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RESEARCH ARTICLE

Production, Purification and Characterization of Protease from Yersinia sp and Staphylococcus sp

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Abstract

Protease is one of the most important enzymes from industrial point of view. The production of protease was studied by submerged fermentation using *Yersinia sp* and *Staphylococcus sp* isolated from fish waste and slaughter house waste. The isolated strains were irradiated by UV rays to increase its enzyme production. Maximum protease production was achieved after 72 hours of incubation at pH 7.0 in 30°C for both parent and mutant strains of *Yersinia sp and* 48 hours of incubation at pH 8.0 in 30°C for both parent and mutant strains of *Staphylococcus sp*. Among the various carbon and Nitrogen sources were tested, lactose and skim milk are the best sources for maximum enzyme production by *Yersinia sp*., glucose and skim milk as the carbon and nitrogen sources for the production of protease by *Staphylococcus sp.,*. The molecular mass of the purified enzyme was determined by SDS-PAGE.

INTRODUCTION

Proteases are, also known as peptidyl or peptide hydrolases (EC 3.4.21-24 and 99). They are said to be industrially useful enzymes, which catalyze the hydrolysis of a peptide bond in a protein molecule (Beg et al., 2003). They are one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sale in the world and they are the leaders of the industrial enzyme market worldwide mainly, because of the industrial applications in the field of brewing, textile, paper, sugar, distilling, food processing, pharmaceuticals, tannery, waste bio degradation, milk clotting, low allergenic infant food formation. Proteases are obtained from plant, animal and microbial sources. Microorganisms are capable of producing these enzymes intracellularly and extracellularly (Pandey et al., 2000; Sudhir et al., 2009).

The extracellular proteases of microbial origin are easy and economical. Microbial proteases are classified as acidic, neutral and alkaline depending on the pH at which they show maximum activity. Several *Bacillus sp, Yersinia sp* and *Staphyloccocal sp* are involved in protease production viz., *B. cereus, B. sterothermophilus, B. mojavensis B. megaterium and B. subtilis* (Soundra Josephine et al., 2012)), *Staphylococcus xylosus S. saprophyticus*, *S. equorum* , *S. carnosus* and *S. simulans* (Annalisa Casaburi et al., 2006). Microorganisms are the most preferred source of these enzymes mainly because of their rapid growth, the limited space required for their cultivation and ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Kumarasamy et al., 2012).

In this investigation, protease producing bacteria were isolated from slaughter house waste, poultry waste and fish waste. The isolates were tested for its production of extracellular protease and its enzyme activity. The isolates were mutated with UV rays and various environmental and nutritional factors were also tested. Finally, the enzymes were purified, characterized and the molecular mass determined by SDS-PAGE.

MATERIALS AND METHODS

Collection of Sample

The samples, viz., slaughter house waste, poultry waste and fish waste were collected from market areas around Tiruchengode, Namakkal District, Tamil Nadu.

Isolation of Proteolytic Bacteria

The proteolytic bacteria were isolated from the waste samples followed by the procedure described by Adinarayana et al (2003)

Identification of the isolated Bacteria

The isolated organisms were identified based on their cultural, morphological, microscopic examination, biochemical characteristics and the data are compared with standard description as given in Bergey's Manual of Determinative Bacteriology (Bergey et al., 1994).

Strain improvement

The selected isolate was strain improved by ultra violet irradiation (UV) as per standard procedure described by Saha and Battacharya (1990).

Determination of Proteolytic Activity

The proteolytic activity of the isolates was determined by the activity of the organism in skim milk agar plates as per the procedure described by Chantawannakul et a1 (2002). A single line streak was made on skim milk agar plates. After inoculation, the plates were incubated at 37°C at 24-48 hours. After incubation, the zone of clearance was observed. The isolates which produce larger area of clear zone were selected for further studies.

Preparation of Crude Enzyme

24 hours old culture was inoculated in the production medium. The P^H of the medium was adjusted to 8.5 and maintained at 27°C for 24-48 hours in an incubator shaker (180rpm). After incubation, the broth was centrifuged at 10,000 rpm at 4°C and the supernatant was recovered. Then, the supernatant was dialyzed against distilled water for 12 hours. The crude enzymes supernatant was subject to further study viz, enzyme activityas described by Secades and Guijarro (1999).

