International Journal of Advanced Technology in Engineering and Science
Vol. No.4, Issue No. 06, June 2016
www.ijates.com
ijates
ISSN 2348 - 7550

ISOLATION AND OPTIMIZATION OF ALKALINE PROTEASE PRODUCING BACTERIAL ISOLATE USING DAIRY EFFLUENT AS SUBSTRATE

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ABSTRACT

A total 15 different alkaline protease producing bacterial strains were isolated by using casein agar medium plates based on zone of clearance. The samples were collected from dairy waste water and sludge of dudhsagar dairy, Mehsana, Gujarat, India. Among them RkNa$_2$ strain gave highest protease activity (453.10 U/mL/min) under submerged fermentation condition. Dairy waste water gave highest protease activity (345.9U/ml/min) among the different substrate studied. Maximum growth and enzyme activity was observed under optimum condition at 37ºC temperature, pH 8.0 (261.55U/mL/min), inoculum size 2%(135.00U/mL/min). Incubation period for maximum protease production was found to be 20 hrs(175.9U/mL/min) with galactose and 1 % peptone as a carbon and nitrogen source with optimum activity (84.35U/mL/min) and (288.12U/mL/min) respectively.

Keywords: Alkaline Protease, Submerged Fermentation.

I. INTRODUCTION

Enzymes are well known biocatalysts that perform many chemical reactions .They are commercially used in the detergent, food, pharmaceutical, diagnostics and fine chemical industries. More than 3000 different enzymes described to date the majority have been isolated from mesophilic organisms. These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the enzymes unrecommendable.[1]. Proteases are the most important group of enzymes produced commercially which are used in detergent, protein, brewing, meat, photographic, leather and dairy industries[2,3]. Microbial proteases are amongst the most important hydrolytic enzymes and have been studied extensively. In bacteria, this enzyme is produced mainly by many members belonging to genus *Bacillus* especially, *B.licheniformis; B. horikoshii, B. sphaericus, Bacillus furmis, Bacillus alcalophilus, Bacillus subtilis*[4].Alkaline proteases possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures[5].

The aim of the study was to isolate and identify alkaline protease producing bacteria, Optimization of physical-chemical parameters for protease activity.

II. MATERIALS AND METHODS

2.1. Isolation and screening of protease producing bacteria

Protease producers were isolated from dairy effluent, dudhsagar dairy, Mehsana, Gujarat, India. Samples were diluted and then different dilutions were applied on casein agar medium (nutrient agar with 1% casein) plates which were incubated 37^0C for 24 to 36 hours. Depending upon size of clearance of zone and protease activity, 15 different isolates were collected. Among them one potent isolate was selected for further study.

Selected isolate was biochemically characterized by Gram's reaction, Motility Test, Spore Staining, carbohydrate fermentation, oxidase test, O-F test, H2S production, IMViC tests, NO2 reduction, starch hydrolysis, KOH Test , Vancomycin Test, as per the standard methods and was confirmed by Vitek 2 systems version:05.04.All the bacterial isolates were preserved on casein agar slant at 4^0 C temperature. Cultures were transferred at 15 days intervals.

2.2.Inoculum preparation

The culture was grown on casein agar plates at 37^0 C temperature for 24 h. The cells were harvested from plates and resuspended in required amount of distilled water. Cell density was measured at 660nm. The cell density of inoculums was adjusted to 1.0 O.D. (approximately 10^6CellsmL^{-1}) to inoculate for enzyme production.

2.3.Production media and culture condition

Cultures were grown on production medium; containng (gL^{-1}), peptone (10.0), meat extract (3.0), Nacl (5.0), casein (10). The casein medium was taken in 250 mL conical flasks. The flasks were sterilized in autoclave at 121^0 C (15 lb) for 15 min and after cooling, the flasks were inoculated with prepared cell suspension adjusted at 1.0 O.D 660 nm (approximately 10^6 CellsmL^{-1}). The inoculated medium was incubated at 37^0 C in shaker incubator for 24 h. At the end of fermentation period, the culture medium was centrifuged at 8000 rpm at 4^0C for 20 min. to obtain crude enzyme.

2.4.Extraction method

After incubation 1 mL broth from flask and 49 mL of (0.1 M) sodium glycinate buffer (pH 8.0) was taken in a 100 mL flask. The sample was mixed properly and centrifuged at 8000 rpm for 20 minutes using Heavy Duty Cooling Centrifuge at 4^0 C. The supernatant was removed and subjected to further protease assay.

