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Optimization of Extracellular Protease Production from Alkali Thermo Tolerant Actinomycetes: *Saccharomonospora viridis SJ-21*

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ABSTRACT
A protease producing actinomycete was isolated from water sample, collected from hot water spring of Gujarat. It could grow in broad temperature range of 35-60 ºC and pH range of 7.0 to 10.0. The optimum conditions observed for protease production were 55 ºC and pH 9.5,with 3% inoculum in the medium containing metal ions like Mg ++ and Ca++. Maximum growth and enzyme production were observed after 120 and 96 hours, respectively, when grown in 100 ml production medium of pH 9.5, under shaking conditions of ~100 rpm at 55 ºC. The best carbon and organic nitrogen sources for the organism were observed to be glycerol and aspargine, respectively, while the most effective inorganic nitrogen source was observed to be sodium nitrate. Among the raw sources used, the maximum protease production was found with wheat bran, 0.5 gm% w/v.
***KEY WORDS**: Actinomycetes, alkaline protease, Saccharomonospora viridis, optimization*

INTRODUCTION

Discoveries of important, novel bioactive compounds depend upon the development of the strategies for isolation and characterization of novel and rare micro-organisms .The capacity of Streptomycetes to produce new compounds remains unsurpassed by the members of other microorganisms. Actinomycetes are becoming increasingly important as a source of novel products and are routinely screened for new bioactive substances like, antibiotics and enzymes [7]. Proteases constitute one of the most important groups of industrial enzymes and have established role in the detergent, food pharmaceutical, leather, waste processing industries and silk industries [13]. Extremophilic, especially alkalophilic, halophilic and thermophilic proteases are preferred due to ease of operation, higher activity, enhanced stability, faster reaction and less proneness to contamination [4]. Extremozymes are now a days replacing chemical catalysts in the manufacturing of chemicals, textiles, pharmaceuticals, paper, food and agricultural chemicals.

Microbial alkaline proteases for industrial uses are produced and studied mainly from Bacillus and Streptomyces. Little is known about proteases from other actinomycetes, and much less from Nocardiopsis spp. Actinomycetes produce extracellular enzymes like proteases, chitinases, amylases etc. Actinomycetes, particularly Streptomycetes are known to secrete multiple proteases in culture medium [14]. With comparison to Bacillus spp, actinomycetes have been less explored for proteases. We have been surveying actinomycetes for protease production and have substantial collection of actinomycetes in the last few years. The aim of this study was to screen actinomycete isolate for alkaline and thermostable protease production, characterization of best strain and optimization of enzyme production.

MATERIAL AND METHODS

Screening of alkalophilic and thermophillic actinomycetes producing protease

Samples collected from hot water spring , soil and other alkaline environments were serially diluted to spread evenly on milk agar medium of pH:8.5 and incubated at 55 ºC for 3-4 days to allow the colonies to grow. At an interval of 24 hrs, the diameter of zone of clearance of casein was measured, which provided a measure of their Proteolytic activity. To study their morphology and cultural characteristics, Gram Staining was performed and colony characters were noted. Out of 21 isolates, colony showing larger zone of casein hydrolysis was selected and designated as SJ-21 .

The isolate was preserved on milk agar slant at 4 ºC and sent to Gujarat Biotechnology Mission, Gandhinagar for 16S r- RNA sequencing.

***Study of biochemical activity**:*

The organisms were inoculated in various biochemical media for their biochemical and enzymatic activity and the results were noted. Media taken were various sugar broths like, glucose, xylose, mannitol, lactose, sucrose, maltose and arabinose, glucose phosphate broth, 1%peptone broth, 2% peptone broth, Moller's medium, phenyl alanine agar slant, urea broth, starch agar plate, casein agar plate, gelatine broth, tributyrine agar plate, etc.