Estimation of Protein Content

The Protein estimation was carried out by Lowry et a1 (1951) by using Bovine Serum albumin (BSA) as the standard. The absorbance was measured at 660nm.

Assay of Protease Activity

Protease activity of the isolated strains was analyzed by Secades and Guijarro (1999). The amount of protease activity was determined using a standard graph prepared using tyrosine. From the standard graph, enzyme units were calculated. One unit of protease was defined as the amount of the enzyme to produce 1 µl of tyrosine/minute.

Environmental Parameters Affecting Protease Production

The effect of environment factors influenced the enzyme activity and it was studied as per the standard procedure mention by Adinaryana et a1 (2003)

Effect of Incubation Period

The effect of incubation time on protease production was carried out. Both parent and mutant strains were inoculated in the production medium and incubated on a shaker (180 rpm) at 37°C for 24, 48, 72 and 96 hours of incubation.

Effect of P^H

To determination of optimum pH for the production of protease enzyme, the pH of production medium was adjusted to various pH ranges from 6 to 10 at 37°C and the cultures were inoculated and incubated.

Effect of Temperature

To determine the optimum temperature for protease production, the production medium inoculated with isolates and incubated at different temperature viz., 10°C, 20°C, 30°C, 40°C and 50°C.

Effect of carbon and Nitrogen Sources

The effect of carbon and Nitrogen sources on protease production was determined by inoculating the parent and mutant strains in different carbon and nitrogen sources containing production medium. Different carbon sources tested were as sucrose, lactose and Glucose, Nitrogen sources like yeast extracts, casein, skim milk powder and beef extract. After incubation, the crude enzyme was collected and the enzyme activity determined by standard procedure Secades and Guijarro (1999).

Partial purification of the protease enzyme

Partial purification of the protease enzyme was carried out by ammonium sulphate precipitation method. 70% ice cold ammonium sulphate added to the cell free extract and incubated at overnight after the incubation the precipitation was found. The precipitate was centrifuged at 6000 rpm for 10 min and the pellet was suspended in 0.1 M Tris HCl buffer. The enzyme was dialyzed using dialysis membrane against Tris HCl buffer Secedes and Guijarro, (1999).

Purification of protease enzyme

The partially purified enzyme was subjected to DEAE-cellulose column using ion exchange chromatography to obtain pure and homogenous enzyme. The molecular weight of the purified enzyme was determined by SDS-PAGE (Laemmli et al., 1970).

RESULT AND DISCUSSION

Isolation and Identification

Proteolytic bacteria were isolated from various samples like slaughter house waste poultry waste, and fishery waste. Totally thirty nine isolates thirteen each from slaughter house waste, poultry waste and fishery waste on skim milk agar plate were isolated based on the enzyme activity. Among the thirty nine isolates, two isolates S3 (12mm) and F2 (12m) were selected (based on the clearing zone) for further studies. The selected isolate S3were identified as, *Staphylococcus sp* and F2 as *Yersinia sp* based on their morphology and standard biochemical characters (Table no 1). Secades and Guijarro (1999) isolated *Y. ruckeri* from enteric red mouth disease affected fish and other fish waste. An alkaline protease production has been reported in *Staphylococcus sp,* (Hasche et al., 1997).

Strain improvement

The selected isolates S_3 and F_1 were treated with UV irradiation for mutagenesis to improve upon their enzyme activity and then the enzyme activity of both parent and mutant strains were analyzed. The enzyme activity of the mutant strains were high (14mm) when compared to that of the parent strains (12mm). Karn and Karn (2014) also reported that the UV mutated *Bacillus* strain RS1 showed maximum (14 – 15 mm) zone compares to parent strain (10- 12 mm). *Bacillus alvei* mutants showed different response to UV radiation for alkaline protease production. These variations are more probably due to the differences induced in their genetic background. Therefore, results obtained revealed variation in gene expression, activity as (Justin et al., 2001). It is suggested that the increase in enzyme productivity might result from damage of genes located in plasmids which have a negative influence on the chromosomal alkaline protease production gene i.e. repression as reported by Solaiman et al (2003).