2.4.1.Enzyme assay method:

100 mL casein broth was inoculated with 1 mL inoculum, containing broth of actively growing culture and incubated at 37^0 C in an orbital shaker, at 100 rpm for 24 h. 1 mL broth from the flask and 49 mL sodium glycinate buffer (0.1m) was taken after incubation in a 100 mL flask and after mixing it properly it was centrifuged at 8000 rpm for 20 min to obtain supernatant to be used as a crude protease enzyme source.

2.5.Optimization of cultural parameters for protease production

The selected bacterium was subjected to further optimization of that particular media by varying its different cultural parameters.

2.5.1.Effect of raw materials on protease production

Fermentation medium was supplemented with different raw materials such as dairy waste water, wheat bran, wheat husk, oil meal waste groundnut cake, groundnut shell powder, maize husk, whey waste, sorghum bran, rice bran at respective concentration. The substrates were sterilized, inoculated and incubated on orbital shaker at 100 rpm for 24 h. Protease activity was measured after centrifugation.

2.5.2.Effect of pH on protease production

Different pH i.e. 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5 were studied to check their effect on protease production. The pH was adjusted using 1 N HCL or 1 N NaOH. The flask was incubated at 37^0 C for 24 h at 100 rpm on orbital shaker. After completion of the fermentation, protease activity was estimated and the optimum pH value was found out.

2.5.3.Effect of substrate concentration on enzyme activity

Fermentation was carried out with 8% to 13% substrate concentration and other optimized parameter kept constant. The enzyme activity measured after fermentation by performing enzymatic assay.

2.5.4.Effect of temperature on enzyme activity

The flasks were incubated at temperature 35°C, 37°C, 40°C, 42°C and 45°C at 100 rpm for 24 hr in shaker keeping the optimized parameters constant .

2.5.5.Effect of incubation time on enzyme activity

The fermentation medium was incubated at 37°C temperature at 100 rpm in environmental shaker. At regular time interval (4 hrs) that was starting from 4 hr to 38 hr, 5 mL of medium withdrawn and analyzed for protease production by protease assay method.

2.5.6.Effect of inoculum size on enzyme activity

Inoculum size ranging from 0.5% to 3.0% were inoculated at optimized temperature for 20 hr in orbital shaker at 100 rpm keeping the other optimized parameters constant. Sample was analyzed for protease production and selected for proper inoculum size.

2.5.7.Effect of nitrogen source on enzyme activity

Different nitrogen sources concentration were used like meat extract and peptone at different concentration. The other components were kept constant. Meat extract as nitrogen source was added at different concentrations i.e. 0.10 %, 0.20 %, 0.3 %, 0.40%, and 0.50 %. Peptone as nitrogen source was added at different concentration i.e. 0.5%,1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5% 4.0%,4.5%, and 5.0%. The inoculated culture media was then incubated in environmental shaker at 100 rpm. The enzyme activity of the centrifuged supernatant was checked and the nitrogen sources which gave highest enzyme production was selected.

2.5.8.Effect of carbon source on enzyme activity

Carbon source like glucose, galactose, mannitol, maltose, sucrose and fructose at 0.05% concentration were chosen for study.Other then carbon source, optimized parameters were kept constant Fermentation media was prepared, inoculated and incubated in the orbital shaker at 100 rpm. The samples were analyzed for enzyme activity.

2.5.9.Effect of Nacl concentration on enzyme activity

0.1 to 1.0 % salt concentration were taken to study its effect on protease production.

III. RESULTS AND DISCUSSION

3.1.Screening and selection of protease producers

Out of the 15 different isolates one isolate ($RkNa_2$) was selected for further study on the basis of its Zone of clearance **(9mm) (Fig 1**) and Protease activity (**453.10 $UmL^{-1}min^{-1}$).**

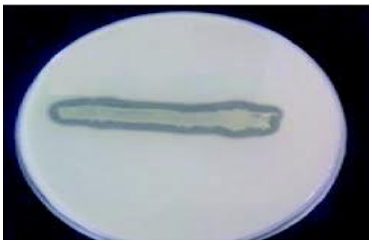

Fig 1: Zone of clearance observed during growth of isolate $RkNa_2$

3.2.Identification of Alkaline Protease producer:-

The bacterial isolate was found to be Gram negative,short rod shaped bacteria. The above results were confirmed by KOH and Vancomycin test. The isolate $RkNa_2$ was identified on the basis of different biochemical tests performed. For further conformation the isolate $RkNa_2$ was sent to Supra Tech laboratory where it was identified as *Aeromonas sorbia* using Installed VITEK 2 Systems Version: 05.04 **(Table 1& 2**).

