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RESEARCH ARTICLE

Optimization of Culture Conditions for the Production of an Extracellular Protease from *Shigella Sp.*

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ABSTRACT

The present study was undertaken to characterize the protease producing potent microbes from the gastro intestinal tract of the estuarine fish species and optimization partial purification of protease production by the isolated bacterium. The pH and temperature optimum for maximizing the protease production by Shigella sp. was registered at 7.0 (18.50 U/ml) and (54.60 U/ml) 40^{o}C respectively. The optimization of protease production was done by using different non-conventional nitrogen source. Among the tested non conventional sources, Bengal gram (37.50 U/ml) was found to be a suitable substrate for maximizing the protease production by Shigella sp.. The different conventional nitrogen sources were also tested in this study, and among them casein (73.00 U/ml) was noticed as the suitable substrate for enhancing the protease production by Shigella sp.. Among the carbon sources tested, it was revealed that, the Shigella sp. prefer glucose (96.00 U/ml) for maximizing the protease production. The result on surfactant-induced protease production revealed that Triton X100 (116.0 U/ml) had the higher influence on enzyme production.

KEY WORDS: *Protease, Agricultural substrates, Shigella sp., Optimization*

INTRODUCTION

Proteases are also known as peptidyl-peptide hydrolases and are industrially useful enzymes which catalyze the hydrolysis of peptide bond from protein molecule. Proteases constitute 50 – 65% of the global industrial enzyme market, most of which are alkaline protease. Given the wide application of this enzyme, it is reported that in year 2005, the global proteolytic enzyme demand will increase dramatically to 1.0 – 1.2 billion dollars [1]. The gut bacteria have shown to produce various substances such as vitamin K, riboflavin, and various enzymes that break down to proteins, carbohydrates and lipids [2]. Microbial activity in some animals may contribute as much as 25 to 35 % of the animal nutritional needs [3]. The normal gut contains many microbes that provide protective barrier against disease causing bacteria. These microbes also transform the components of the diet that have important consequences for overall health. As the result the gut is an important site for the immune system [4].

The gastro intestinal tract of fish can influence nutrition, growth, and disease susceptibility. The micro flora may be essential in fish that feed on recalcitrant material or on material lacking vitamins, which the microflora can synthesis [5]. The gastro intestinal micro flora has a role in the nutrition, growth and disease susceptibility of the fish [5]. Symbiotic bacteria in an animal's digestive tract often produce complement enzymes for digestion of plant foods as well as synthesize compounds that are assimilated by the host [6]. The biological diversity of marine and estuarine provides a wide array of enzymes with unique properties. In recent years, proteases from the gut of fishes received much attention [7]. Studies relating to such properties of thee enzymes are imperative for the successful application of these respective industries [8]. Though the protease productions by bacterial strains were documented well, the protease productions by fish gut bacterial isolates are still stand as lacunae. Here the present work was undertaken to investigate the protease production by the fish gut bacterial isolate.

MATERIALS AND METHODS

Isolation and screening of proteolytic bacterial strain

The s experimental fish was collected from Thengapattinam estuary and identified as *Mugil cephalus* Kanyakumari district, south west coast of India and identified by standard procedures described in Bergy's manual of determinative bacteriology. The identified organism was tested for protease production on skim milk agar plates. After 24 h of incubation at 37°C, the proteolytic activity was confirmed by clear zone formation around the bacterial growth. The protease activity in the liquid medium was assessed first by enriching the bacteria in enrichment medium containing beef extract (0.3 %), peptone (0.5 %), NaCl (0.5 %) and glucose (0.5 %) at pH 7 for 24 h and then 10 % of enriched culture was inoculated in 250 mL flask containing 45 mL Basal medium containing (g/l) - $(NH_4)_2SO_4$ – 2g ; K_2HPO_4 – 1g ; KH_2PO_4 – 1g ; $MgSO_4.7H_2O$ – 0.4g ; $MnSO_4.H_2O$ – 0.01g ; $FeSO_4.7H_2O$ – 0.01g ; Yeast extract – 1g ; Peptone – 10g at pH 7. The culture was then incubated for 2 days by reciprocal shaking at 32°C. The cells were then harvested by centrifugation at 10000 rpm for 15 min and the supernatant was used for further protease assay.