Environmental Parameters Affecting Protease Production

Optimal culture conditions for protease productivity were studied by many investigators The environmental conditions of the fermentation batch play a vital role in the growth and metabolite production of a microbial population (Kim et al., 2001; Miyaji et al., 2006). In this study, mutant strains showed maximum enzyme production compare to parent strain.

Effect of Incubation Period on protease production

The activities of the crude enzyme by parent and mutant strains of *Staphylococcus sp* and *Yersinia sp* were determined at different incubation period (24,48,72 and 96 hours) and the results are depicted in Figure – 1 and 2. The enzyme activity was maximum 198U/ml and 372U/ml at 48 hours of incubation for both parent and mutant strain of *Staphylococcus sp* respectively. Another organism of *Yersinia sp* shows the maximum enzyme activity 190U/ml and 368U/ml at 72 hours of incubation for both the parent and mutant strains respectively. A gradual decrease in enzyme production was observed with increasing incubation period clearly suggesting the enzyme role as a primary metabolic, being produced in the log phase of the growth the bacteria and fungus for utilization of proteins present in the substrate (Alagarsamy et al., 2006). Bacterial growth increased with an increased incubation period similar to protease production suggesting that enzyme production was growth associated in nature. These observations made by Qader et al (2009).

Effect of Temperature on protease production

The protease activity of *Staphylococcus sp* and *Yersinia sp* recorded at different temperature were carried out and the results are presented in figure 3 and 4. The maximum enzyme activity was recorded at 20°C (286U/ml) for *Staphylococcus sp.*, and at 30°C (225U/ml) for *Yersinia sp.* Similar results reported by Secades and Guijarro (1999) stated that the optimum temperature for protease production by *Y.ruckeri* was at 30˚C. Higher temperature is saidto have some adverse effect on metabolic activities of microorganisms. The enzyme gradually becomes unstable at about 42°C.

Effect of P^H on protease production

The protease production by the isolated strains at different p^H showed (Figure 5 and 6) that the maximum enzyme activity of 268.1U/ml and 186U/ml was recorded by mutant and parent strain of *Staphylococcus sp* at p^H 8.0, whereas *Yersinia sp* showed higher production of 251U/ml by mutant and 181.6U/ml by parent strain at p^H 7.0. In both *Staphylococcus sp* and *Yersinia sp* enzyme activity was minimum at p^H 6.0. Secades and Guijarro (1999) reported maximum activity was exhibited at pH 8.1 for *Y.ruckeri* under assay condition. The enzyme activity and productivity was higher at 7.6 to 8.5 and 6.1 to 9.5, very little activity was recorded at or below 6.1, but there is a sharp increase occurred at 6.7 and enzyme activity decreased at 8.6.Enzyme production was affected if pH level was higher or lower compared to the optimum level (Tunga et al., 1998).

A notable decline in the enzyme productivity occurred at both higher and lower p^H values. Similar results observed by Teufel and Gotz (1993) that the neutral metalloprotease from *Staphylococcus epidermidis* has p^H

optimal in the range 5.0 -8.0. Most of the *Bacillus sp* reported has optimum pH from 7.0 to 11.0 for the production of protease (Suganthi et al., 2013). Prasad et al (2013) also reported the optimization studies on *Bacillus licheniformis* have showed maximum enzyme production at pH 8.0.

Effect of Carbon Source on Protease Enzyme in Production Medium

The effect of various carbon sources on enzyme production was carried out and the results are presented in figure 7 and 8. In case of *Staphylococcus sp,* maximum enzyme production was observed in mutant (292U/ml) and parent (198U/ml) strains using glucose as a carbon source. *Yersinia sp* showed maximum enzyme production of both parent (194U/ml) and mutant (288U/ml) strains in the production medium containing lactose as a carbon source. The study of assimilation of carbon sources by Saha and Bhattacharyya (1990) observed that the presence of glucose and maltose increased the protease production by the mutant strain of *Pseudomonas sp.* These findings indicate that glucose is the best carbon source for protease production by *Bacillus sp* has been observed in earlier investigations (Shafee et al., 2005). It was found that protease production increased as the concentration of glucose increased. Some other researchers also found considerable increase in protease production by *Bacillus sp.,* with glucose as a carbon source (Adinarayana et al., 2003 and Prakasam et al., 2006)