Measure of proteolytic activity

- Measure of relative activity of protease by strain SJ-21: Fresh culture of Actinomycetes SJ-21 was taken and a small drop was put in the middle of the milk agar plate (pH 8.5, N-agar with 20% sterilized milk) and incubated for 5-6 days and at an interval of 24 hrs. Zone of casein hydrolysis and diameter of growth were measured and the relative enzyme activity (REA) was calculated by using the formula:
- REA =Diameter of zone of casein hydrolysis/ Diameter of colony in mm.
  Based on REA test, organism can be categorized into three groups showing excellent (REA>5), good (REA>2.0 to, 5.0) and poor (REA<2) (5)
- Measure of protease activity in terms of enzyme units: Here SJ-21 is inoculated in production medium at specific environmental conditions and enzyme produced is measured in terms of activity.

PRODUCTION MEDIUM AND CULTURE CONDITIONS:

The culture was inoculated in 250ml Erlenmeyer flasks containing 100 ml of production medium, consisting of: Glucose150mg,$K_2HPO_4$20mg,$KH_2PO_4$20mg,$MgSO_4$10mg,$CaCl_2$10mg,casein200mg, $NaNO_3$100mg pH-8.5 and put on an environmental shaker at 100 rpm at 55 ºC for 168 hours and checked for enzyme activity at an interval of 24 hours. The supernatant was collected after centrifugation at 15000 rpm , at 4 ºC temperature for 15 minutes and was used as crude enzyme source.

PRPROTEASE ASSAY:

Proteolytic activity in the supernatant was determined by using spectrophotometer method, given by Hagihara [6] with minor modification. 0.5 ml of crude enzyme solution was allowed to react with 2.0 ml of 0.6% casein in glycine-NaOH buffer (0.2M, pH 9) at 70 ºC for 20 minutes and the reaction was terminated by the addition of 2.5 ml 10% trichloroacetic acid. The reaction mixture was allowed to stand for 15 minute before centrifugation. The mixture was then centrifuged at 10000 rpm for 10 minutes at 4 ºC and 0.5 ml of supernatant was taken as the enzyme source and the activity was determined according to Lowry [11].Absorbance was measured at 670nm. Activity of enzyme was measured in terms of unit [6]. One unit of enzyme is defined as the quantity of enzyme required to release 1 micrograms of tyrosine per minute, under the assay conditions .

PROTEIN CONTENT:

Protein content was measured by Lowry's [11] method with Bovine serum albumin as a standard protein

Study of growth curve

Actinomycetes SJ-21 were inoculated in the broth containing glycerol 0.5%,yeast extract 0.2%, K_2HPO_4 0.1%, $NaNO_3$ 0.1%, (pH 8.5) and incubated at 55 ºC and optical Density was measured at 680 nm wavelength at regular interval, to obtain the growth curve .

***Optimization of medium conditions and ingradients**:*

The effect of temperature on growth of organism was determined by inoculating the growth medium (milk agar plates of pH 8.5) and incubating the plates at various temperatures viz: 25, 37, 45, 55, and 60 ºC for two to three days and then observing the growth.

The effect of temperature on enzyme production was determined by growing the isolate in production medium at different temperatures (25-65 ºC) for 96 hours and then measuring the enzyme activity.

The effect of salt concentration on growth of the isolate was determined by inoculating the isolate in N- broth supplemented with glycerol and varied salt concentrations (0.3-30.0% NaCl). The effect of pH on growth of isolate was checked by inoculating the isolate in N-broth of different pH values.

The effect of pH on protease production of isolate SJ-21 was determined by growing the isolate in production media of different pH in the range of 6.0-11.0, using appropriate buffers for 96 hours and then measuring the enzyme activity.

***Optimization of inoculum size and incubation period*:**

Protease production and alkaline protease activity was measured and monitored at 24 hr intervals over 120 hr fermentation period by growing the isolate in production medium for different time periods viz: 24, 48, 72, 96 & 120 h at 55 ºC. The different inoculum sizes of 1, 2, 3, 4, 5% v/v of 48 hours old inoculum (optical density 0.75 at 660 nm), were inoculated in the production media and protease production was determined after 96 hours of incubation at 55 ºC on an environmental shaker at 100rpm.

OPTIMIZATION OF VARIOUS CARBON AND NITROGEN SOURCES AND RAW MATERIALS:

Carbon sources taken for the study were Glucose, starch, sucrose, sodium citrate, lactose at 150mg% w/v and glycerol 0.5% v/v. Sources for Nitrogen selected were inorganic and organic nitrogen and amino acids. Nitrogen sources were $NaNO_3$, casein; yeast extract, aspargine, ammonium sulphate, lysine and urea at 150 mg% w/v. various raw materials checked for protease production by SJ-21 were oat meal, wheat bran, gram flour, wheat flour and rice flour in 0.5% w/v along with salts.

