



Evaluation of the effect of Mutations on the Protease enzyme production by *Lactobacillus delbrueckii*

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Received: July 20th 2013, Accepted: Aug 13th 2013, Published: Sep 1st 2013

Abstract:

Proteases are the enzymes produced in most organisms. Proteases produced by enzymatic methods are more environment friendly when compared to the chemical process and it has tremendous potential in the leather and several other industries. However optimization of protocol for the production of protease could involve several variables such as temperature, pH and incubation period. In the current work *lactobacillus delbrueckii* has been employed for the production of protease. Further the strain was subjected to mutations and the mutant strain was also employed in the production of the enzyme so as to check the effect of mutations on enzyme production. For the surprise it was found that the biochemical properties exhibited by the mutant strain of *lactobacillus delbrueckii* were exactly the same as shown by *Neisseria flavescens*. In other words the exposure of *lactobacillus delbrueckii* to UV Irradiated mutations made the bacteria to exhibit the properties of *Neisseria flavescens*. The current work includes the Isolation, Identification and Production of mutant strains of *lactobacillus delbrueckii* followed by the application of these strains in the production of the enzyme. The protocol also involves the quantitative estimation of the enzyme produced by the two strains.

Keywords: Spread plate technique, pure culture, Biochemical test, Lowry's method, Salt precipitation, Dialysis, Enzyme kinetics.

Introduction:

Enzymes are functional proteins, found within each of the cells, which help the cell to carry out its specialized functions. Proteases are enzymes that catalyze the breakdown of proteins. Protein breakdown is a normal process necessary to maintain cellular homeostasis. Active proteases can be found throughout your body, including the digestive tract, inside cells and circulating in the blood^[1]. Proteases play a critical role in many physiological and pathological processes such as protein catabolism,

blood coagulation, cell growth and migration, tissue arrangement, morphogenesis and development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes^[2].

Materials and Methods:

Collection of the Bacteria from Soil Source

Protease is an enzyme that is specifically performing protein hydrolysis which is the most common process required in the pathways of soil bacteria. Thus soil has been chosen in the current work as a source for the isolation of the bacterial species expected to produce the enzyme protease.

Preparation of Pure cultures

The bacteria were isolated onto the nutrient agar plate by using serial dilution and spread plate methods resulting in the production of a master plate that contains mixed bacterial cultures. These master plates were used to prepare pure cultures of the selected bacterial colonies by the method of streak plate. Thus finally producing the pure culture plates for the selected bacterial cultures.

Fig 1: Master plate showing the mixed colonies of the bacteria culture as obtained from the soil sample.



Screening for the production of the enzyme Protease:

To select the organisms that are capable of producing the extracellular protease enzyme a screening test is performed. The test involves the use of Protease screening medium that essentially contains the basic nutrients with the major carbon source to be any form of protein. If the bacteria are capable of producing the protease enzyme they can utilize the proteins and grow on the media plates.

In the other case due to the lack of alternative nutrient source and the enzyme protease to use the existing medium the bacteria fails to grow. Thus depending upon the presence or absence of the growth of bacteria in the Screening medium the enzyme producing ability of the bacteria can be assessed.

Categorization of bacteria based on Grams Nature:

Based upon the ability of the bacteria to respond to the gram's staining the preliminary identification can be done. Those cultures that gave positive response to the screening test were selected and were subjected to gram's staining for the identification of their gram's nature.

Biochemical Identification of bacteria based on Bergey's manual:

Based upon the grams nature of the selected colonies the bacteria are further subjected to the biochemical tests to identify them at the species level. Based upon the results of the gram's test and other series of tests as per the Bergey's manual the tests performed for the bacterial identification were Catalase test, Starch hydrolysis test, Glucose fermentation test and Mannitol fermentation test.

Induction of Mutation by UV Irradiation:

One of the replicas of pure plate is kept under the UV hood in the laminar air flow. The plates are exposed for a period of 60 seconds. The culture is thus mutated. The mutant culture is further used for the production of the enzyme followed by enzyme extraction, purification and enzyme estimation.

Production of the extracellular enzyme Protease:

Once the Screening and the conformation of the organism is completed, the next step is to proceed for the production of the enzyme. Production of the

enzyme requires the special media called production media whose major property is the presence of the substrate i.e. protein in high concentrations than the screening media. This protein substrate would enable the high quantity production of the required enzyme.

Extraction of protease enzyme:

To extract the enzyme that has been produced in the production medium 5ml of production medium was centrifuged at 6000rpm for 10 mins. The supernatant has to be collected. This is called the crude enzyme that has to be stored at 4⁰C until use.

Purification of enzyme by salt precipitation & Dialysis:

The crude extract collected in the above step has to be purified by salt precipitation approach. To the crude extract 44.4% of Ammonium sulphate salt must be added pinch by pinch for saturation of the solution maintained under ice cold conditions. Once the saturation point is reached the solution must be incubated at 4⁰C for complete precipitation of the pure protein. Once the protein is precipitated out than it must be stored in TE buffer for further procedure. The pure enzyme precipitate still contains some salt impurities that can be removed by Dialysis. The precipitate is collected into the TE buffer and stored till the next step.