Effect of Nitrogen Source on Protease Enzyme in Production Medium

The effect of nitrogen source on crude enzyme production by *Staphylococcus sp* and *Yersinia sp* (parent and mutant strains) were studied and results are presented in figure 9 and 10. *Staphylococcus sp* showed the maximum (198.0 U/ml, 278.0 U/ml) enzyme in the medium containing beef extract as a nitrogen source, and *Yersinia sp* shower maximum (197.0 U/ml , 272.2U/ml) in the medium containing skim milk. And lower enzyme (127U/ml, 161U/ml) production observed in the medium containing beef extract as a nitrogen source. Skimmilk act as a best nitrogen source for the enzyme production by *Yersinia sp*., (Secades and Guijarro 1999). Beef extract was found to be the best organic nitrogen source for alkaline protease enzyme production for the *Bacillus* species (Pedge et al., 2013)

Purification of Protease Enzyme

The crude protease enzyme was dialyzed and purified by DEAE cellulose ion exchange column chromatography. The molecular weight of the enzyme under denaturing conditions was estimated and it is approximately 40-50 KDa for both *Yersinia sp* and *Staphylococcus sp.* This observation was in accordance in with (Secades and Guijarro 1999) who reported that the molecular weight of extracellular protease from *Y. ruckeri* was 47KDa. The molecular weight of the alkaline protease from *Staphlococcus aureus* was 40 KDa (Maeda et al., 2000).

Table-1 Morphological and Biochemical characters of the isolated proteolytic bacteria

Test	Isolate S_3	Isolate F_2
Colony morphology on nutrient agar	Colonies are circular, rough, and crystal like appearance. Produce dark yellow color pigment.	Colonies are small, transparent discs, opaque and delicate
Gram's reaction	Positive cocci	Negative rod
Motility	Non motile	Non motile
Catalase	Positive	Positive
Oxidase	Negative	Negative
Indole	Negative	Negative
MR	Positive	Positive
VP	Positive	Negative

Citrate	Negative	Negative
Gelatin	Positive	Negative
Glucose	Positive	Positive
Galactose	Positive	Positive
Manitol	Negative	Positive
Arabinose	Positive	Positive
Fructose	Positive	Positive
Identified organism	*Staphylococcus sp.,*	*Yersinia sp.,*

Figure 1- Effect of incubation period on protease activity of *Staphylococcus sp*.

