International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **6(2),** 36-41, February **(2017)** *Int. Res. J. Biological Sci.*

Screening of protease produced by bacterial isolates of bakery industry

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Available online at: www.isca.in, www.isca.me
Received 16th January 2017, revised 4th February 2017, accepted 7th February 2017

Abstract

Quantitative estimation of protease produced by bacterial isolates of bakery industry was performed. Primary screening of protease production was carried out using gelatine agar plates followed by quantitative estimation of protease was conducted for five bacterial isolates named as TKMFT8,TKMFT22,TKMFT25, TKMFT39 and TKMFT53 using MRS broth inoculated with incubated over a 10 days period in a rotary shaker at 37 ^{o}C. The activity of TKMFT8, TKMFT22, TKMFT25, TKMFT39 and TKMFT53 was found to be 215.19±0.90 U/mL, 208.23 ± 1.50U/mL, 205.76 ± 0.75U/mL, 198.53 ± 1.98U/mL, 103.56 ± 1.98U/mL respectively. Among the five isolates studied, the highest activity (215.19±0.90 U/mL) was recorded in TKMFT8 strain.

Keywords: Protease, MRS broth, Enzyme activity.

Introduction

Proteases are defined as enzymes which catalyze hydrolytic bonds breakdown in proteins into amino acids and/or peptides[1]. Proteases representing one of the important classes of enzymes which are in 60% of total enzyme market[2]. Proteases have tremendous applications in various fields such as protein hydrolysate, baking, brewing, leather processing, waste treatment, detergent formulations, cheese manufacture, meat tenderization, peptide synthesis, silk industry, organic synthesis, soy sauce production, pharmaceutical industry etc[3, 4]. In bakery industry, wheat flour is commonly used for making most of the bakery products which consists of gluten protein which is insoluble in nature. Gluten protein can be hydrolyzed by protease enzyme[3].

Proteases are classified into proteinases and peptidases. Degradation of protein into smaller peptide fractions catalyzed by proteinases; while peptidases catalyze the breakdown of peptides to amino acids[5-7]. Based on the site of action of protease on protein substrates, proteases are classified as endo- or exo-enzymes[8]. Another classification based on their catalytic mechanism as cysteine proteases, aspartic proteases, metalloproteases or serine proteases[9]. Further proteases are classified into, alkaline, neutral and acidic proteases based on their pH optima and of these alkaline protease is the most important one for industrial application[10].

Major sources of proteases composed of animals, plants and microorganisms. Among these microorganisms serve as the important source of protease. Due to their rapid growth, cost effectiveness and ease with producing genetically modified strains with high yielding nature, the world demand for proteases of microbial origin is increasing[11].

In the present work, bacterial isolates from bakery industry were screened for their ability of protease production and the produced proteases were quantitatively determined. Based on the results, the best protease producing bacterial isolate can be further used for the degradation of protein rich waste discarded from bakery industry leading to food industry waste recycling.

Materials and methods

Isolation of protease producing bacteria: Isolation of protease producing bacteria from soil and water samples collected from bakery industry was carried out using serial dilution method[12]. A total of 5 isolates (TKMFT8, TKMFT22, TKMFT25, TKMFT39 and TKMFT53) were selected based on zone diameter on primary screening of protease production using gelatin agar medium. Observed zone diameter for TKMFT8, TKMFT22, TKMFT25, TKMFT39 and TKMFT53 was 26mm, 25mm, 20mm, 23mm and 15mm respectively.

Quantitative estimation of protease: Five conical flasks with 100 ml MRS (De Man, Rogosa and Sharpe) broth containing (g/l) Yeast extract 5.0, Peptone 10.0, Polysorbate 80 1.0, Beef extract 10.0, Sodium acetate 5.0, Dextrose 20.0, Ammonium citrate 2.0, magnesium sulphate 0.1, Manganese sulphate 0.05, Dipotassium phosphate 2.0 broth each were inoculated separately with the selected bacterial isolates followed by incubation at 37^{0}C in a rotary shaker for a period of 10 days. A control of uninoculated MRS broth was maintained. The five bacterial isolates were inoculated separately to five conical flasks containing 100 ml MRS and incubated over a 10 days period at 37^{0}C in a rotary shaker (120rpm). MRS broth without bacterial inoculation kept as control. Crude enzyme was collected from the fermented broth by centrifuging the contents at10000 rpm at 4^{0}C for 10 minutes. Fermentation day with

maximum enzyme production was determined by conducting protease assay from first day of incubation to the last day (day10).