Protease assay

The assay system consists of following ingredients such as 1.25 ml Tris buffer (pH 7.2), 0.5 ml of aqueous solution and 0.25 ml culture supernatant. Approximate controls were also made. The mixture was incubated for 30 min at 30 C. Then 3 ml 0f 5 % TCA was added to this mixture and it forms precipitate and it was placed at 4C for 10 min. Then it was centrifuged at 5000 rpm for 15 min. From this, 0.5 ml of supernatant was taken and 2.5 ml 0f 0.5 M Na_2CO3 were added and mixed well and incubated for 20 min. Then it was added with 0.5 ml of folins phenol reagent and absorbance was taken at 60 nm using UV- Vis Spectrophotometer. The amount protease produced was found out with the help of tyrosine standard graph.

Effect of pH and Temperature on protease production

Optimum pH for protease production by the experimental microorganism was determined by using different pH buffers in the assay medium. The assay was carried out individually at various pH such as 4, 5, 6, 7, 8, 9 and 10. The effect of temperature on protease production was studied by incubating the enzyme and substrate solution at various temperatures such as 10, 20, 30, 40, 50, 60, 70, 80 and 90°C The assay was carried out individually at these temperature.

Optimization of low cost agriculture substrates

To achieve the maximum protease production, soy meal in the basal medium was substituted with other three nitrogen sources. They were corn flour, bengal gram and tamarind seed meal. For this, 50 ml of each of culture medium was taken in 250 ml Erlenmeyer flask containing four selected nitrogen sources respectively (I, II and III) including a control flask(C) (Table 1). Then the individual medium was added with the positive culture and incubated for 3 days. After the culture period, the supernatant was taken through centrifugation and assayed for protease activity.

Table 1. Composition of basal medium and experimental media substituted with various non-conventional nitrogenous sources.

Media components	Control (g/l)	Experimental medium (g/l)		
		Corn flour	Bengal gram	Tamarind seed
Soy meal	20	-	-	-
Casein	10	10	10	10
Na_2CO_3	4	4	4	4
K_2HPO_4	5	5	5	5
$MgSO_4$	0.18	0.1	0.1	0.1
Corn flour	-	20	-	-
Bengal gram	-	-	20	-
Tamarind seed flour	-	-	-	20

Optimization of Bengal gram

From the above experiment, with three different nutrient sources, bengal gram was identified as a maximum producer of protease. Hence it was then optimized by varying the concentration in the individually prepared medium (Table 2).

Table 2: Composition of experimental medium substituted with various concentrations of Bengal gram.

Media composition (g/l)	Experimental media (g/l)				
	I	**II**	**III**	**IV**	**V**
Bengal gram	20	30	40	50	60
Casein	10	10	10	10	10
Na_2CO_3	4	4	4	4	4
N_2HPO_4	5	5	5	5	5
$MgSO_4$	0.1	0.1	0.1	0.1	0.1

Optimization of nitrogen sources on protease production

The effect of nitrogen sources on protease production by using the various nitrogen sources such as casein, skim milk, beef extract, yeast extract and tryptone. The enzyme assay was carried out individually after 48h incubations

Table 3. Composition of experimental medium substituted with various concentrations of nitrogen sources.

Media composition (g/l)	Experimental media (g/l)				
	I	**II**	**III**	**IV**	**V**
Bengal gram	30	30	30	30	30
Na_2CO_3	4	4	4	4	4
N_2HPO_4	5	5	5	5	5
$MgSO_4$	0.1	0.1	0.1	0.1	0.1
Casein	10	-	-	-	-
Skim milk	-	10	-	-	-
Peptone	-	-	10	-	-
Beef extract	-	-	-	-	-
Yeast extract	-	-	-	10	-
Tryptone	-	-	-	-	10

Optimization of casein on protease production

From the above experiment, casein was found to influence the maximum protease production and hence it was then optimized by using varying concentrations in the production media (Table .4).

