IJASRM

International Journal of Advanced Scientific Research and Management, Volume 4 Issue 3, Mar 2019

www.ijasrm.com

ISSN 2455-6378

Optimization of Culture conditions for Protease production from *Staphylococcus sciuri* (TKMFT 8)

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Abstract

Proteases are a group of enzymes catalyze the hydrolysis of polypeptide chains into smaller polypeptides or free amino acids. Microbial proteases are one of the important groups of industrially and commercially produced enzymes. The protease production was studied by using shake flask fermentation using *Staphylococcus sciuri* (TKMIT8) isolated from soil and water samples collected from different halwa manufacturing units located in Kollam and Karunagappally, Kerala, India. The fermentation conditions (Incubation time, pH, Temperature, Carbon sources, Metal salts, and Nitrogen sources) were optimized to enhance protease production and activity by *Staphylococcus sciuri* (TKMFT 8). The results showed that protease production by *Staphylococcus sciuri* (TKMFT 8) was increased under optimized conditions with a medium containing glucose as carbon source, beef extract as the nitrogen source, NaCl as the metal salt, and pH 8 at 20ºC for 48 h of incubation of fermentation broth. The results in the optimization study on different factors will be useful during further production of protease by *Staphylococcus sciuri* (TKMFT8).

Keywords: *Protease, Screening, Optimization, Staphylococcus sciuri (TKMFT8).*

1.Introduction

Proteases can be defined as enzymes which catalyze the breakdown of hydrolytic bonds in proteins thereby releasing amino acids and peptides (Theron and Divol, 2014) [25]. Proteases are a distinct subgroup of hydrolytic enzymes which catalyze the peptide bond breakdown in proteinous substrates (Sankeerthana et al., 2013[19], Ghasemi et al., 2011[6], Bhatnagar et al., 2010[5], Yadav et al., 2011) [34]. On account of their broad substrate specificity, proteases have extensive applications in various fields (Maal et al., 2009[14], Sumantha et al., 2006[24], Ward et al., 2009) [33]. Moreover, baking industries use bakery dough or wheat flour and this compound consists of an insoluble protein called gluten which is hydrolyzed by protease (Sumantha et al., 2006) [24]. Also, the addition of proteases decreases the mixing time and results in the four increased loaf volumes (Leng and Xu, 2011) [12].

Generally, proteolytic enzymes (proteases) are categorized into peptidases and proteinases. Proteinases catalyze protein degradation into smaller peptide fractions; while hydrolysis of specific peptide bonds or completely breakdown peptides to amino acids are catalyzed by peptidases or peptide bond hydrolase (Anita, 2010[4], Vasantha and Subramanian, 2012) [30]. Proteases are classified under the subgroup 4 of Group 3 (hydrolases) (International Union of Biochemistry, 1992) [8]. Based on the pH optima, there are three different types of proteases such as acidic, neutral and alkaline protease (Tiwari et al., 2015) [25]. The environmental conditions of the fermentation batch play a major role in the growth and metabolite production of a microbial population (Miyaji et al., 2006[15], Kim et al., 2001) [10].

Proteases are seen in all forms of life such as plants, animals and microorganisms. Vadlamani and Percha (2011) [29] reported that proteolytic enzymes are widely found in all living organisms. Microorganisms represent an important source of enzymes including protease due to their broad biochemical diversity (Godfrey and West, 1996) [7]. Several *Bacillus* sp, *Yersinia* sp, and *Staphyloccocal* sp are involved in protease production (Josephine et al., 2012) [9]. 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 the properties that are desirable for their various applications (Kumarasamy et al., 2012) [11].

The study aims to optimize the culture conditions of protease production by *Staphylococcus sciuri* (TKMFT 8) isolated from soil and water samples collected from different halwa manufacturing units located in Kollam and Karunagappally, Kerala, India.

2.Materials and Methods

2.1 Sample collection

Protease producing microbes were isolated from, soil and water samples collected from different halwa manufacturing units located in Kollam and Karunagappally, Kerala, India. Soil samples for the study were collected from different areas in halwa production units whereas water samples collected comprised of wastewater from the food processing units.

2.2 Isolation of Protease producing bacteria

The technique used for the isolation of bacteria from soil and water samples was the serial dilution agar plate technique (Sjodahl et al., 2002) [22].