RESULTS AND DISCUSSION

Protease is an industrially important enzyme having wider applications in pharmaceutical, leather, laundry, food and waste processing industries. Industrial enzyme production would be effective only if the organism and the target enzyme are capable of tolerating different variables of the production processes.

1. Primary screening for protease producing actinomycetes was done on Milk agar medium based on the zone formation due to casein hydrolysis. Totally, 21 strains showing protease activity were isolated from the various samples. Among the twenty one strains, the best one (SJ 21) was selected based on the zone of casein hydrolysis (Figures 1 and 2). The morphological (figure-3), physiological and biochemical characteristics of the protease producing strain SJ-21, tested in the present study, are given in Table-1 and Table-2 . From all these results as well as 16S r-RNA analysis, the organism was identified as *Saccharomonospora viridis .*
2. Study of growth in the culture medium suggested that maximum growth of the isolate SJ -21 was obtained at 120 hours when the isolate was inoculated in the medium containing glycerol 0.5%,yeast extract 0.2%, K2HPO4 0.1%, NaNo3 0.1%, (pH 8.5) and incubated at 55 ºC . The optical density was measured at 680 wavelength at regular interval .(figure-2) Maximum growth was obtained at 120 hours while the enzyme production was maximum after 96 hours indicating, that protease is produced maximally in the late exponential phase of growth. The result was quite similar to that of Streptomyces fradiae described by Galas and Kaluzewsk, [5]. Keila et al demonstrated that the beginning of protease production by Streptomyces clavuligerus occurred during the post-exponential phase of growth [9]. The reports of Jigisha T Thumar and Satya P Singh showed that the synthesis of Protease from Streptomyces starts in early Stationary phase of growth [7], Mary E. Upton and William Fogarty 1976 reported that Thermomonospora viridis produces the protease (60 Hrs) earlier to mesophilic organisms (e.g. Aspergillus spp in 3 days) [12].
3. Effect of Production Temperature: The growth and enzyme production is greatly influenced by different incubation temperatures. The effects of different incubation temperatures on protease production were evaluated and it was found that 55 ºC was the most favorable temperature for protease production. Below 45 ºC and above 70 ºC, protease production was negligible (FIGURE-5). This result matches with the reports of Aliya Siddique , 2001, that extracellular protease was secreted in the culture medium during second lower phase of biphasic growth, at 50 ºC, for S. thermoviolaceus [1].
4. Effect of pH, inoculum size and salt concentration on enzyme production and growth: pH of the production medium greatly affects enzyme production [2].The results showed (figure-6) that the enzyme production was maximum at pH 8 and 9.5. Interestingly, the enzyme seems to have two pH optima for production. This may be due to the presence of mixture of proteases (our current study was done with crude enzyme). This report matches with that of Debananda S. Ningthoujam

[4], who reported the same type of two pH optima with Alkalithermotolerant strains of actinomycetes HA-4.

Inoculum size also affects the enzyme production greatly. Five different inoculum sizes represented graphically (Figure-7) were investigated for their effect on productivity of the protease by SJ-21. The results indicated that the use of 3.0 ml of 48 hours old inoculum (optical density 0.75 at 660 nm), gave the highest yield. The effect of salt concentration on the growth of isolate was determined by inoculating the isolate in nutrient broth containing varied salt concentration (0.3-30.0%). The results showed that the growth range of the Isolate SJ-21 was 0.3gm% to 2.0 gm% of NaCl, and above 2.0 gm%, the isolate could not grow. The organism was also inoculated in the nutrient broth of various pH values and the results indicated that the organism grows best at pH 9.2. (TABLE 3). The isolate was able to grow at pH range of 6.8 to 10, indicating that the strain was alkalotolerant.