Table 4. Composition of experimental media substituted with various concentration of skim milk.

Media composition (g/l)	Experimental media (g/l)				
	I	**II**	**III**	**IV**	**V**
Bengal gram	30	30	30	30	30
Casein	15	20	25	30	35
Na_2CO_3	4	4	4	4	4
N_2HPO_4	5	5	5	5	5
$MgSO_4$	0.1	0.1	0.1	0.1	0.1

Influence of carbon sources on protease production

Carbon source induced production of protease by *Shigella sp.*. was studied by adding different sugars in individually prepared medium. The carbon sources selected were glucose, fructose, starch, maltose, sucrose and lactose (Table 5).

Table 5. Composition of experimental media substituted with various concentrations of carbon sources.

Media composition	Experimental medium (g/l)					
	I	II	III	IV	V	VI
Bengal gram	30	30	30	30	30	30
Casein	10	10	10	10	10	10
Na_2CO_3	4	4	4	4	4	4
N_2HPO_4	5	5	5	5	5	5
$MgSO_4$	0.1	0.1	0.1	0.1	0.1	0.1
Glucose	5	-	-	-	-	-
Starch	-	5	-	-	-	-
Sucrose	-	-	5	-	-	-
Fructose	-	-	-	5	-	-
Lactose	-	-	-	-	5	-
Maltose	-	-	-	-	-	5

Optimization of glucose on protease production

Among the sugars tested glucose was found to influence maximum protease production than others. Hence it was then optimized by using varying concentrations in the individually prepared medium (Table 6).

Table 6. Composition of experimental media substituted with various concentration of glucose.

Media composition (g/l)	Experimental media (g/l)				
	I	II	III	IV	V
Bengal gram	30	30	30	30	30
Casein	15	20	25	30	35
Na_2CO_3	4	4	4	4	4
N_2HPO_4	5	5	5	5	5
$MgSO_4$	0.1	0.1	0.1	0.1	0.1
Glucose	1.0	1.5	2.0	2.5	3

Surfactants induced production

To test the effects of surfactant on production of protease by *Shigella sp.*. three different surfactants were used. For this, the previously optimized medium was individually added with selected surfactants (I to III) and at the same time medium without surfactant was also used as control (Table 7).

Table 7. Composition of experimental media supplemented formulated with various surfactants.

Media composition (g/l)	Experimental media (g/l)				
	I	II	III	IV	V
Bengal gram	30	30	30	30	30
casein	15	15	15	15	15
Na_2CO_3	4	4	4	4	4

N_2HPO_4	5	5	5	5	5
$MgSO_4$	0.1	0.1	0.1	0.1	0.1
Glucose	15	15	15	15	15
Triton x 100	-	1	-	-	-
Tween 20	-	-	1	-	-
Tween 80	-	-	-	1	-
Polyethylene glycol 100	-	-	-	-	1

Optimization of Triton X 100 on protease production

From the above experiment, Triton X 100 added medium enhanced the maximum protease production; hence it was then selected for optimization by using varying its concentrations in the individually prepared medium (Table 8).

Table 8. Compositions of experimental media supplemented with various concentrations of Triton X 100.

Media composition (g/l)	Experimental media (g/l)				
	I	**II**	**III**	**IV**	**V**
Bengal gram	30	30	30	30	30
Casein	15	15	15	15	15
Na_2CO_3	4	4	4	4	4
N_2HPO_4	5	5	5	5	5
$MgSO_4$	0.1	0.1	0.1	0.1	0.1
Glucose	15	15	15	15	15
Triton X	1 ml	2 ml	3 ml	4 ml	5 ml

RESULTS

Identification of bacteria

Based on the morphological, physiological, biochemical characteristics of the protease positive bacteria, *Shigella sp.*. was identified as a potent strain with the diameter of 16mm zone, based on the comparison with Bergey's Manual of Determinative Bacteriology.