2.3 Screening for best strain produced protease

A total of 87 dissimilar colonies from nutrient agar plates were selected and each isolate was given a reference number (TKMFT 01 to TKMFT 87) and subjected to primary screening by plate assay using protease specific medium where gelatin was used as the substrate. The clear zone diameters were measured by flooded the plates with mercuric chloride solution (Abdel Galil, 1992) [1].

2.4 Preparation of Crude Enzyme

The culture was inoculated in the production medium and incubated over ten days at 37^0 C in a rotary shaker. The contents of the flasks were centrifuged at 10000 rpm for 10 minutes at 4^0 C to get the cell-free supernatant containing the crude enzyme. The protease assay was carried out from the first day to 10^{th} day to find out the day with maximum enzyme production.

2.5 Measurement of activity of enzyme

Protease activity was carried out using the supernatant solution (Tsuchida et al. (1986) [28]. The amount of protease activity was determined using a standard graph prepared using tyrosine. One unit of protease is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute under the standard conditions of supernatant solution. Enzyme activity was expressed as U/ml.

2.6 Quantitative assay of protein

The total protein content was determined by the Lowry method using Bovine Serum Albumin (BSA) as standard (Lowry et al, 1951) [13]. OD was measured at 660 nm and expressed in milligram per milliliter (mg/ml).

2.7 Identification of Protease producing bacteria

The isolated organisms were identified based on their colony morphology (Aneja, 2003) [3], microscopic observation using Gram's staining (Todar et al., 2005) [27], biochemical identification using VITEK 2 compact-Biomerieux automatic system, and molecular identification using 16S Ribosomal RNA Sequencing (Vimal et al., 2016) [31].

2.8 Optimization of culture conditions for maximum protease production

The culture conditions viz., incubation time, p^H, temperature, carbon sources, nitrogen sources and metal salts were optimized to enhance protease production by the isolate.

2.8.1 Effect of incubation time on protease production

Production medium at pH 7.0 was inoculated with 12h grown selected bacterial cultures. The broth was kept for shaker fermentation at different time period of 24-240 hours (Day1 to Day10). At the end of incubation, the cell free culture filtrate is obtained and used as enzyme source.

2.8.2 Optimization of Protease production of TKMFT8 at different p^H

The effect of p^H on enzyme activity was determined over a pH range of 4, 5, 6, 7, 8, 9, and 10 using 1N HCl and 1N NaOH and culture flasks were incubated at 37ºC for 48h. Protease activity was determined in the supernatant.

2.8.3 Optimization of Protease production of TKMFT 8 at different Temperatures

Production medium was inoculated with overnight grown selected bacterial strain. The broth was incubated at different temperatures from 10, 20, 30, 40, 50 and 60ºC for 48h. At the end of the incubation period, the cell-free culture filtrate is used as an enzyme source.

2.8.4 Effect of Carbon sources on protease production of TKMFT8

The effect of different carbon sources for protease production was determined with carbon sources such as Glucose, Sucrose, Maltose and Lactose. Each source was used at a concentration of 1% (w/v). The protease assay was carried out to determine the concentration of the enzyme.

2.8.5 Effect of Nitrogen sources on protease production of TKMFT8

The effect of various nitrogen sources such as peptone, yeast extract, beef extract and ammonium citrate on protease production was determined. Protease activity in cell-free supernatant was determined.

2.8.6 Effect of various metal salts on protease production

Effect of various metal ions on the production of protease enzyme was determined by incubating the medium with different metal ions such as NaCl, $MgSO_4$, $BaCl_2$, $CaCl_2$, and $CuSO_4$ at a concentration of 0.2% which contributed the metal salts Na^+, Mg^{2+}, Ba^{2+}, Ca^{2+} and Cu^{2+} respectively.

3. Result and Discussion

3.1 Isolation and screening of protease producing bacteria

Protease producing bacteria were isolated and showed a clear zone around colonies on gelatin agar plate as shown in Fig.1. Among 87 bacterial isolates, 27 isolates were protease producer and out of them TKMFT 8 was selected for secondary screening as the highest zone diameter was observed for TKMFT 8 (26mm). Secondary screening was carried out and the results are inferred that bacterial strain TKMFT 8 produces maximal protease on the second day of incubation (215.66±1.98 U/mL). At the end of the incubation period, the cell-free culture filtrate is used as an enzyme source.