Estimation of the enzyme by Lowry's method:

To estimate the enzyme present in the product produced Lowry's method of protein estimation based on colorimeter is applied. This method involves the use of Folin's reagent that imparts blue colour to the proteins. Based upon the concentration of the protein present the blue colour would develop that is measured using a colorimeter.

Results and Discussion:

Screening for Protease enzyme Production:

As per the results of Protease enzyme screening it has been found that the selected bacteria was capable of digesting the proteins thus resulting in the growth of the culture in the screening medium.

Biochemical tests and Gram's test for the identification of Bacteria

TEST	RESULT
Gram's Staining	Gram's Positive rods
Catalase Test	Positive
Starch Hydrolysis Test	Negative
Glucose fermentation Test	Negative
Mannitol fermentation Test	Negative

Based on the biochemical tests the organism is identified to be *lactobacillus delbrueckii*

Production of the Enzyme Using specific production medium:

To produce the extracellular enzyme in huge quantities the gene must be stimulated by the addition of more concentration of the substrate in the medium. The concentration must any way be limited to prevent feed back inhibition. Using the specific substrate in the inoculation medium enables the production of enzyme into the medium which can be later extorted and purified.

Extraction and Purification of the Enzyme:

Once the production of the enzyme in the medium is completed the product must be extracted out to separate it form the bacterial cells and other components of the media. The extraction can be done by Centrifugation at the optimum speed. The centrifugation step will make the protein get separated from the other components.

Purification by Salt Precipitation and Dialysis:

The solution containing the soluble protein is subjected to purification by Salt precipitation [4]. The Ammonium sulphate salt is used to perform the precipitation so as to collect the pure protein pellet. The pellet thus obtained is dissolved in the suitable buffer and subjected to dialysis [6] for the separation of pure protein. Thus the protein has been extracted from both mutant strains and wild strains and the concentration of protein synthesized was evaluated using Lowry's method.

Quantitative estimation of protein by Lowry's method:

The Mutant and wild strains were subjected to protein production in the previous step and the

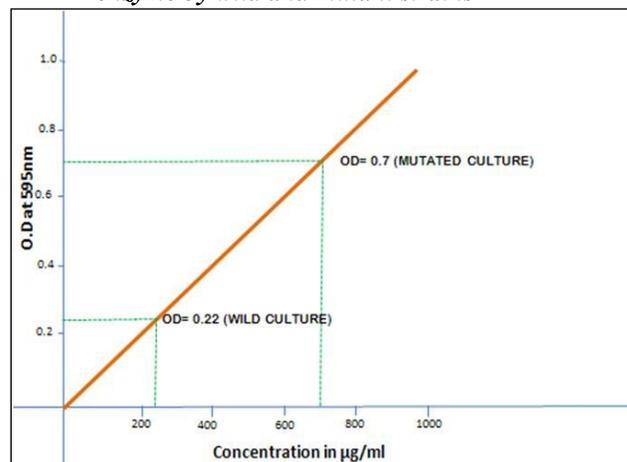
protein thus produced was purified. The next step is to estimate the amount of protein produced from wild and mutant strains and the comparison of the enzyme producing capability. Lowry's method [5] showed that the enzyme produced by **Wild strain** was **0.23mg/ml** whereas the concentration produced by **mutant strain** was **0.70mg/ml**. These results show that the exposure of the inoculums to UV irradiations and induction of mutations before using it for the enzyme production would be a suitable approach to enhance the rate of production of enzyme. With the induction of the mutations in the test bacteria the enzyme producing ability has been increased 3 fold.

Calculations based on the Graph:

In the above protein standard calibration graph, the OD value obtained for the concentration of wild and mutant strains have been plotted. According to the graph it can be inferred that the OD of 0.7nm as shown for the mutated strain corresponds to the concentration of 710 micrograms/ml, and the concentration of the enzyme produced by wild strain is 220micro grams/ml corresponding to the OD 0.22

From the evaluation graph it can be concluded that the induction of UV mutations has increase the production of enzyme from 220 to 710 micrograms/ml i.e. there is an increase of three fold in the concentration of the enzyme produced. Thus UV irradiations have a positive effect on enzyme production.

Graph showing the comparative production of enzyme by wild and mutant strains



Conclusion:

The current work aimed to standardize and propose the method to improve the enzyme producing ability of *Lactobacillus delbrueckii*. The wild strain of the bacteria was used to prepare the pure plates in two replicas. One of the replica was directly used in the production of the enzyme whereas the other is subjected to mutations using UV irradiations.

In the next step both the mutant and wild strain were used for the production of the enzyme in vitro conditions. After extraction and purification of the enzymes produced by respective cultures the work was extended to compare the concentration of the enzyme produced by both the strains. The method employed for the enzyme estimation was Lowry's method. The results showed that the enzyme produced from wild strain was 3 fold less than the mutant strain. The wild strain produced **0.23mg/ml** of enzyme into the broth whereas the mutant strain produced **0.70mg/ml**.

These results conclude that the enzyme concentration produced by the mutant strains was much higher than the wild strains. Thus induction of mutations would be a positive approach to enhance the yield of the enzyme by the cultures. Further the biochemical characterization of mutant strains showed that they exhibited the properties that were exactly identical to the properties of the bacteria *Neisseria flavescens*.

This data can be used to precede the research towards the comparison of the similarities exhibited by **two strains** *Lactobacillus delbrueckii* and *Neisseria flavescens*.

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