Effect of pH on protease production by *Shigella sp.*.

The pH optima of protease activity by *Shigella sp.*. was studied using different buffer system. The pH tested was 4, 5, 6, 7, 8, 9 and 10. The maximum activity found in pH 7.0 (18.5 U/ml). Hence the pH optimum for enhancing the protease activity by *Shigella* was 7 – 8 (Fig-1).

Effect of temperature on protease production

The maximum amount of enzyme production was recorded at 40^0C (19.8 U/ml). Similar to that of pH effect, it also showed an increasing trend from the tested starting temperature of 10^0C and increased up to 40^0C. Beyond that, the enzyme production decreased and accordingly the optimum temperature was 30 – 40^0C for protease production by *Shigella sp.*. (Fig-2)

Optimization media components for maximum production of protease

Protease production by *Shigella sp.*. was altered by optimization of media components. Alternations were done in this medium to obtain maximum synthesis. Here soybean meal in the basal medium was substituted with the cheapest sources such as corn flour and bengal gram and tamarind seed. Among the tested nitrogen sources, the maximum protease synthesis was recorded in Bengal gram (37.5 U/ml) substituted medium, where as the control medium (18.8 U/ml) and corn flour substituted medium (28 U/ml). Obviously tamarind seed substituted medium registered the synthesis of 26 U/ml (Fig-3)

Optimization of Bengal gram concentration for maximum production

Among the tested concentrations, 30 g/l Bengal gram was found to be optimum for enhancing the maximum protease synthesis. In this concentration, the protease synthesis was recorded in 54.6 U/ml. Increasing the concentration of Bengal gram to 40g/l resulted the decrease in protease synthesis by *Shigell sp* (Fig-4)

Effect of nitrogen sources on protease production

At the optimum bengal gram concentration, the protease synthesis was continued with other nitrogen source such as skim milk, tryptone, peptone, beef extract and yeast extract. Table- shows the influence of other nitrogen sources tested in the place of casein. Among these tested sources, casein showed maximum (65 U/ml) protease production than the other nitrogen sources including casein (control) was recorded in 54.6 U/ml (Fig-5).

Optimization of skim milk on protease production

The effect of various concentrations of skim milk on protease synthesis by *Shigella sp.* indicated that the 15 g/l of casein was optimum to maximize the protease production. At this concentration, the protease synthesis was recorded in 73 U/ml. (Fig-6)

Influence of carbon source on protease production

Fig-7 shows the influence of sugars on protease synthesis by fish intestinal microbe *Shigella sp.*. Among the sugars tested, the simple sugar glucose influenced the high protease synthesis and the recorded value was 88 U/ml. the lowest protease synthesis of 76 U/ml was registered in sucrose supplemented medium. The medium devoid of sugar showed the synthesis of 73 U/ml.

Optimization of glucose concentration

Fig.8 shows the effect of various concentrations of glucose on protease synthesis by *Shigella sp.*. Among the concentration tested, the amount of 15 g/l glucose displayed the maximum protease synthesis of 96 U/ml.

Influence of surfactants on protease production

Surfactants influenced production of protease was studied by using three different surfactants such as Triton X 100, Tween 80, Tween 20 and poly ethylene glycol – 100. Among these tested surfactants, protease production of Triton X 100 was found to be (116 U/ml) high than the other surfactants. (Fig.9)

Optimization of Triton X 100 on protease production

Among the tested concentrations, 3 ml/l of Triton X 100 was found to be the maximum for enhancing protease production than the other concentrations tested. The synthesis recorded was 136 U/ml. (Fig.10)