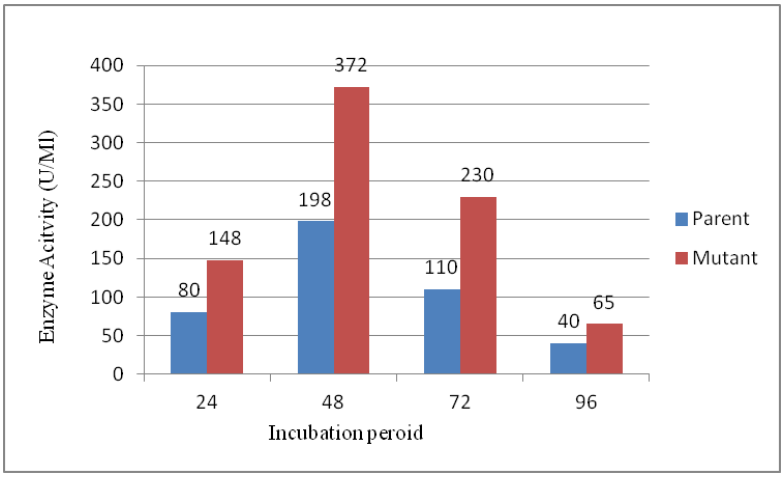


Figure 2- Effect of incubation period on protease activity of *Yersinia sp*

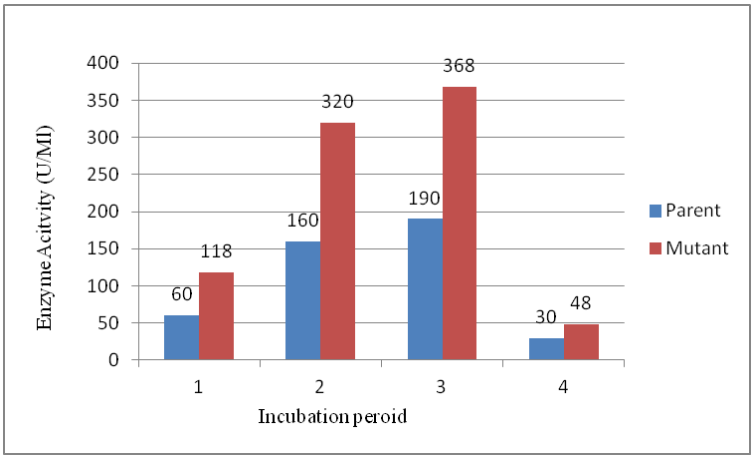


Figure 3- Effect of temperature on protease activity of *Staphylococcus sp*

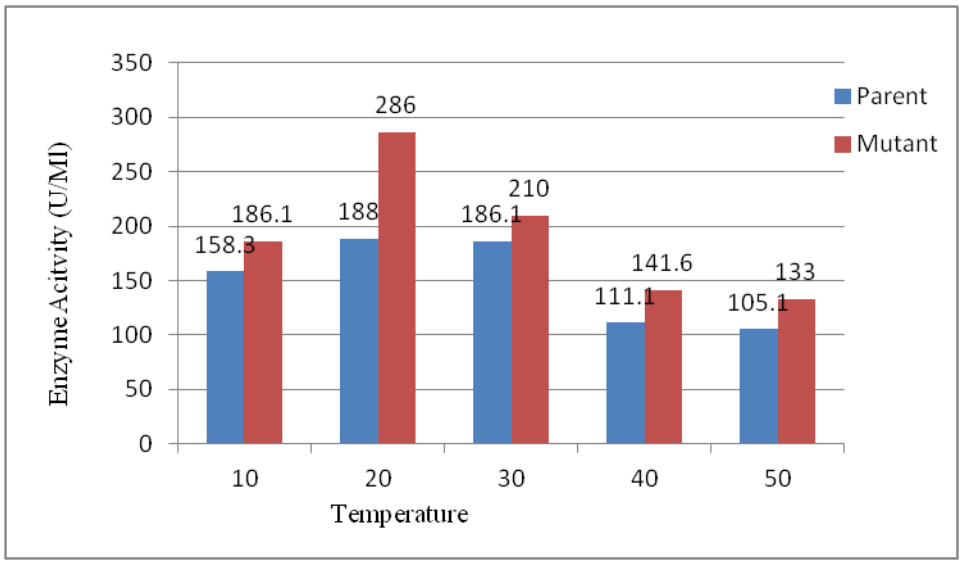


Figure 4- Effect of temperature on protease activity of *Yersinia sp*