5. Effect of carbon sources, nitrogen sources and raw materials:

Cultural and environmental conditions play important role in the microbial growth and enzyme production. The present study was aimed for optimization of medium components for the maximum production of thermostable alkaline protease. The result showed that the best carbon source was glycerol (figure-8).Lactose and glucose were found superior to sucrose and starch. Similar reports were published by Mary Upton and William Fogarty for *Thermomonospora viridis* [12]. Glycerol was better source of carbon for actinomycetes, also reported by Aliya Sidique [1]. Among the sugars, in our study lactose was the best source. Similar observations were made by Chi and Zhao, Kathiresan and Minivanan[3,8]. Thumar and Singh reported that, glucose is best for growth but protease production is optimal with sucrose for actinomycetes [7]. Studies have also indicated a reduction in protease production due to catabolite repression by glucose (10).In commercial practice, high carbohydrate concentrations repressed enzyme production [2].

Alkaline protease is comprised of 15.6% nitrogen and its production depends heavily on the availability of nitrogen sources, production was optimum with aspargine (70.14 units/ml), followed by peptone (53.44units/ml), we also tried combination of casein+peptone (232 units/ml) and casein +urea (108 units/ml)as nitrogen source and the combinations were better than single nitrogen source (Figure-8). In contrast, Thumar and Singh reported that, aspargine was best for growth but did not support enzyme production [7]. Among raw sources used, wheat bran supported protease production significantly; when used as the sole carbon and nitrogen source (Figure-9). These findings are similar to an alkaline protease from *Streptomyces clavulgerus* [9]. The production of the enzyme with these sources would be economically attractive one.

Figure: 1 SJ-21 on milk agar showing zone of casein hydrolysis after 72 hours

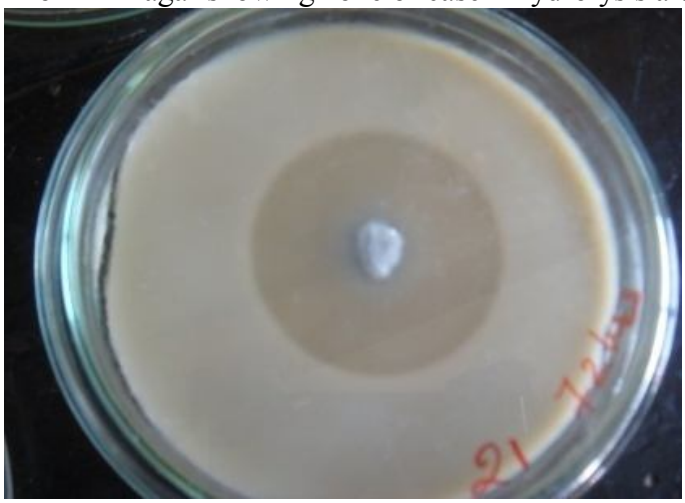


Figure: 2 Reverse of same milk agar plate showing violet pigment produced by SJ-21.

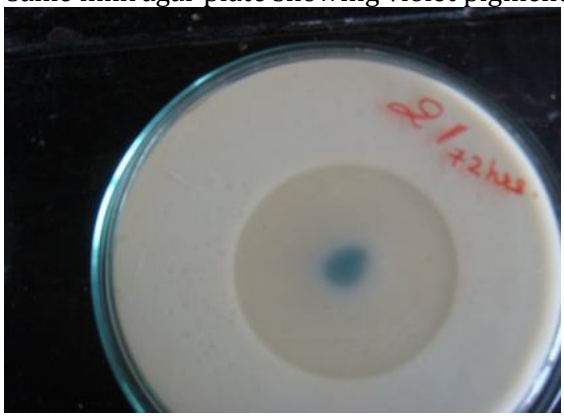


Figure: 3 Result of gram staining for morphology study.