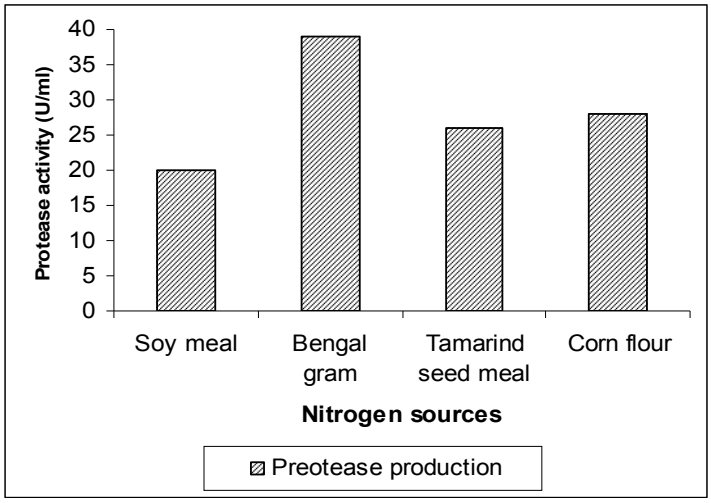

Fig. 1- Experimental media substituted with various non-conventional nitrogenous sources.

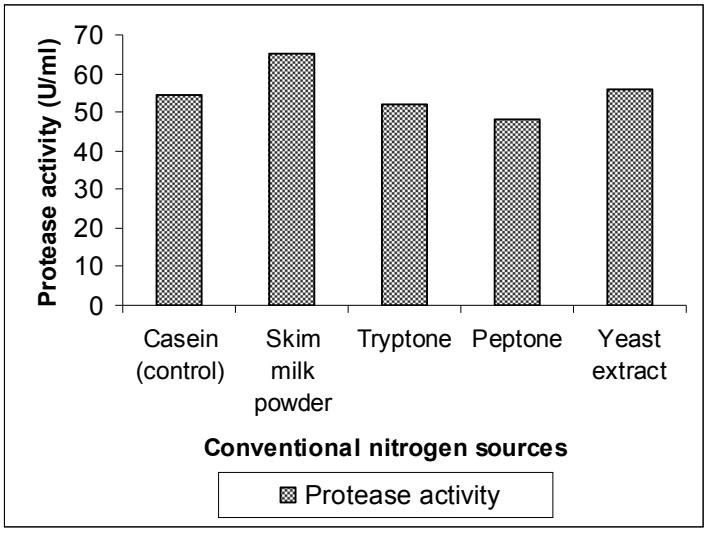

Fig-2. Experimental media substituted with various conventional nitrogenous sources.

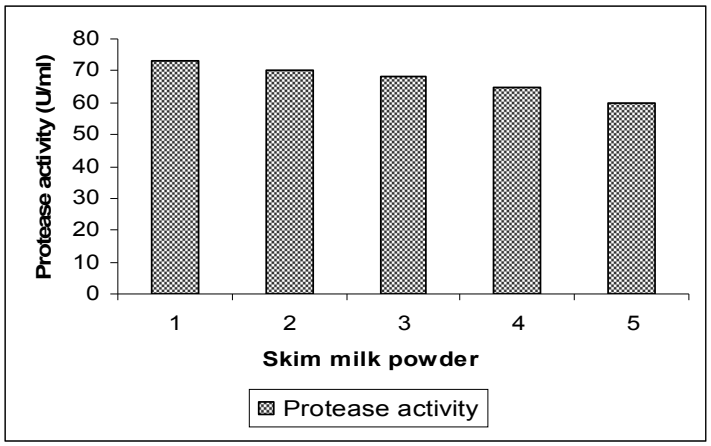

Fig.3 Experimental media substituted with various concentration of skim milk.