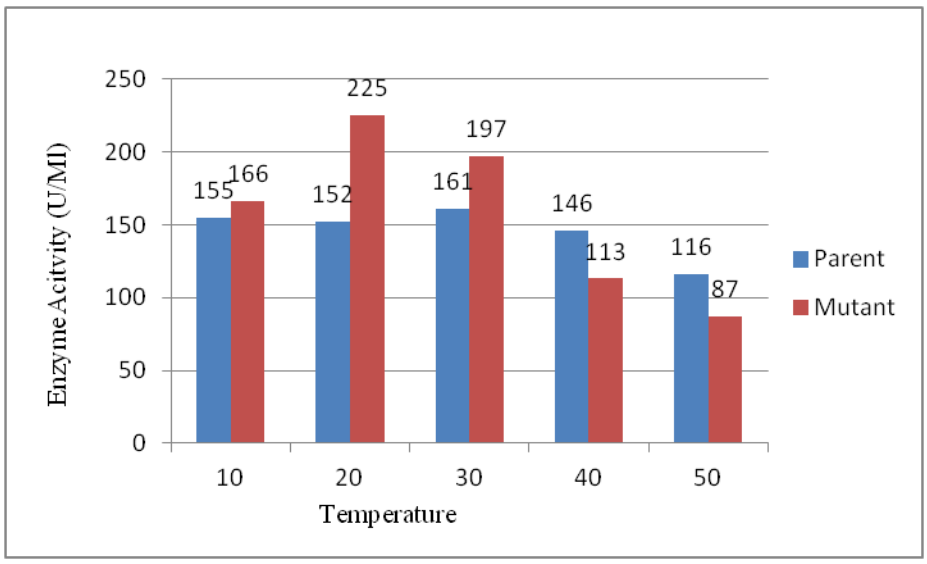


Figure 5 -Effect of pH on protease activity of *Staphylococcus sp*

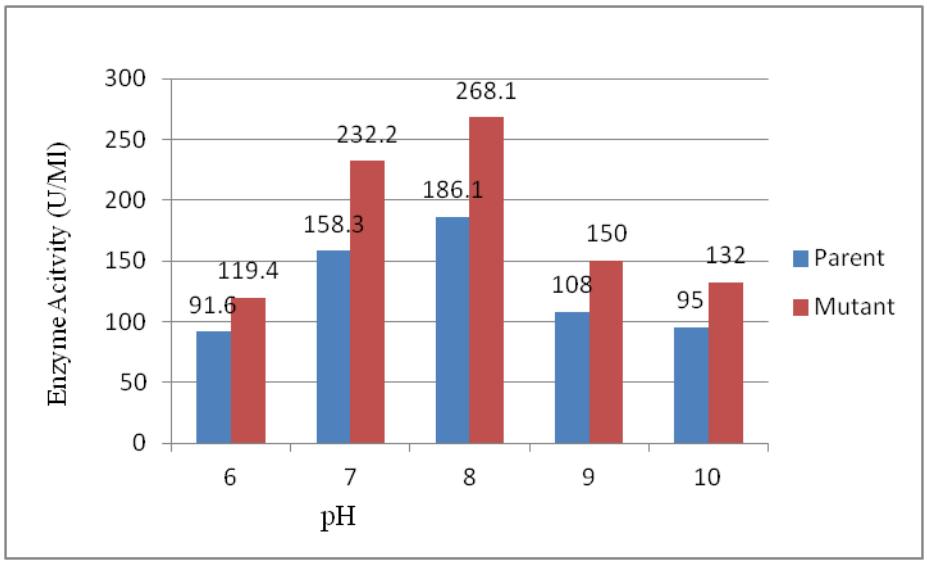


Figure 6- Effect of ph on protease activity of *Yersinia sp*

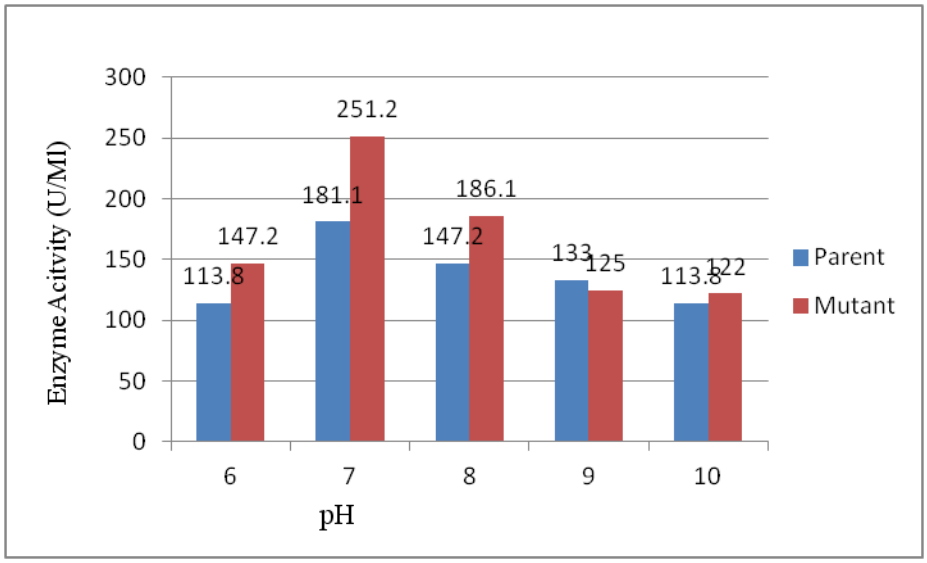


Figure 7 –Effect of Various carbon sources in protease production of *Staphylococcus sp* on production medium