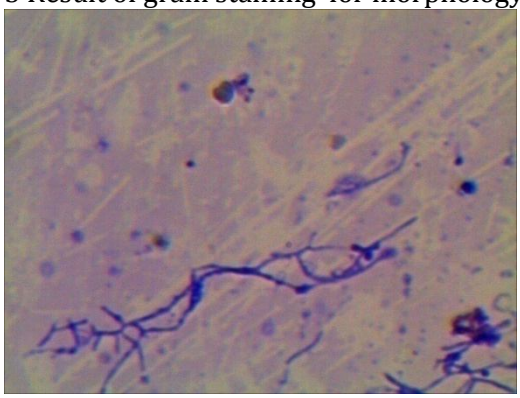


Table:1 Relative activity, colony characters and morphological characters of SJ-21

ISOLATE	ZONE SIZE IN mm / colony size in mm							GRAMS REACTION
	24Hrs	48Hrs	72Hrs	96	120	144	COLONY CHARACTERS	
SJ-21 (hot water spring) RE (RELATIVE ACTIVITY)	12 02	35 07	44 08	50 08	63 08	63 14	Very big, flat, grayish white colonies which turn violet in two three days. Violet pigment is temperature sensitive and diffusible too.	Gram positive thin filaments turn gram negative on incubation. Spores are observed in grams staining. Figure:3
	6.0	5.0	5.5	6.25	7.87	4.5		

Table-2 : Results of biochemical activity of SJ-21 *(Saccharomonospora viridis)*

	NAME OF BIOCHEMICAL TEST	STRAIN 21
1	INDOLE PRODUCTION	NEGATIVE
2	METHYL RED TEST	NEGATIVE
3	V. P. TEST	NEGATIVE
4	CITRATE UTILIZATION	NEGATIVE
5	NITRATE REDUCTION	NEGATIVE
6	AMMONIA PRODUCTION	POSITIVE
7	CATALASE TEST	NEGATIVE
8	UREASE PRODUCTION	NEGATIVE
9	GELATIN HYDROLYSIS	POSITIVE

10	AMYLASE PRODUCTION	POSITIVE
11	H2S PRODUCTION	NEGATIVE
12	DEHYDROGENASE TEST	NEGATIVE
13	GRWTH PATTERN IN N- BROTH	GROWTH WITH GREYISH VIOLET PIGMENT
14	CARBOHYDRATE UTILIZATION Glucose broth Xylose broth Lactose broth Sucrose broth Maltose broth Mannitol broth	 NEGATIVE NEGATIVE NEGATIVE NEGATIVE NEGATIVE NEGATIVE