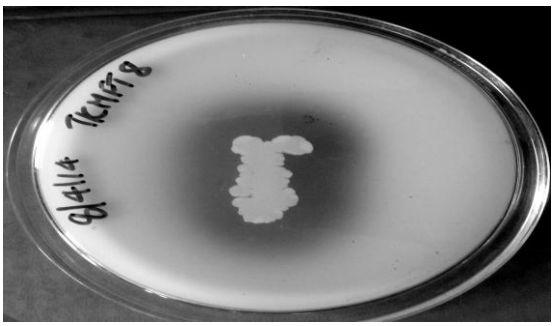

Fig1: Zone of hydrolysis of bacterial isolate (TKMFT 8) on gelatin agar plate

3.2 Identification of protease producing bacteria

Identification was carried out using cultural characteristics (presented in Table 1) followed by biochemical identification (presented in Table 2) and molecular identification. Summary of the BLAST result presented in Table 3. Being highly selective and sensitive, molecular identification methods are currently used for the identification of microorganisms (Vimal et al., 2016) [31]. VITEK 2 is an automated microbial identification system that provides highly reproducible and accurate results as shown in multiple independent studies. Wallet et al. (2005) [32] reported the performances of VITEK 2 colorimetric cards for identification of gram-positive and gram-negative bacteria. A similar study conducted by Simgamsetty et al. (2016) [21] found to achieve 90-95% probability of identification. In the present study, it was found to achieve 99% probability of identification for *Staphylococcus sciuri* (TKMFT 8).

Table 1: Colony morphology and microscopic observation of TKMFT 8

Bacterial isolate (TKMFT8)	
Gram staining	Gram Positive cocci
Colony characters on Nutrient agar	
Size	Medium
Pigmentation	Dark yellow colour
Form	Circular
Margin	Entire
Elevation	Flat
Texture	Rough

Table 2: Microbial identification using Biomerieux VITEK 2 system

Strain Ref. No.	**Species identified**	**Test method**
TKMFT 8	*Staphylococcus sciuri*	VITEK/GP CARDS

Table3: Summary of the BLAST result of TKMFT8

Sequence length blasted (bp)	**% identity (Accession no.)**	**Identified name of sample**
817	99 (AB 233331)	*Staphylococcus sciuri*

3.3 Optimization of culture conditions for maximum protease production of *Staphylococcus sciuri* (TKMFT 8)

3.3.1 Effect of incubation time on protease production

Production medium was inoculated with 12h grown selected bacterial cultures. The broth was kept for shaker fermentation at different time period of 24-240 hours (Day 1to Day10) at 37ºC. At the end of incubation period the cell free culture filtrate is used as enzyme source. The results are presented in Table 4.

Table 4: The effect of period of incubation on protease production

Incubation Day	**Protease Activity (U/ml)**
1	121.49±0.54
2	**215.19±0.90**
3	157.64±1.31
4	137.12±1.28
5	108.5±1.87
6	89.73±1.39
7	82.2 ±1.52
8	70.16±1.59
9	59.43 ±1.8
10	46.09 ±1.53

The results presented in Table 4 showed that *Staphylococcus sciuri* (TKMFT 8) under study secreted protease enzyme at varied levels. The protease production graph proceeded at a slower rate after which it increased sharply attaining a maximum value at 48 hours of incubation and the activity of enzyme gradually decreased after 48 hours of incubation. The highest protease activity of *Staphylococcus sciuri* (TKMFT 8) has been observed on the second day of incubation (215.66±1.98 U/mL). A gradual decrease in the production of enzyme was observed with increasing incubation period clearly indicating the role of the enzyme as a primary metabolite, being produced in the log phase of the bacterial growth and utilization of proteins in the substrate (Alagarsamy et al., 2006) [2]. Similar to protease production, bacterial growth increased with an increased incubation period suggesting that the production of enzyme was growth associated in nature (Qader et al., 2009) [17].

3.3.2 Optimization of Protease production of *Staphylococcus sciuri* (TKMFT 8) at different p^H

From the pH optimization study shown in Table 5, it was observed that *Staphylococcus sciuri* (TKMFT 8) was able to produce 239.71±0.75 U/ml at pH 8.0. It was observed that there was a gradual increase in protease production from pH 4.0-8.0. Subsequently, the production capacity was shown a downward trend after pH 8.0. A decline in the productivity of enzyme was observed at both higher and lower pH values. A similar study conducted by Suganthi et al. (2013) [23] observed most of the *Bacillus* sp reported has optimum pH from 7.0 to 11.0 for protease production.