Table 1:Biochemical tests performed for the isolated $RkNa_2$

Sr. No.	Test	Result
1.	Sugar utilization	
	Glucose	+
	Sucrose	+
	Ribose	+
	Maltose	+
	Lactose	-
	Fructose	+
	Mannose	+
	Mannitol	+
	Galactose	+
	Arabinose	-
	Raffinose	-
	Trihalose	+
	Xylose	-
	Rhamnose	-
	Cellobiose	-
	Inositol	-
	Sorbitol	-

	glycerol	-
2.	Indole production	+
3.	MR test	+
4.	Citrate utilization test	+
5.	VP test	-
6.	H_2S production	-
7.	Nitrate reduction	+
8.	Starch hydrolysis	+
9.	Casein hydrolysis	+
10.	Catalase test	+
11.	Gelatin hydrolysis	+
12.	Triple sugar iron test	+
13.	Motility test	+

Key + positive, - negative.

Table 2: Biochemical test on basis of VITEK 2 system

Well	Test	Results
1	Ala-phe-pro- ARYLAMIDAS**E**	+
2	ADONITOL	-
3	L-Pyrrolydonyl-ARYLAMIDASE	+
4	L-ARABITOL	-
5	D- CELLOBIOSE	+
6	BETA-GALACTOSIDASE	-
7	H2S PRODUCTION	-
8	BETA -N-ACETYL-GLUCOSAMINIDASE	+
9	Glutamyl Arylamidase pNA`	-
10	D-GLUCOSE	+
11	GAMMA-GLUTAMYL-TRANSFERASE	+
12	FERMENTATION /GLUCOSE	+
13	BETA - GLUCOSIDASE	+
14	D-MALTOSE	+
15	D-MANNITOL	+
16	D-MANNOSE	+
17	BETA-XYLOSIDASE	-
18	BETA-Alanine arylamidase PNA	-
19	L-Proline ARYLAMIDASE	-
20	LIPASE	-
21	PALATINOSE	+

22	Tyrosine ARYLAMIDASE	+
23	UREASE	-
24	D-SORBITOL	-
25	SACCHAROSE/SUCROSE	+
26	D-TAGATOSE	-
27	D-TREHALOSE	+
28	CITRATE(SODIUM)	+
29	MALONATE	-
30	5-KETO-D-GLUCONATE	+
31	L-LACTATE alkalinisation	-
32	ALPHA-GLUCOSIDASE	-
33	SUCCINATE alkalinisation	+
34	Beta-N-ACETYL-GALACTOSAMINIDASE	+
35	ALPHA-GALACTOSIDASE	+
36	PHOSPHATASE	-
37	Glycine ARYLAMIDASE	+
38	ORNITHINE DECARBOXYLASE	-
39	LYSINE DECARBOXYLASE	-
40	L-HISTIDINE assimilation	+
41	CORMARATE	+
42	BETA-GLUCORONIDASE	--
43	O/129 RESISTANCE (comp.vibrio.)	+
44	Glu-Gly-Arg-ARYLAMIDASE	+
45	L-MALATE assimilation	+
47	ELLMAN	-
48	L-LACTATE assimilation	-

3.3.Optimization of cultural parameters for protease production

3.3.1.Effect of raw materials on protease production

In the present study, dairy waste water, whey waste, wheat bran, wheat husk, groundnut oil meal waste cake, groundnut shell powder, maize husk and sorghum bran were used as substrate for production of protease from isolate $RkNa_2$. Maximum production of protease (345.9 U\mL\min) was observed in case of dairy waste water(**Fig 2)**.