Measurement of activity of enzyme: Determination of protease activity was carried out using the supernatant solution[13]. A mixture of 200 µl crude enzyme extract and 500 µl of 1% (w/v) of casein in 50 mM phosphate buffer at pH 7 were incubated at 40°C for 20 minutes in a water bath. After 20 minutes, enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) TCA (Trichloroacetic acid) and was kept at room temperature for 15 minutes. Unreacted casein was separated from the reaction mixture by centrifugation for five minutes at 10,000 rpm followed by mixing the supernatant with 0.44M Na_2CO_3 (2.5 ml). To this, Follin Ciocalteus phenol reagent (1 ml of 3-fold diluted) was added and incubated at room temperature for 30 min in the dark. The absorbance of the blue colour developed was measured against a reagent blank at 660 nm using a tyrosine standard[13]. One protease unit is defined as the amount of enzyme which releases 1 µg of tyrosine per ml per minute under the standard conditions of supernatant solution. Enzyme activity was expressed as U/ml.

Figure-1: Bacterial isolates inoculated MRS broth for quantitative estimation of protease.

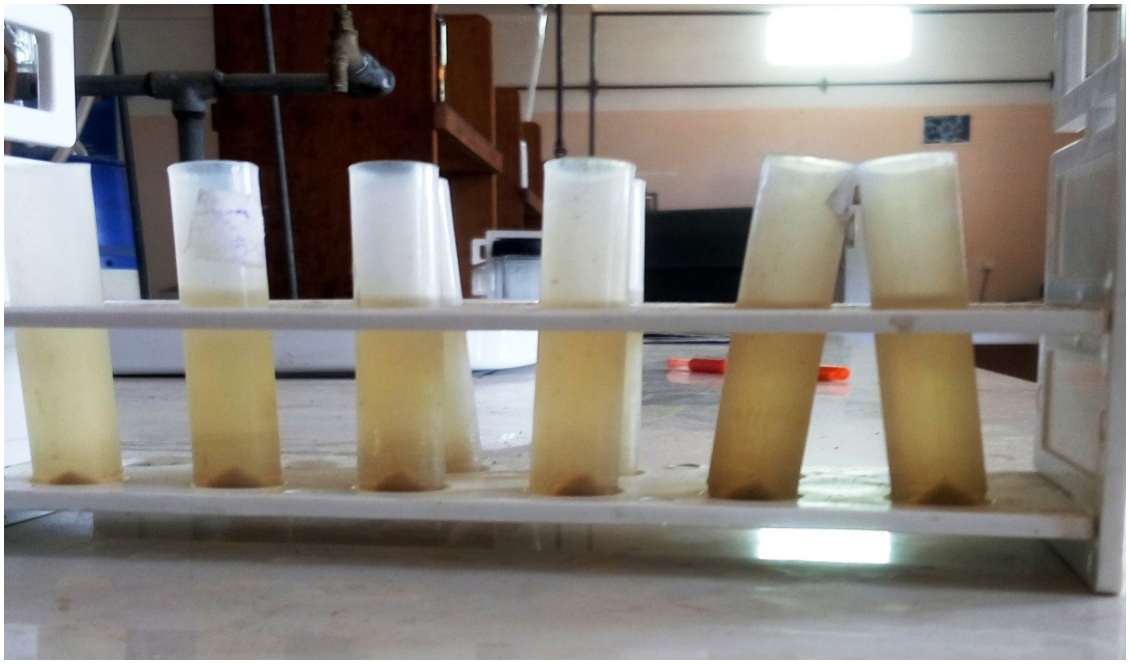

Figure-2: Crude protease enzyme.

Quantitative assay of protein: Determination of total protein content was carried out by following Lowry method using BSA (Bovine serum Albumin) as standard[14]. Optical Density was measured at 660 nm and expressed in mg/ml (milligram per millilitre).

Preparation of reagents: reagent A (alkaline sodium carbonate) : Sodium carbonate in sodium hydroxide (0.1 N) 2.0 % (w/v).

Reagent B (copper sulphate solution): Copper sulphate in distilled water 0.5 % (w/v).

Reagent A (Rochelle's sal): Sodium potassium tartrate solution in distilled water 1.0 % (w/v).

Working solution (Prepared fresh before use): Reagent A (100ml) added with Reagent B and Reagent C (1 ml each).

Folin and Ciocalteau's phenol reagent (1:1) was used after diluting with distilled water on the day of use.