Figure-4 Effect of incubation period and growth on enzyme production

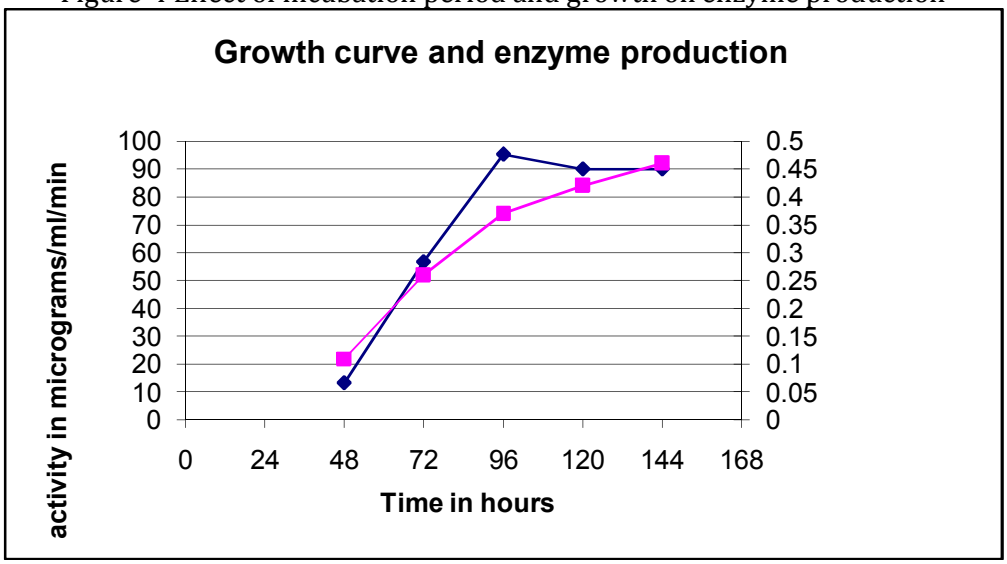


Figure-5: Effect of incubation temperature

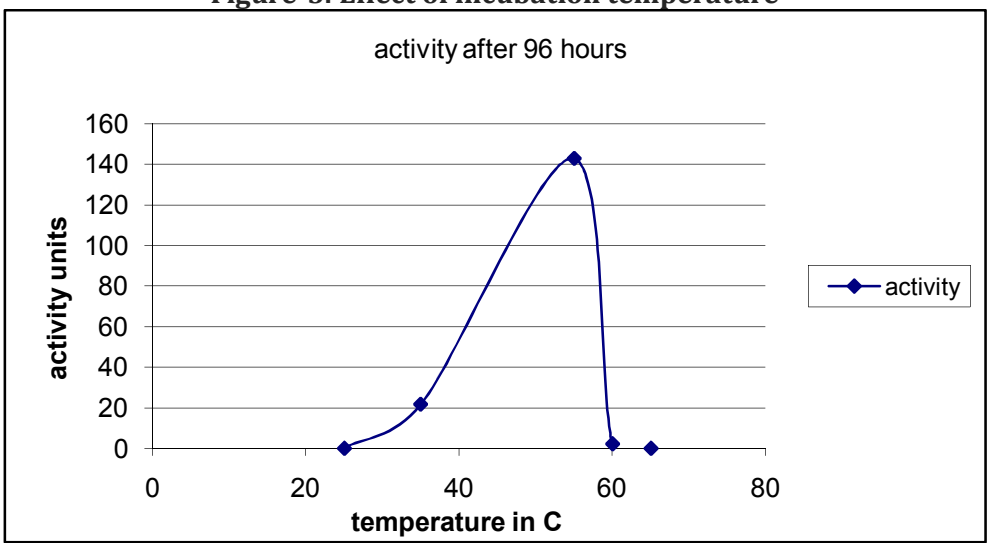


Figure-6: Effect of pH of Production Medium

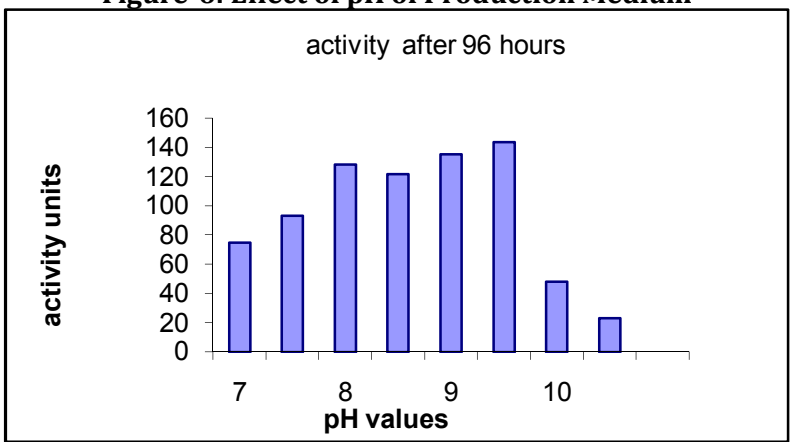


Figure-7: Effect of inoculum size

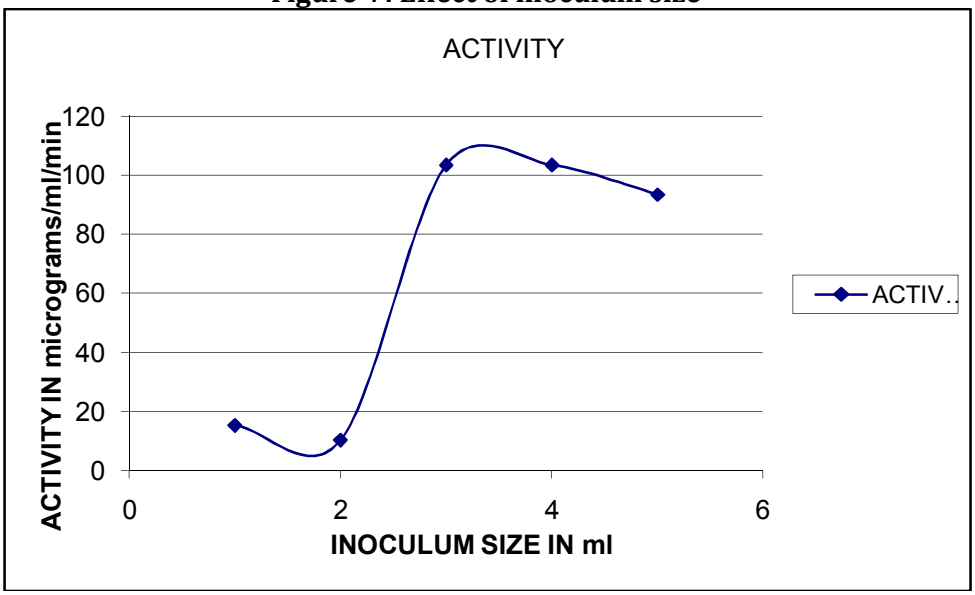


TABLE: 3 Effect of pH, salt concentration and temperature on growth of SJ-21 *Saccharomonospora viridis*

pH	Growth in terms of visible turbidity	NaCl Gm%	Growth in terms of visible turbidity	Incubation Temperature ºC	Growth on Milk- agar plates
2.8	__	0.3	++	25 ºC	No growth
3.6	__	0.5	+++	37 ºC	Little growth
4.4	__	1.0	+	45 ºC	Excellent growth
5.2	__	2.0	+	55 ºC	Fair growth
6.0	__	5.0	__	60 ºC	Poor growth
6.8	+	10.0	__		
7.6	++	20.0	__		
8.4	++				
9.2	+++				
10.0	++				