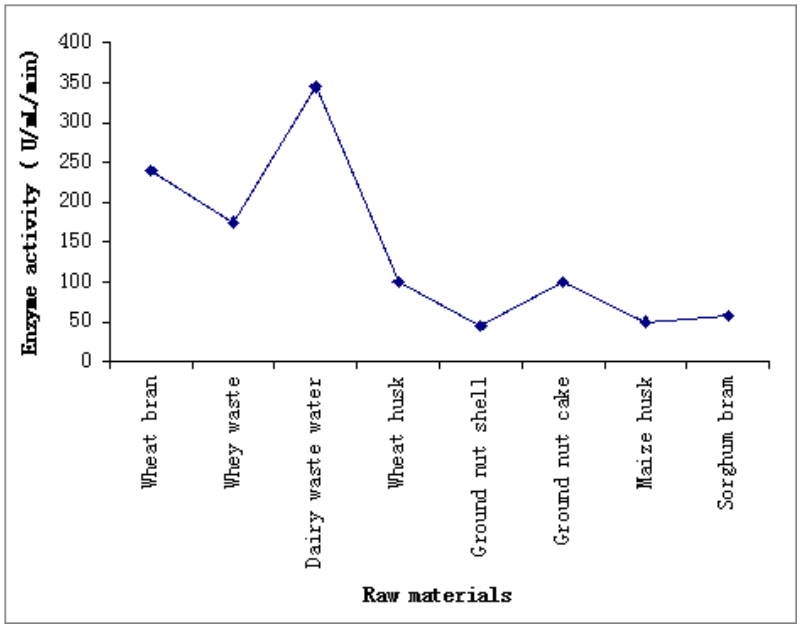

Fig 2: Effect of raw materials on protease production

3.3.2.Effect of pH on protease production

The optimum pH was found to be 8.0 at which isolate $RkNa_2$ showed maximum activity 261.5U/mL/min **(Fig 3)**. But, the organism showed very less enzyme activity i.e. 42.15 U/mL/min at pH 7.0 and no activity at pH 10.0. Our obtained results proved that the alkaline protease was produced in neutral to alkaline pH. The metabolic activities of microorganisms were very sensitive to the change in hydrogen ion concentration of their environment. Protease production was affected if pH level was higher or lower compared to the optimum value.Maximum activity for protease production was obtained by *Bacillus spp.* at pH 8.0[6,7].

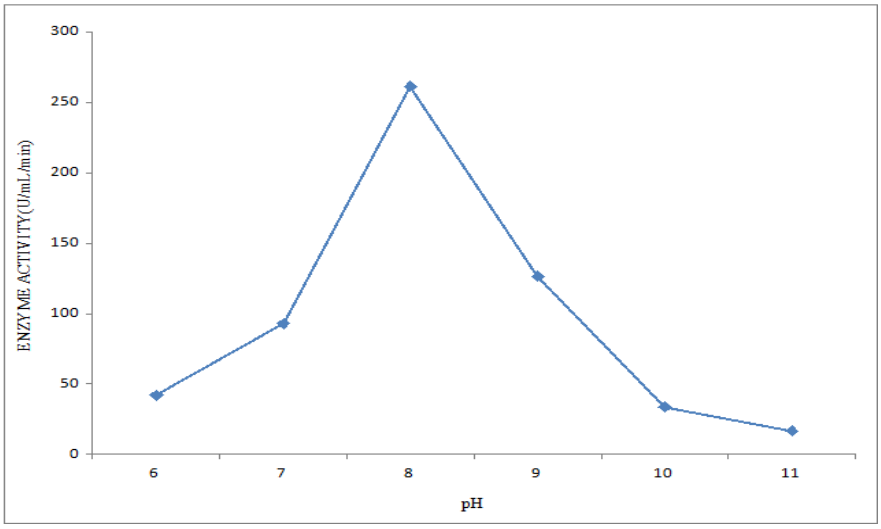

Fig 3: Effect of pH on protease production

3.3.3.Effect of temperature on protease production

Isolate $RkNa_2$ showed highest activity at 37^0C **(Fig 4)**. Our results indicate that the organism was mesophilic in nature.