Estimation: To 100µl of protein sample, distilled water was added and made upto 1ml and 5ml of working solution was added and incubated for 10 min after thorough mixing. To the above mixture, Folin Ciocalteau reagent (0.5ml) was added, mixed well and incubated for 30 min. The absorbance of the blue colour developed was measured at 660 nm in a UV-Visible spectrophotometer (UV 1800 Shimadzu, Japan).

Results and discussion

Isolation of protease producing bacterial isolates: Isolation of bacteria from samples collected from bakery industry was performed. Primary screening of protease production was conducted using gelatin agar medium. A total of 87 isolates were evaluated for proteolysis ability using gelatine agar medium which was indicated by zone formation around the colony. A total of 27 isolates were protease producer and out of them 5 isolates (TKMFT 8, TKMFT22, TKMFT25, TKMFT 39 and TKMFT 53) were selected for further experimental studies.

Protease production by selected bacterial isolates: Screening of protease production by the bacterial isolates during their growth on the medium was carried out[15]. The selected bacterial isolates were screened for their ability of producing extracellular protease in the medium (MRS broth) and highest protease producing isolate was selected. Determination of enzyme activity in the crude extract was performed[16].

The results shown in Table-1 revealed that selected bacterial isolates secreted protease enzyme at varied levels. Protease production graph proceeded at a slower rate after which it increased sharply attaining a maximum value at 48 hours for the isolates TKMFT8 and TKMFT39. It was also clear from the results that the activity of enzyme gradually decreased from third day of incubation to tenth day of incubation. It was found that the highest protease activity of these isolates has been observed on second day of incubation and it was found to be 215.66±1.98U/mL and 198.53±1.98/mL respectively. The enzyme activity exhibited by TKMFT22 and TKMFT25 was gradually increased from first day of incubation to third day of incubation as shown in Table-1. From there onwards gradual decrease in the production was observed. The highest activity by TKMFT22 and TKMFT25 was found to be 208.23±1.50U/mL and 205.76±0.75 respectively. The enzyme activity of TKMFT53 has been increased from first day of incubation to fourth day of incubation and after that it started decreasing and the highestactivity was observed on fourth day of incubation and it was found to be 103.56±1.98U/mL. Among all studied bacterial isolates, the maximum protease activity (215.66±1.98U/mL) was recorded in TKMFT8. From the above result, TKMFT8 strain was selected for further optimization study for maximum protease productivity.

