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Screening of biologically active microbial strains having therapeutic applications

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Natural sources, particularly microbes yield active molecules that have wide application in food and pharmaceutical industries, degradation of hazardous bacterial biofilms, etc. Safety and acceptability of such drugs attract researchers' attention for new drug discovery. Here, we explored biologically active microbial strains having therapeutic applications isolated from five different geographical areas of India. On screening, we found 10 strains capable of producing chitinase (Chi), seven cholesterol oxidase (COD), five glutaminase (Gln) and two heparinase (Hep) producing strains. Most of the isolated strains were found to be actinomycetes. Morphological and biochemical characterization of the strains suggest that the selected 13 isolates belong to the genus *Streptomyces*. Out of which, four were characterized through 16S ribosomal RNA gene analysis as *Streptomyces xanthochromogenes* MTCC 11937 (S1), *Streptomyces violascens* (N1), *Streptomyces xanthopheus* MTCC 11938 (H1) and *Streptomyces rimosus* MTCC 10792 (Ay). Results suggest that the soil isolated *Streptomyces* strains continue to act as a fascinating source of clinical and commercially importance enzymes. Partially purified enzymes were found to possess a broad range of pH and temperature stability indicating their capability to be used in clinical and pharmaceutical fields.

Keywords: Actinomycetes, Chitinase, Cholesterol oxidase, Glutaminase, Heparinase, Soil isolates,*Streptomyces*

Biologically active molecules obtained from microbial sources have applications in several fields, such as food and pharmaceutical industries as well as degradation of hazardous bacterial biofilms[1-3]. Microbial extracts have continued to be a potential source of new biologically active molecules for drug discovery[1,2]. It is estimated that more than 30% of the drugs in human consumption worldwide have compounds from natural sources as their origin[4].

Enzymes are the central dogma of life which catalyzes thousands of biochemical reactions both inside and outside the cell in the environment and assumed greater significance in the day-to-day activities of mankind. In the recent past, it has emerged as one of the major industrial use in the global market. Enzymes are frequently used as a biocatalyst for process improvement to enable utilization of new types of raw materials, flavour improvement of food (glutaminase), food processing, pharmaceutical field (cholesterol oxidase, heparinase, glutaminase, chitinase, etc), waste treatment, etc.[5,6]. Many enzymes play important role in cancer therapy such as glutaminase and asparaginase. Cholesterol oxidase plays important role in obesity and cardiovascular disorders.

The present work focuses on the microbes capable of producing cholesterol oxidase (COD), glutaminase (Gln), heparinase (Hep) or chitinase (Chi). During the investigations, different soil samples collected from various geographical areas were screened. Further we focused on selected microbial strains producing pharmaceutically/clinically important enzymes related with tumor inhibition activity, blood clot inhibition, antifungal activity and cholesterol diagnosis.

Material and Methods

Isolation and screening of microorganisms

A small amount of the soil sample, collected from basin of Gomti river (Lucknow, 26°50'27"N, 80°56'48"E), rhizosphere (Lucknow: 26.7°N, 80.9°E), forest (Thinmala range Kerala: 8.5074° N, 76.9730° E), slaughter house (Delhi:

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28.6100° N, 77.2300° E) and the Himalayan Mountain region (Badrinath: 30.7440° N, 79.4930° E), was suspended in sterile 0.85% NaCl solution and a portion of the suspension was spread on cholesterol enrichment medium (CEM) containing glucose 20, yeast extract 10, KH_2PO_4 0.05, KNO_3 1, $MgSO_4$ 0.5, $FeSO_4$ 0.01, NaCl 0.5, cholesterol 1 (dissolved in 0.5% Triton X-100) and agar 15 g/L; colloidal chitin agar (CCA) (chitin 0.45 and agar 1.5%); glutamine enrichment agar (GEA); heparin selective media (HSM) (heparin 10.0, sodium acetate 2.05, calcium acetate 0.48 g/L and pH 7.1±0.2) and incubated at 30°C for 7 days. Single colonies appearing on the plates were used for secondary screening and streaked on different indicator plates and incubated at 30°C for 2-4 days. Colonies that formed pigments around them were selected as specific enzyme producing cultures and maintained on YMG agar medium (yeast extract 4, glucose 4, malt extract 10 and agar 20 g/L) at 4°C for secondary screening and further studies. Secondary screenings for Chi, COD and Gln producing strains were performed by indicator plate methods. Heparinase producers were further confirmed by heparinase plate assay as described by Zimmermann *et al*.[7]. Colonies developed on HSM medium were lysed by exposure to chloroform vapors for 20 min followed by 1 h incubation at 28°C. Subsequently, 2% protamine sulfate (Sigma-Aldrich, USA) solution was added to the plate and incubated at 30°C for 1-2 h.