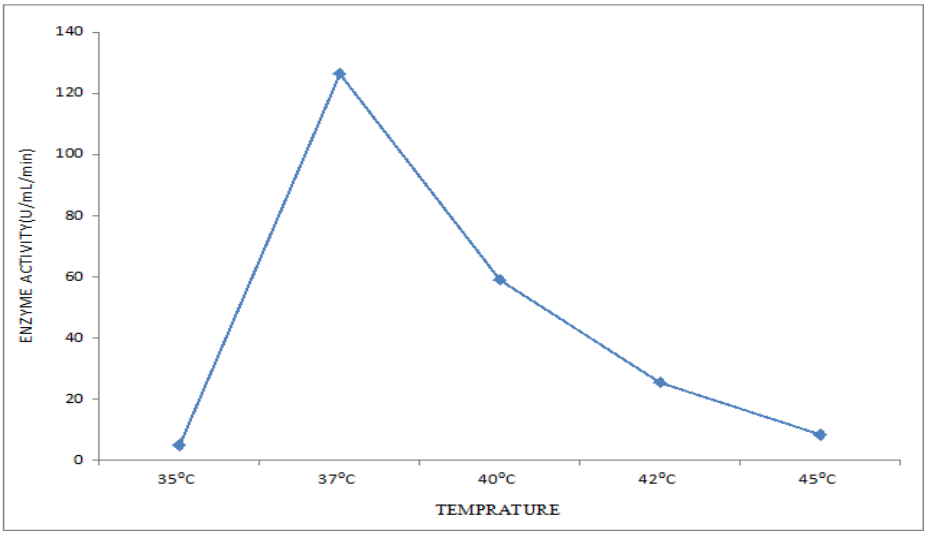

Fig 4: Effect of temperature on protease production

Maximum activity has been obtained for *Bacillus subtilis* grown at 37^0C temperature. *Pseudomonas spp* has been reported secerete maximum extra cellular protease at 34^0C and 37^0C[8,9].

3.3.4.Effect of incubation time on protease production

The highest protease activity was observed at 20hr with optimum activity 124.25 U/mL/min **(Fig 5)**.Isolate $RkNa_2$ showed gradually decreased protease production with increase in incubation time (20 h). A gradual decrease in enzyme activity was observed with increased incubation time. The duration needed for incubation might depend on the growth rate of microbes and its enzyme production might be due to depletion of nutrient of fermentation medium. Thus the time required for enzyme production in case of bacteria is less because of their faster doubling time

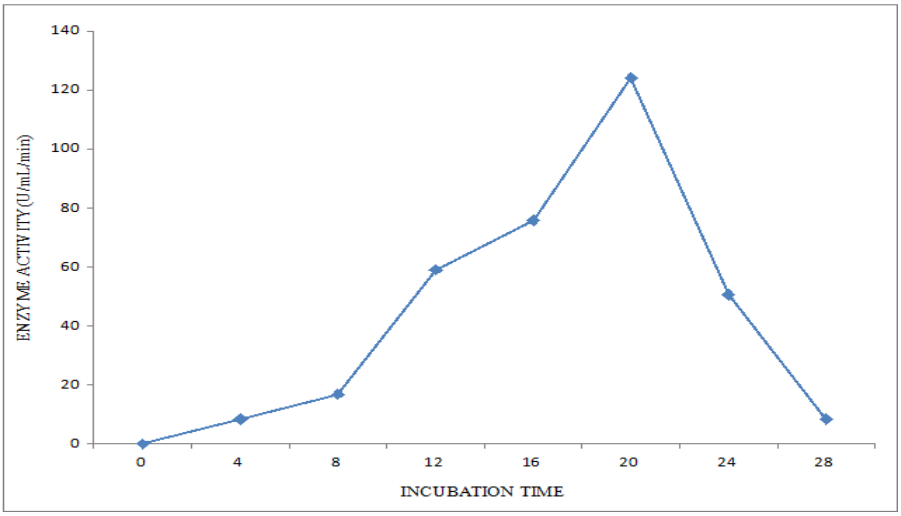

Fig 5: Effect of incubation time on protease production

3.3.5.Effect of inoculum size time on protease production

Different 6 inoculums size were studied to find out the effect of productivity on protease enzyme by Maximum protease activity for isolate $RkNa_2$ was observed at 2% inoculums size with 135.00 $UmL^{-1}min^{-1}$ protease activities **(Fig 6)**.If the inoculums sizes is too small, insufficient number of bacteria leads to reduced amount of

protease secreted. At higher percentage of inoculums size, protease activity was also found to be decreased even though luxurious growth was observed. studies have suggested that higher inoculums sizes resulted in reduced dissolved oxygen and increased competition towards nutrient and therefore decreased the protease production.Maximum protease activity has been obtained for *Bacillus* sp. MPTK 712 isolated from Dairy Sludge at inoculum size 2%. [10]