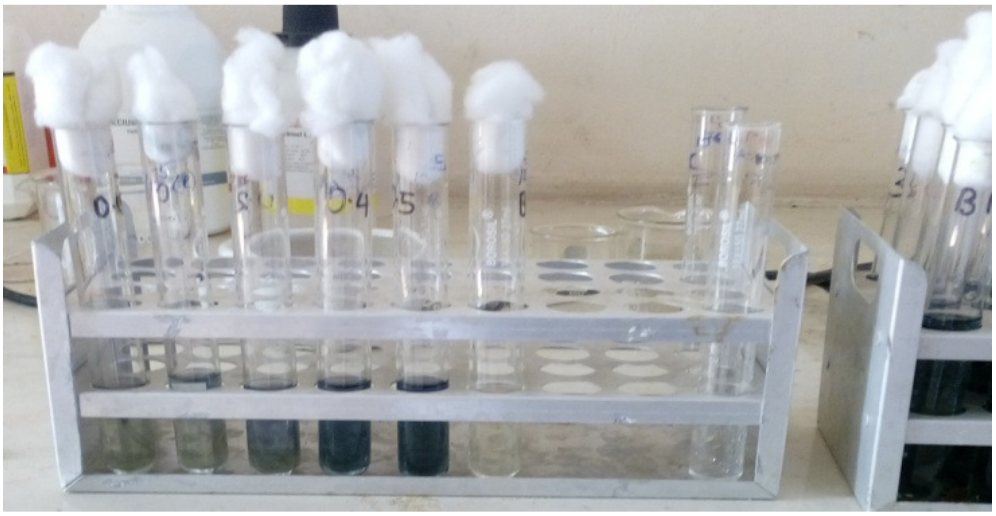

Figure-3: Standard Protein assay by Folin Lowry's method

There is a relation between protease activity and protein content that increase in protease activity decreases protein content as shown in Table-1. The decrease in the total protein content was resulted from the degradation of protein due to the action of proteolysis enzymes secreted by the selected bacterial isolates. Among the five selected bacterial isolates studied for protease activity, the highest activity (215.66±1.98U/mL) was recorded in strain named as TKMFT8. Similar screening method[17] in which that highest activity (380 U/mL) was exhibited by *Bacillus sp*. In another study[18], bacterial isolate No. 2 exhibited highest activity (37.94 U/mL) at 37ºC for 72h incubation. The highest production of extracellular protease by *Bacillus sp* (243 U/ml) without optimization of culture conditions was reported earlier[19]. Similar study[20] reported maximum enzyme activity (124.2 U/ml) shown by *Bacillus sp* .Sp-5 at 25ºC for 24 h of incubation time with nitrogen and carbon sources like yeast extract and sucrose respectively. Highest protease activity (66.23 U/ml) after 48 h at 37ºC of incubation by *Serratia liquiefaciens* was reported in an earlier investigation[21]. These reports are in agreement with the present findings that bacterial isolates capable of producing protease are widespread in nature and can be able to produce protease without optimization of the culture conditions.

Table-1: Protease activity (U/mL) of bacterial isolates and protein content (mg/ml).

Incubation day	Protease activity(U/ml) and protein content mg/ml					
		TKMFT8	TKMFT22	TKMFT25	TKMFT39	TKMFT53
1	PA	121.49±0.54	100.37±0.77	104.25±1.87	106.88±0.67	40.09±0.83
	PC	1.34±0.02	1.15±0.03	1.76±0.01	1.14±0.03	2.73±0.03
2	PA	215.19±0.90	141.57±0.9	154.95±2.09	197.55±1.1	46.42±1.28
	PC	0.77±0.02	0.97±0.02	1.77±0.02	0.86±0.01	1.63±0.01
3	PA	157.64±1.31	207.49±1.01	206.43±0.82	143.42±1.23	83.55±0.62
	PC	1.10±0.10	0.84±0.01	0.83±0.01	0.97±0.02	1.21±0.01
4	PA	137.12±1.28	123.62±1.26	127.77±0.75	116.16±0.9	104.37±0.83
	PC	0.75±0.01	0.79±0.02	0.87±0.01	0.81±0.02	1.13±0.04
5	PA	108.5±1.87	90.45 ±0.81	114.90±0.64	87.8±0.42	97.93±0.46
	PC	0.73±0.02	0.80±0.01	0.89±0.01	0.75±0.02	0.97±0.02
6	PA	89.73±1.39	84.61±0.68	107.72±1.76	62.71±0.8	92.09±0.62
	PC	0.71±0.02	0.75±0.02	0.83±0.02	0.72±0.02	0.97±0.02
7	PA	82.2 ±1.52	77.47 ±1.18	104.67±1.55	53.18±0.64	73.73±1.08
	PC	0.68±0.01	0.70±0.01	0.86±0.01	0.70±0.02	0.97±0.01
8	PA	70.16±1.59	43.7±1.47	42.13±0.98	17.28±0.43	61.46±0.62
	PC	0.65±0.01	0.73±0.01	0.83±0.01	0.63±0.03	0.93±0.03
9	PA	59.43 ±1.8	16.98 ±0.42	35.81±1.32	14.73±0.62	54.81±1.01
	PC	0.66±0.01	0.71±0.01	0.82±0.01	0.62±0.02	0.93±0.02
10	PA	46.09 ±1.53	6.36±0.68	16.23±1.03	7.09±0.29	32.36±1.18
	PC	0.53±0.01	0.73±0.01	0.83±0.02	0.64±0.01	0.87±0.02

PA: Protease Activity, PC : Protein Content

Protease activity expressed in U/ml: Protease activity and Protein content are related. It was clear from the data presented in Table-1 and Figure-4. Protein Content in Figure-5 that increased protease activity decreased the protein content in the inoculated media. The decrease in the total protein content was resulted from the degradation of protein due to the action of proteolysis enzymes secreted by the selected bacterial isolates.

Statistical analysis: Three replicate determinations of each sample were used for statistical analysis which was carried out using a SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. 17, 2008). The Pearson correlation analysis was performed between protein content (dependant variable) and incubation days and bacterial isolates (independent variables). The statistical analysis shown in Table-2 revealed that type of bacteria had shown a positive correlation (0.327) with the protein content, on the other hand, the incubation period exhibited a high degree negative correlation with (-0.603) the protein levels. However the impact was more predominant in case of different bacterial isolates.