Figure-8 Effect of carbon sources

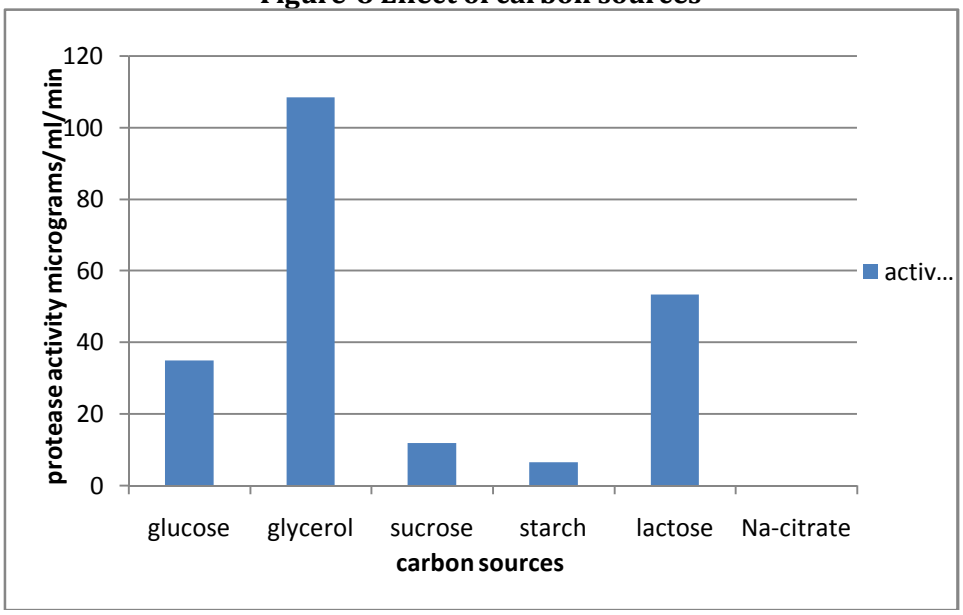


Figure- 9 Effect of various nitrogen sources on protease production

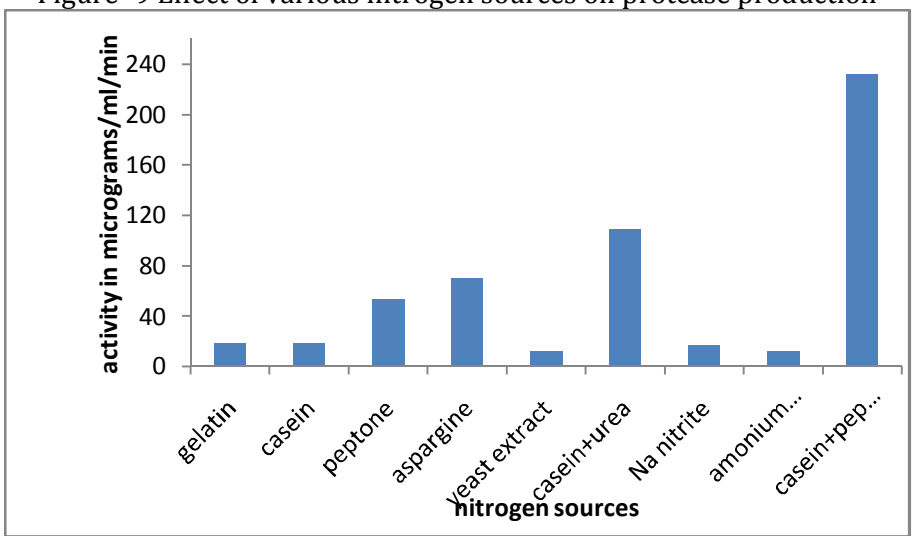
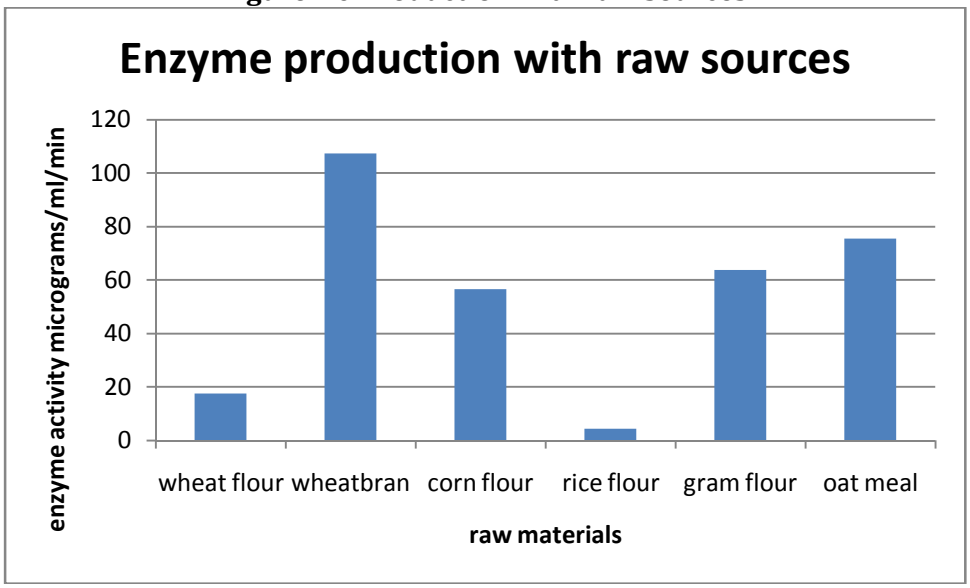


Figure-10 Production with raw sources



CONCLUSION

- ✓ Based on the relative enzyme activity and protease production, strain SJ-21 was selected for study. It was thermophilic and can grow and produce extra cellular protease at high temperatures above 55ºC. From cultural characters, biochemical activity, morphology, spore arrangements, and 16S r-RNA sequence analysis, isolate SJ-21, was identified as *Saccharomonospora viridis*.
- ✓ Maximum production of protease is observed after 96 hours at 55 ºC and, suitable pH for maximum enzyme production was 9.5, indicating that the protease is alkaline as well as thermostable.
- ✓ Glycerol was found to be the best carbon source. The best organic nitrogen source was found to be asparagine but the maximum production could be achieved by employing mixture of casein and peptone as nitrogen source.
- ✓ Wheat bran can serve as the best source of carbon as well as nitrogen and can be used at larger scale because of its low cost and ease of availability.

All these information could be used for construction of production media, for maximum protease production by this organism. As the organism is alkalophilic and thermophilic both, it could be a potential source of thermostable alkaline protease, with novel Industrial applications.

ACKNOWLEDGEMENT

Gujarat State Biotechnology Mission, Gandhinagar, Gujarat, India provided the16 S r-RNA sequencing of the isolate SJ-11 and helped in the identification of the isolate.

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