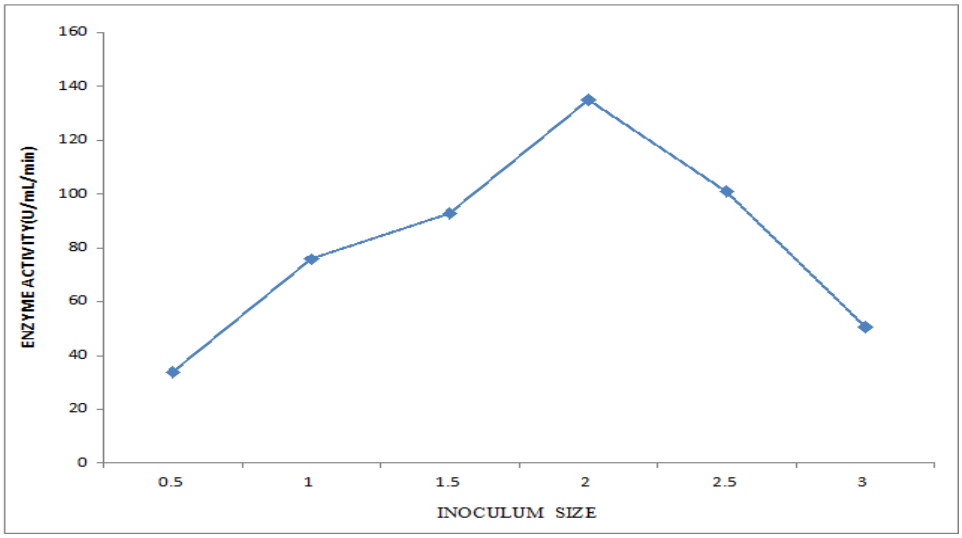

Fig 6: Effect of inoculum size on protease production

3.3.6.Effect of substrate concentration time on protease production

Isolate $RkNa_2$ showed maximum protease production at 9 mL of substrate concentration with 92.80 $UmL^{-1}min^{-1}$ protease activities**(Fig 7)**. Enzyme activity increased or decreased on increasing or decreasing the substrate concentration. It was found that application of dairy waste water as crude protein source influenced the yield of protease.

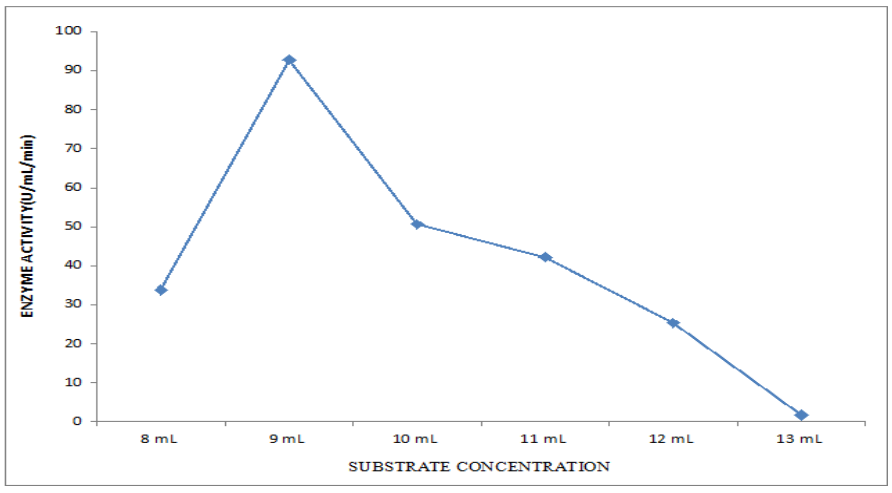

Fig 7: Effect of substrate concentration on protease production

3.3.7.Effect of carbon source on protease production

It was observed that Isolate $RkNa_2$ was capable of using a wide range of carbon sources, but protease production varied according to the various carbon source. The isolated strain $RkNa_2$ showed high enzyme activity (84.35 $UmL^{-1}min^{-1}$), when galactose was used as carbon source and less protease activity (16.85 $UmL^{-1}min^{-1}$) was

observed with fructose as carbon source **(Fig 8)**.

