tryptophan

Urea

7% Nacl

It has been reported that the production of extracellular protease by different micro organisms can be strongly influenced by the culture conditions. Bacillus sp, was found to be predominant in the sample. In the present study three bacterial isolates shown proteolytic activity which are *Bacillus galactodidilyticus, Bacillus gelatine, Bacillus gibsonii.* The protease producer was identified as genus Bacillus Sp. It is Gram positive, motile, catalase positive bacteria

References

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Date of submission:_9th September 2019_ Total Marks obtained: _____/20

Grading Rubric:

	GRADED ITEM	Max. Score	Obtained Score
1.	Format Guidelines followed? (margins, font, spacing, title pg, etc.), Neatness, spelling, grammar, nomenclature • Report written in professional, objective manner--no personal pronouns • Have all scientific names been written using proper nomenclature? • Is grammar and spelling proper and accurate? Is report prepared in a tidy fashion according to requirements?	**2**	
2.	Introduction Purpose of study clearly stated	**2**	
3.	Materials and Methods • Was each technique used described in appropriate detail? • Was streak plate isolation described and any problems discussed? • Were isolation and biochemical testing procedures performed correctly? • Was isolation accomplished and pure cultures maintained regularly.	**4**	
4.	Results Tables - • Flow Chart: Is path of action indicated for the unknown, including any extra work? • Is there a complete table of test results showing visual AND interpretive information? • Were results correctly interpreted (info in notebook and in discussion section)	**4**	
5.	Correct ID • Was the unknown correctly identified? • If unknown ID incorrect, was it the result of student error (technique or judgement) or a factor out of the student's control?	**1**	
6.	Discussion • Has student avoided repetition of methods and results in this section? • Have rationales for identifications been adequately described? • Has student demonstrated he/she can make appropriate conclusions? • Has students identified any errors in technique or judgment and suggested alternatives for future work? • Is discussion written in a professional manner? • Have all changes in original plan been discussed?	**3**	
7.	References • Have all required components been included in proper scientific format? • Have all authors been cited, in the order in which they appear?	**1**	
9.	Laboratory working and discussions • Participation in discussions, following proper techniques, following up with results and suggesting logical steps for further tests.	**3**	
10.	Report submitted late (-10% per day)		
	TOTAL	**20**	

Teacher-in-charge