Fermentation studies

The X-media was used for the growth of actinomycetes ($MgSO_4.7H_2O$ 0.5, $(NH_4)_2HPO_4$ 0.5, NaCl 3, K_2HPO_4 1, soybean meal 10, $CaCO_3$ 3 g/L and glycerol 15 mL). Samples were withdrawn at 24 h interval and cells were separated by centrifugation at 11086 g for 20 min. The microbial growth was measured by pellet weight (obtained by drying it at 40°C for 48 h) and the supernatant was used for the enzyme assay. All experiments were performed in triplicate.

Enzyme assays

The biochemical tests for extracellular enzyme(s) production were carried out in triplicates using the following assays:

Chitinase assay

Chitinase activity was determined by a dinitrosalicylic acid (DNS) method[8], using colloidal chitin as a substrate. One mL of broth (enzyme source) was incubated with 1 mL of 0.45% colloidal chitin in a 0.1 M phosphate buffer (pH 7.0), in shaking water bath for 30 min at 37°C. The reaction was terminated by adding 2 mL DNS reagent and kept in a boiling water bath for 5 min to develop the colour. The tubes were cooled to room temperature (25ºC), centrifuged at 5000 g for 10 min to settle down unused chitin and absorbance was measured at 575 nm against the blank (0.1 M phosphate buffer and 0.45% colloidal chitin without enzyme). One unit of chitinase is defined as the amount of enzyme, which releases 1 micromole of N-acetylglucosamine per h under the reaction condition[9].

Cholesterol oxidase assay

Extracellular COD production was determined by using Allian *et al*. method[10] based on the conversion of cholesterol into 4-cholesten-3-one. In a 3.03 mL reaction mixture, containing 94 mM potassium phosphate, 0.35% Triton X-100, 3.4 mM taurocholic acid, 0.9 mM cholesterol, 19.8 mM phenol, 1.5 mM 4-aminoantipyrine and 19 units' horseradish peroxidase. The reaction mixture (3 mL) was incubated with 50 µL of enzyme solution at 37°C for 5 min after that enzyme reaction was terminated by heating in boiling water for 5 min. After cooling to room temperature (25ºC) absorbance of the reaction mixture was observed at 500 nm. One unit of COD is defined as the amount of enzyme required to produce 1 µmol of 4-cholesten-3-one/min at pH 7.0 and temperature 37°C.

L-Glutaminase assay

Extracellular glutaminase activity was measured by estimating the amount of ammonia released during the hydrolysis of glutamine[11]. The reaction mixture consisted of 0.5 mL glutamine (40 mM), 0.5 mL 0.1 M phosphate buffer (pH 7), 0.5 mL distilled water and 0.5 mL enzyme solution was incubated at 37ºC for 30 min for enzyme reaction which was stopped by adding 0.5 mL of TCA (Trichloro acetic acid). 0.1 mL of the reaction mixture was added to 3.7 mL of distilled water and 0.2 mL of Nessler's reagent was added to measure the amount of ammonia released. The absorbance was taken at 450 nm. One unit (U) of L-glutaminase was defined as the amount of enzyme required to liberate one µmol of ammonia in one minute under experimental conditions.