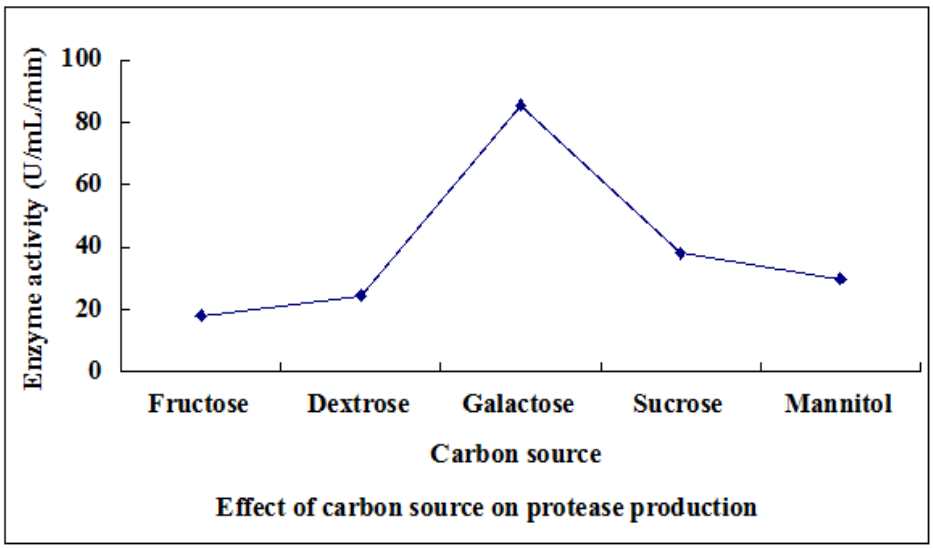

Fig 8: Effect of carbon source on protease production

3.3.8.Effect of nitrogen source on protease production

Peptone concentration was studied from 0.5% – 4.0%. The isolated strain $RkNa_2$ showed high protease activity (288.12 $UmL^{-1}min^{-1}$)at 1% concentration of peptone (**Fig 9)**.

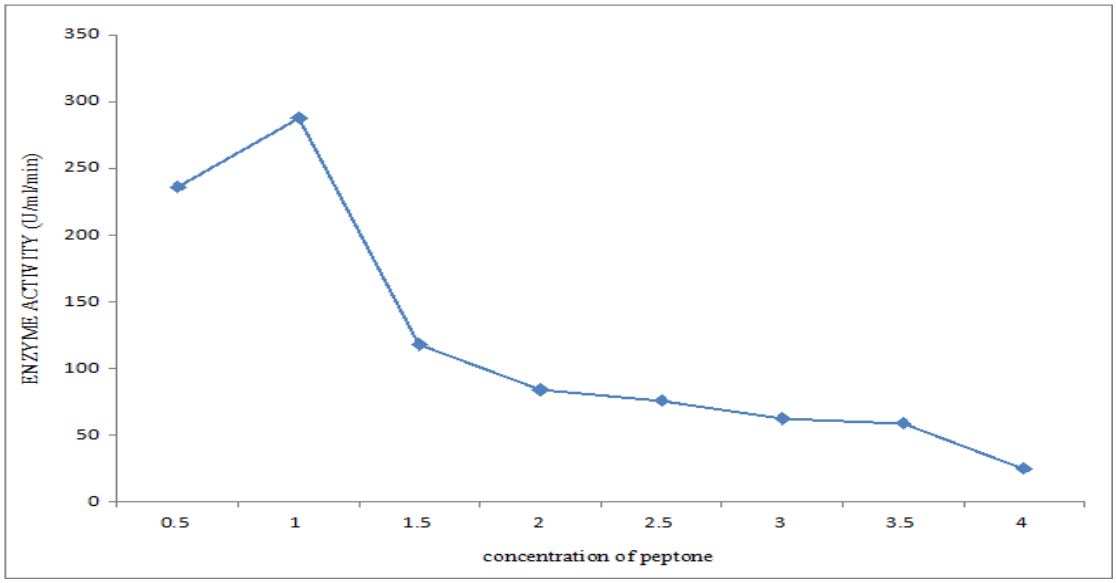

Fig 9: Effect of concentration of peptone on protease production

3.3.9.Effect of different concentration of meat extract as nitrogen source

Meat extract concentration from 0.1% – 0.5% was studied and it was observed that the isolated strain $RkNa_2$ showed high protease activity (236.25 $UmL^{-1}min^{-1}$) at 0.3 % concentration meat extract (**Fig 10)**.