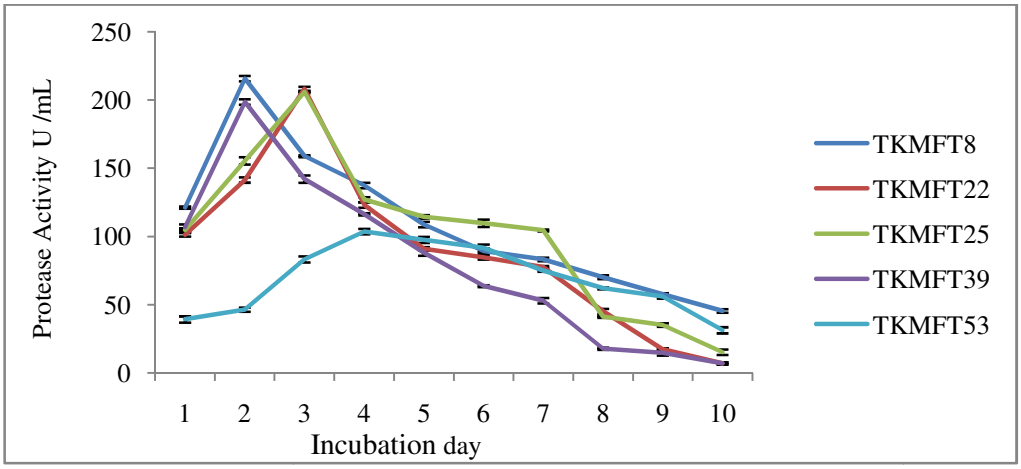

Figure-4: Comparison of Protease activity (U/mL) by different bacterial isolates.

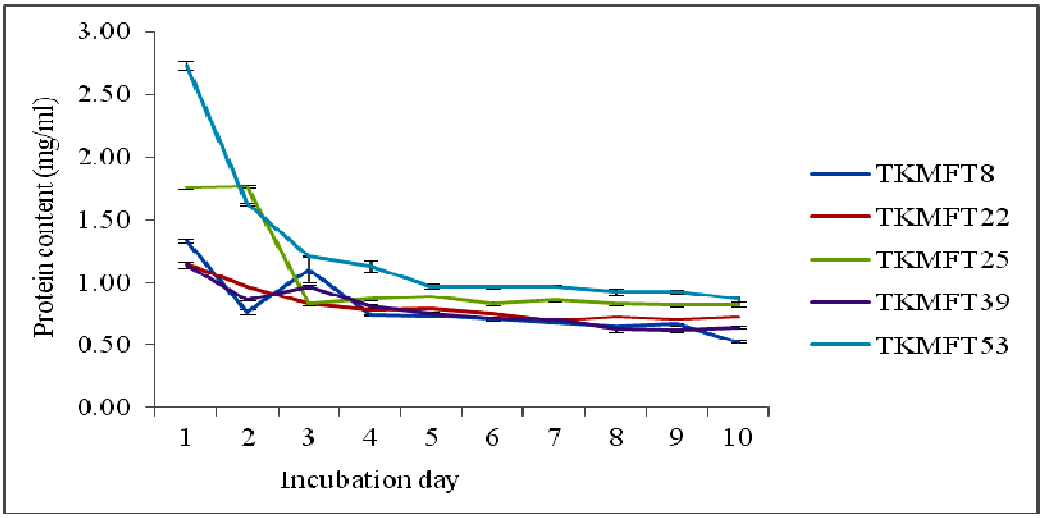

Figure-5: Comparison of protein content in samples to detect the effect of protease activity.

Table-2: Pearson's correlation coefficients between the variables.

	Protein	Incubation period	Bacterial Isolates
Protein	1	-0.603**	0.327**
Incubation period	-0.603**	1	0
Bacterial isolates	0.327**	0	1

**Correlation is significant at the 0.01 level (2-tailed).

Conclusion

Among the five bacterial isolates studied, the highest protease activity (215.19±0.90) was exhibited by TKMFT8 followed by TKMFT22, TKMFT25, TKMFT39 and TKMFT53 and the activity was found to be 208.23±1.50U/mL and 205.76±0.75 U/mL, 198.53±1.98 U/mL and 103.56±1.98 U/mL respectively. The highest protease producing isolate can be selected for the degradation of protein rich waste material discarded from bakery industry leading to food industry waste recycling.

Acknowledgement

The author is thankful to The Head, Cashew Export Promotion Council of India (CEPCI), Mundakkal, Kollam, Kerala, India for providing experimental facilities to perform the research work.

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