Heparinase assay

Heparinase activity was measured by azure-A dye metachromasia, where one unit of heparinase activity was defined as, amount of the enzyme required for the degradation of 1 mg of heparin in 1 h[12]. About

200 µL of enzyme extract was incubated with 100 µL of heparin assay mix (heparin 25 g/L in 0.025 M sodium acetate and 0.0025 M calcium acetate) at 30°C. At 10 minutes intervals, 10 µL samples were withdrawn from assay tube and added to 10 mL azure-A dye solution. Azure-A metachromasia is produced by a dye-dimer reaction with the polyanionic heparin in a loose electrostatic manner. The dimerization results in the decrease in electron delocalization of the dye molecules, which leads to a shift (increase/decrease) in the absorbance at 620 nm[13]. The change in optical density was measured within 1 h at 620 nm and compared with a standard curve of 0 to 8 µg/mL heparin in azure-A solution.

Protein estimation

Extracellular protein estimation was performed according to the method described by Lowry[14]. The biuret reaction of interaction of proteins with copper under alkaline conditions and the Folin-Ciocalteau phosphomolybdic phosphotungstic acid reduction leads to the formation of hetero molybdenum blue by the copper catalysed oxidation of amino acids. The protein standard was prepared with bovine serum albumin (BSA)[15].

Purification steps

Each enzyme was purified from the centrifuged culture broth by precipitating with 30-80% ammonium sulphate. The solution was left overnight at 4°C, centrifuged at 10000 rpm for 20 min and the precipitate was obtained in the form of a pellet. The pellet was dissolved in phosphate or citrate buffer (10 mM, pH 7) and stored in a tube at 4°C. The pellet obtained was dialyzed against same buffer (10 mM, pH 7) overnight on a magnetic stirrer for desalting. After desalting, the enzyme solution was concentrated against 40% sucrose solution for 3-5 h at 4°C. The concentrated enzyme thus obtained was subjected to chromatographic separation and purification. The purified enzymes obtained were resolved on SDSPAGE.

Results

Screening of active strains

Enzymatic productions by microbes were confirmed by observing microbial growth on plates enriched with specific components (heparin, cholesterol, chitin or glutamine) (Fig. 1) indicating the production of respective enzymes. Soil samples from five different geographical niches were screened and fifteen active strains were isolated. Out of which ten strains were found to produce extracellular Chi; seven were COD producers, two Hep and five Gln. All the isolates were subjected to the secondary screening on indicator plates.

All the 10 strains showing chitinase production during primary screening, as indicated by the presence of dense growth and bigger clear zone on CCA plates, were subjected to secondary screening performed with the culture filtrates using well diffusion method on colloidal chitin (0.45%) agar plates and indicator plates. After 3-4 days of incubation at 37°C five strains YE21, YE23, HE32, H1 and N1 were found to be strong producers on the basis of a clear zone around the well of CCA plate. It was observed due to the dissolution the colloidal chitin of agar medium through the chitinase present in the sample (Fig. 2A). Results were further confirmed by the appearance of reddish purple colour on the indicator plate (Fig. 2B)[16]. For screening of the L-glutaminase producing cultures, indicator plates contained L-glutamine as the sole carbon and nitrogen source and phenol red dye (pH range 6.8-8.2) were used. Indicator plate turning from yellow to pink confirmed the presence of Gln (Fig. 2C) and COD (Fig. 2D). Out of the four microorganisms, those found heparinase positive during primary screening were subjected to secondary screening in HSM agar plates. Two strains (V1 and V4) showed marked utilization of heparin, as observed by the