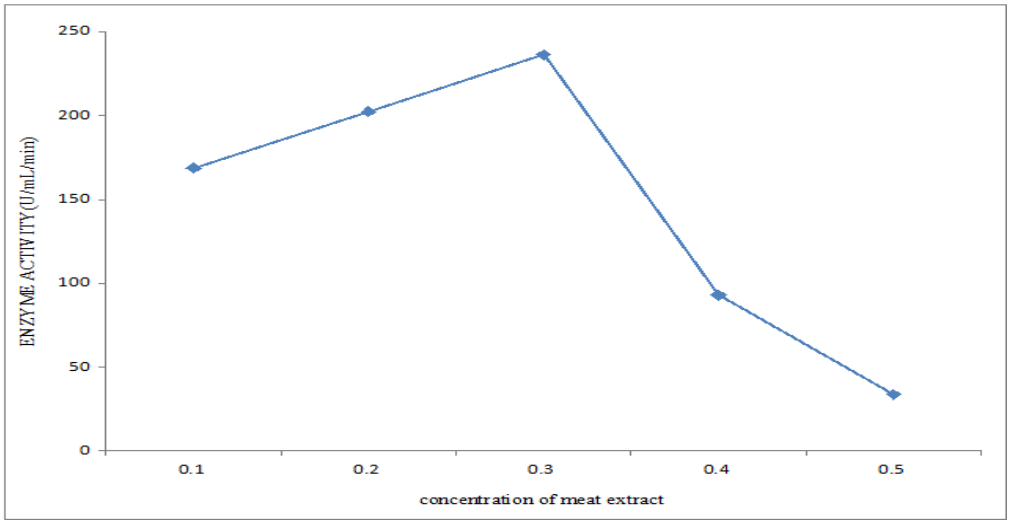

Fig 10: Effect of concentration of meat extract on protease production

3.3.10.Effect of Nacl concentration on protease production

On studying the effect of different NaCl concentration on protease activity it was observed that at 0.5% NaCl concentration Protease activity increased **(Fig 11)**. By changing upper and lower NaCl concentration protease production negatively affected as compared to 0.5% NaCl concentration.

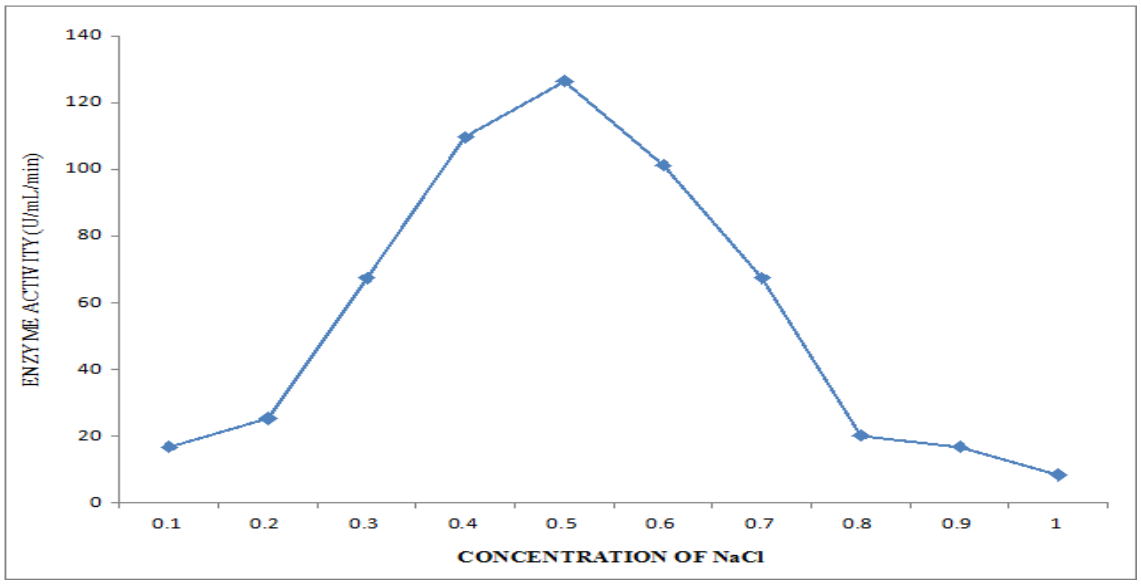

Fig 11: Effect of concentration of Nacl on protease production

IV. CONCLUSION

Isolate $RkNa_2$ was identified as a potent protease producing bacterial species from dairy sludge of "Dudhsagar dairy", Mehsana, which was used as substrate. Further study related to compatibility of Protease with various detergents.and purification of protease can also be done.

V. ACKNOWLEDGEMENT

I am thankful to the Head, Department of Microbiology, Gujarat Vidyapith, Sadra for providing necessary facilities to carry out my dissertation work.

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