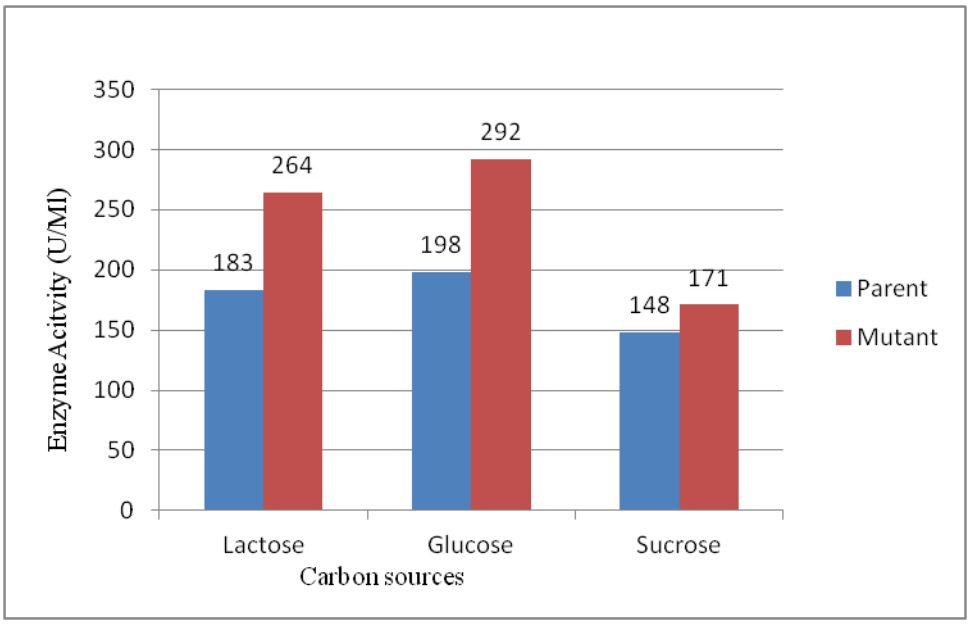


Figure 8- Effect of various carbon sources in protease production of *Yersinia sp* on production medium

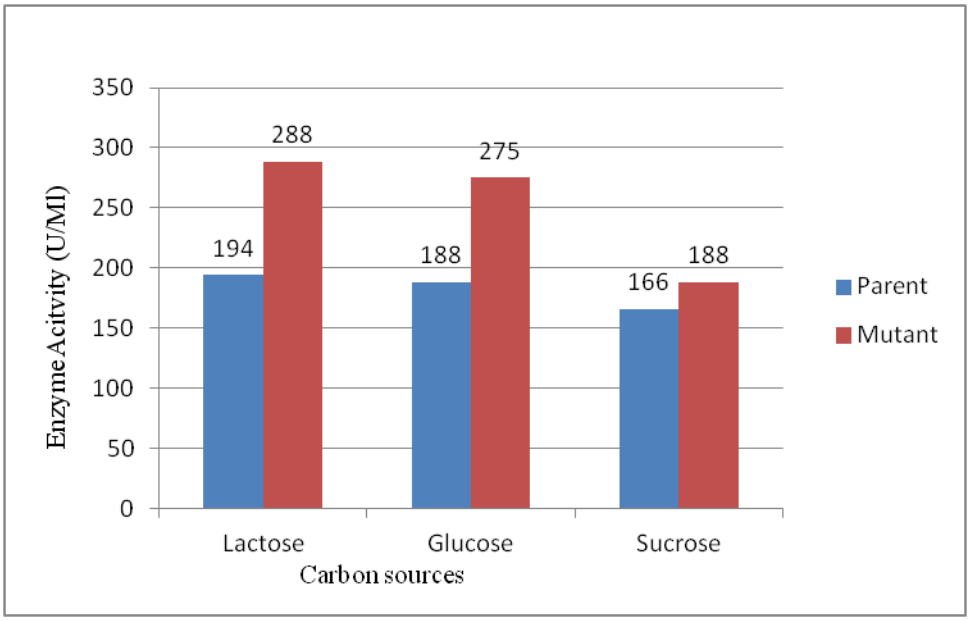


Figure 9- Effect of various nitrogen sources in protease production of *staphylococcus sp* on production medium