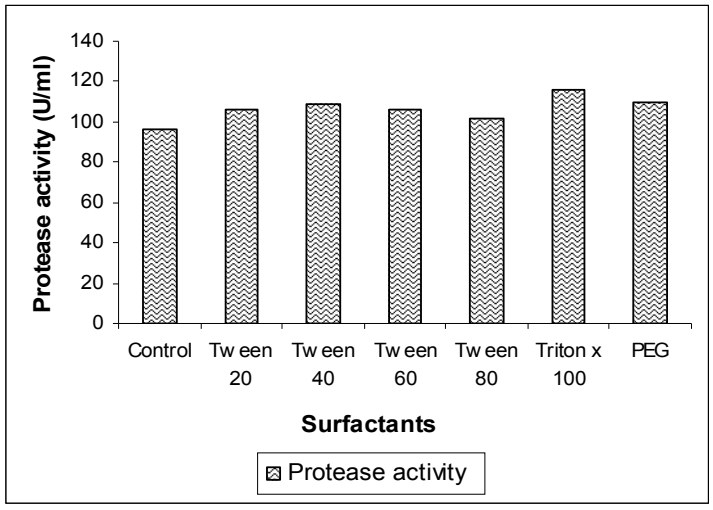

Fig.4 Effect of surfactants on protease production

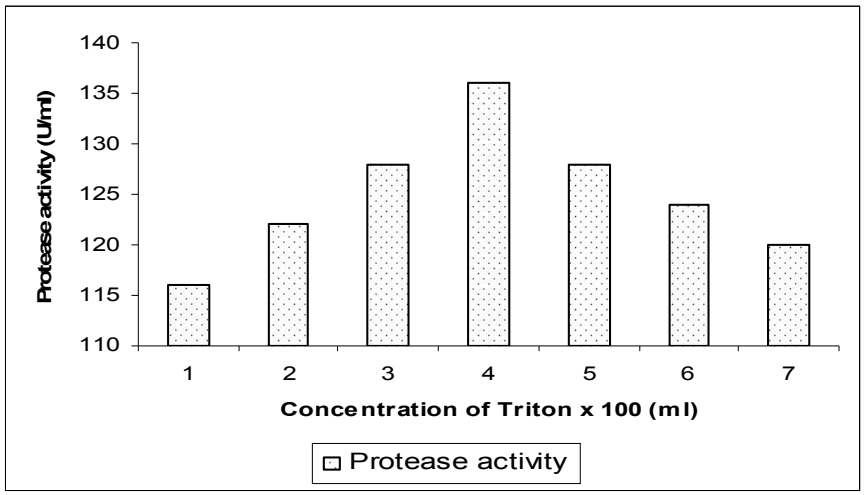

Fig.5 Optimization of Triton X100

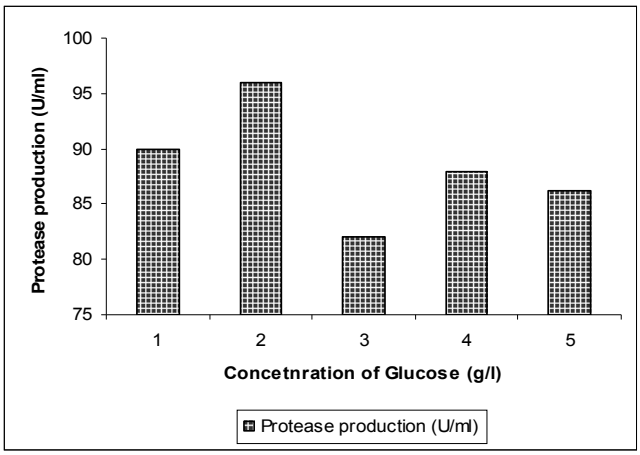

Fig.6. Experimental media substituted with various concentrations of glucose

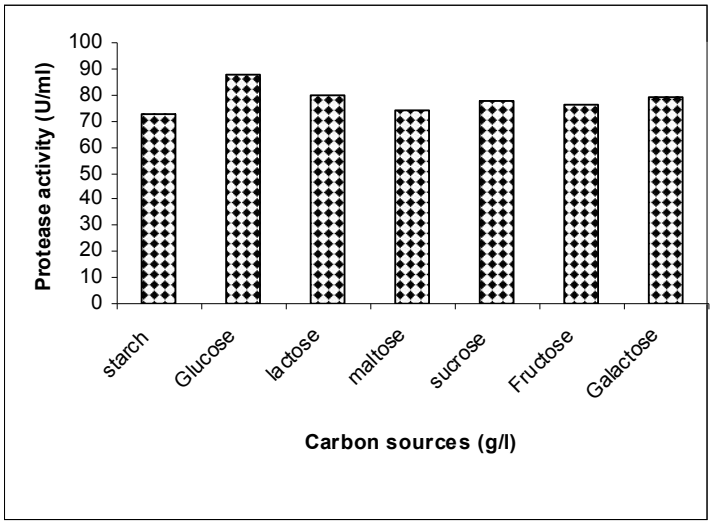

Fig.7 Effect of carbon sources on protease production**.**

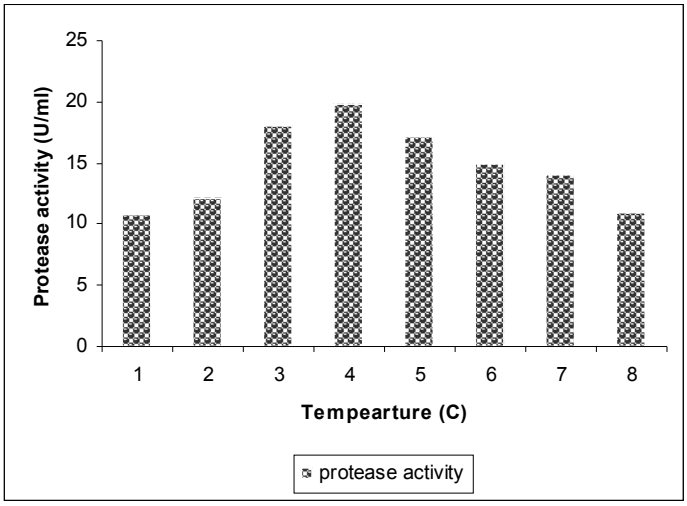

Fig. 8 Effect of temperature on protease production

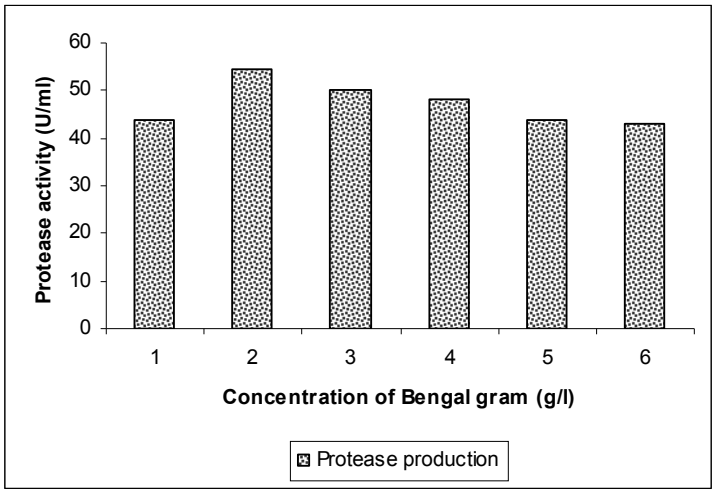

Fig.9 Effect of Bengal gram on protease production

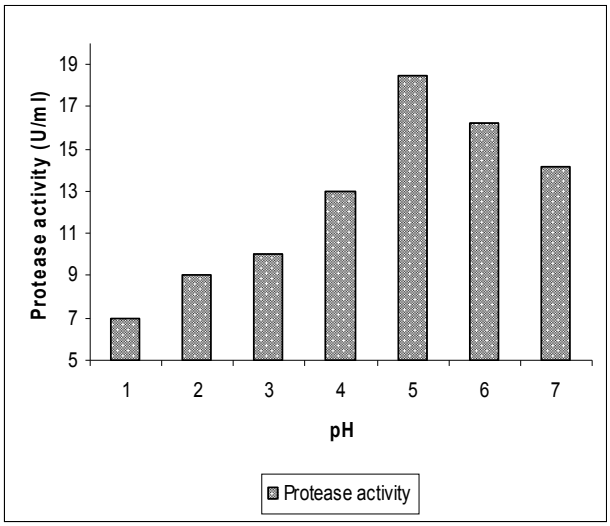

Fig.10 Effect of pH on protease production

DISCUSSION

The bacterial flora that exists in the digestive tract represents a very important and diversified enzymatic potential [9]. But sufficient information is not much available on their role in digestion and hence the present study was carried to isolate and characterize the potent protease positive bacteria from the gastro intestinal tract of estuarine fish *Mugil cephalus*. The results indicated that the digestive tract of *M. cephalus* contains four protelytic bacteria and among the four positive isolates only one was the dominant protease producer with 16mm diameter zone and it was identified as *Shigella sp.*. In the present study, pH on protease activity by *Shigella sp.*. revealed that, it displayed maximum protease activity at pH 7 (18.5U/ml). This present result was supported by the studies of Hoshino *et a*l., [10] on the protease enzyme from fish intestinal isolate *Pseudomonas* sp was optimum at pH 7. Results on the effect of temperature on protease production by *Shigella sp.