Table 5: The effect of P^H on protease production

P^H	Protease activity (U/ml)
4	103.63±0.54
5	13.59±1.95
6	168.15±1.09
7	203.27±1.8
8	**241.55±0.68**
9	136.45±0.56
10	94.43±1.19

3.3.3 Optimization of Protease production of *Staphylococcus sciuri* (TKMFT 8) at different Temperatures

Enzyme activity recorded at different temperatures revealed that between a range of 20ºC-60ºC, the enzyme production was found to be maximum at 20ºC (235.23±0.98 U/mL).

Table 6: The effect of temperature on protease production

Temperature	Protease activity (U/ml)
10	152.21±1.2
20	**235.74±0.44**
30	212.70±0.36
40	112.84±0.54
50	81.75±0.38
60	72.74±0.46

3.3.4 Effect of Carbon sources on protease production of *Staphylococcus sciuri* (TKMFT 8)

Results presented in Table 7 showed that glucose was found to be the best carbon source when compared to other carbon sources. Protease production was 195.21±1.3 U/mL on 48 h incubation at pH 8. Shafee et al. (2005) [20] observed that protease production increased as the concentration of glucose as the carbon source increased.

Table 7: The effect of Carbon sources on protease production

Carbon sources	Protease activity(U/ml)
Glucose	**194.5±0.95**
Sucrose	148.8±0.57
Maltose	94.76±0.43
Lactose	122.05±0.83

3.3.5 Effect of Nitrogen sources on protease production of *Staphylococcus sciuri* (TKMFT 8)

Results given in Table 8 showed that among the various nitrogen sources tested, beef extract was found to be the excellent nitrogen source for the highest protease activity by *Staphylococcus sciuri* (TKMFT 8) under previously optimized conditions. The beef extract was found to be the best organic nitrogen source for the production of alkaline protease for the *Bacillus* species (Pedge et al., 2013) [16].

Table 8: The effect of Nitrogen sources on protease production

Nitrogen sources	Protease activity(U/ml)
Peptone	146.86±0.43
Yeast Extract	182.23±0.92
Beef extract	**227.26±0.67**
Ammonium citrate	111.49±1.42

3.3.6 Effect of various metal salts on protease production of *Staphylococcus sciuri* (TKMFT 8)

The various metal ions tested in the present study showed considerable variation concerning their effect on enzyme production. Among the various metal salts evaluated, NaCl increases protease production followed by $CaCl_2$, $MgSO_4$, $BaCl_4$, and $CuSO_4$. The results are depicted in Table 9.

Table 9: The effect of metal salts on protease production

Metal salts	Protease activity(U/ml)
NaCl	**212.73±0.37**
$CaCl_2$	198.57±0.34
$MgSO_4$	153.19±0.46
$BaCl_2$	98.16±0.90
$CuSO_4$	23.16±0.16

The culture grew in the pH range 4-10, with optimum protease secretion at pH 8 at specific incubation time (48 h) and temperature (20°C) and glucose and beef extract as the carbon and nitrogen source respectively. Among the various metal salts evaluated for their effect on protease productivity, NaCl increases protease production. These observations are comparable with the findings of Revathi and Palanisamy (2015)[18] using

Staphylococcus sp where the optimum pH, temperature, incubation time, carbon and nitrogen sources of the medium were 8, 20°C, 48h, glucose and beef extract respectively. Protease yields vary considerably with temperature and pH which may be attributed to other components of the medium and their combined influence on the metabolism of the bacterial species.

The culture can be used for large-scale production of alkaline protease to meet present-day needs in the industrial sector. It is also determined the optimum growth parameters for cultivating the organism. As alkaline protease has more applications than another protease in industry, alkaline protease production is recommended.

4. Conclusion

The fermentation conditions (Incubation time, pH, Temperature, Carbon sources, Metal salts and Nitrogen sources) were optimized in order to enhance protease production and activity by *Staphylococcus sciuri* (TKMFT 8). The results showed that protease production by *Staphylococcus sciuri* (TKMFT 8) was increased under optimized conditions with a medium containing glucose as carbon source, beef extract as nitrogen source, NaCl as the metal salt, and pH 8 at 20ºC for 48 h of incubation of fermentation broth. The results in the optimization study on different factors will be useful during further production of protease by *Staphylococcus sciuri* (TKMFT8).

Acknowledgment

I gratefully acknowledge the research facility given by The Cashew Export Promotion Council of India (CEPCI), Kollam, and Kerala, India.

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