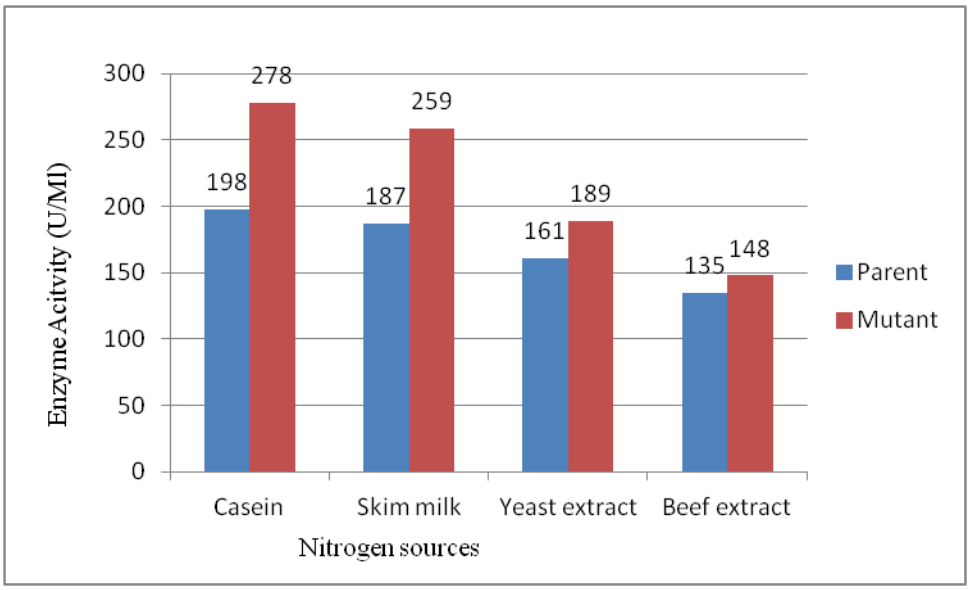
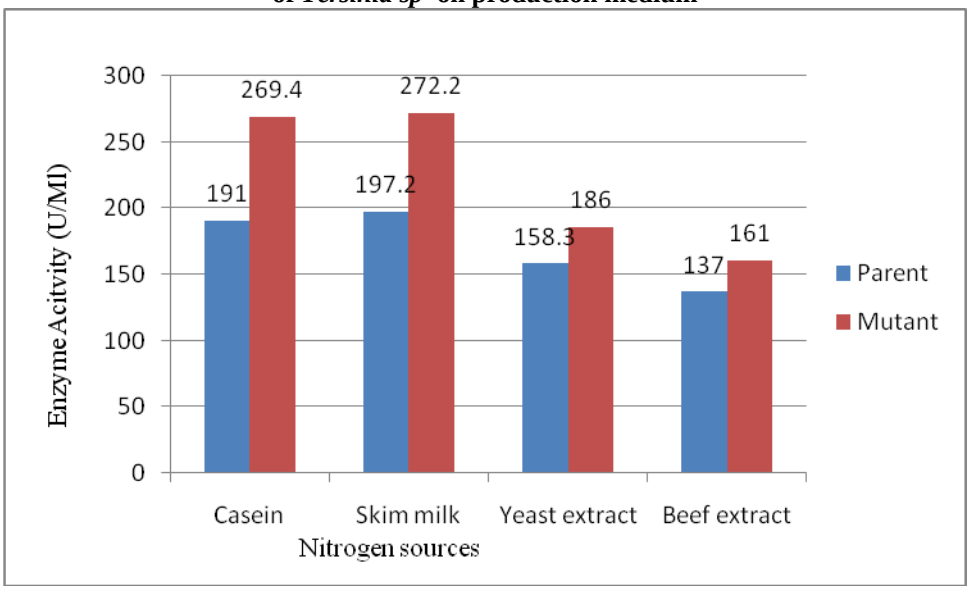


Figure 10-Effect of various nitrogen sources in protease production of *Yersinia sp* on production medium



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