* indicated that the optimum temperature for protease activity was 40^{o}C (19.8U/ml). The effect of various incubation temperatures on protease production resulted that 30 – 40^{0}C was found to be a suitable temperature range for this bacterium. This was supported by the studies of Gupta *et al.* [11] and Samarntarn *et al.* [12], where the protease from *Virgibacillus pantothenticus* and *Aspergillus oryzae* U1521 has its maximum activity with the temperature range between "30 – 50^{0}C". Optimization of protease production by *Shigella sp.*. was done by alternations with the basal medium proposed by Han-Seung Joo *et al*.[13]. In optimization of nitrogen source, the basal medium nitrogen sources such as Soy meal and casein were targeted. Further additions to this Bengal gram in basal medium in the place of soy meal significantly reduce the cost of production. This result is consistence with the earlier report of Krishna Suresh, Babu naidu and Lakshmi Devi [14] on optimization of protease production by using rice bran. They also obtained higher protease production in basal medium substituted with rice bran. In this study other sources used were, tamarind seed meal and corn flour meal. These sources were also gave considerable protease production in comparison with potent substrate-Bengal gram. The optimization of Bengal gram study indicated that the concentration of 30g/ l augment the protease production (54.6U/ml). Addition of Bengal gram above it is optimum concentration, the enzyme production declined. After optimization of Bengal gram, the optimization process continued by means of substitution of casein with other conventional nitrogen sources. The results indicated that skim milk (65 U/ml) concentration of 15g/l was found to be optimum to produce maximize the protease in combination with Bengal gram. The present results revealed that the maximum amount of protease production was recorded in casein (73.00 U/ml) added medium. This is because casein and yeast extract not only serve as nitrogen sources but also they have energy sources such as carbohydrates, free amino acids. The results on the casein improved protease production by *Shigella sp.* also supports the previous studies on casein aided higher protease production by *Virgibacillus pantothenticus* [11], *Chromohalobacter* sp. TVSP101 [15]. The effect of various carbon sources on protease production indicated that maltose (88 U/ml) gave higher production when compared the other carbon sources supplied. In consistence with this present study, bacteria such as *Bacillus* sp. [16] and *Bacillus clausii* [17] were also reported to produce higher protease in maltose supplied medium. The effect of surfactants on protease production was studied by using four different surfactants such as Tween 20, Tween 80, Triton X100, and Polyethylene glycol-300. The results indicated that all the added surfactants have positively influenced the protease production. Among the tested surfactants Triton X100 added medium produced maximum protease. This is correlated with the report of Joo and Chang [17,18] that the protease from *Bacillus clausii* and *Bacillus* sp. retain their activity with different surfactants such as Triton X 100, Tween 20 and SDS.

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