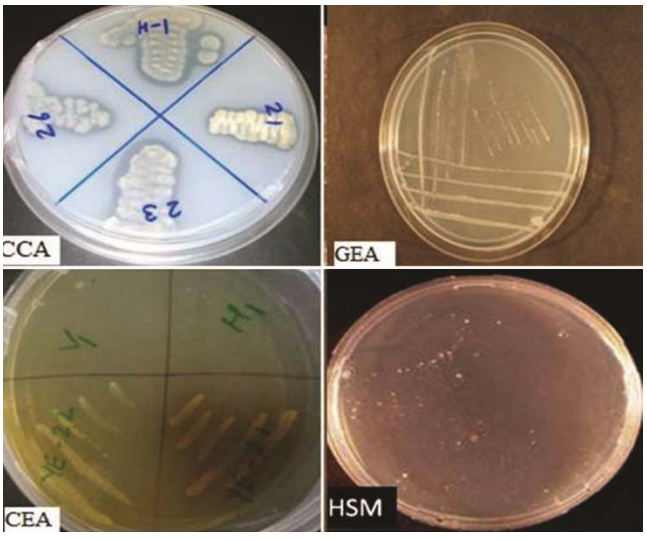

Fig. 1 — Primary screening for selection of chitinase (colloidal chitin agar: CCA), glutaminase (glutamine enrichment plate: GEA) producing microorganisms, cholesterol oxidase (cholesterol enrichment plate: CEA) and heparinase (heparinase selection medium: HSM)

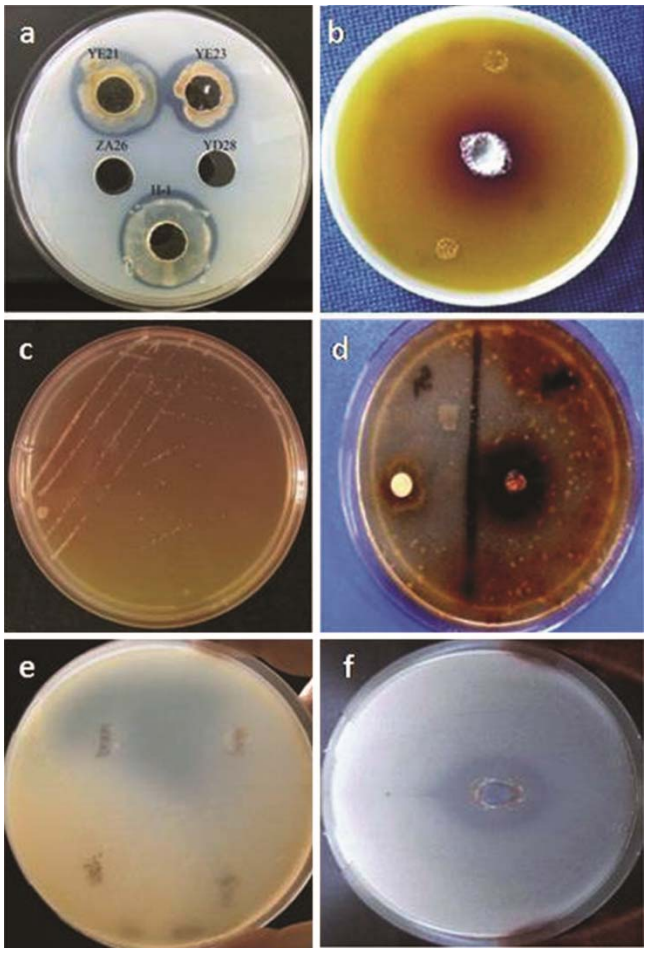

Fig. 2— Secondary screening for (A) chitinase using supernatant (B) chitinase indicator plate (C) glutaminase on indicator plate (D) cholesterol oxidase on indicator plate (E) heparinase through heparin utilization (F) heparin protamine reaction indicates heparin hydrolyzing activity of heparinise enzyme present in the cell extract.

presence of clearance zone around the microbial growth due to protamine sulphate precipitation (Fig. 2 E-F).

Quantitative studies

Final selection of the microorganisms was based on quantitative measurements of the enzyme activity under submerged culture conditions. The bacterial strains were studied for the enzyme production in shake flask conditions. Enzyme production during growth was evaluated in production media at different time interval. The cultures were incubated at 28°C except heparinase (32°C) on a rotary shaker at 180 rpm for 120 h. Results of the enzyme production from the selected strains are summarised in Table 1.

Strains HE32, YE23, YE21, H1 and N1 produced chitinases in higher concentration (0.2-0.36 U/mL) as compared to the rest (0.03-0.16 U/mL). Enzyme production started during the exponential phase of growth. However, due to accumulation of enzyme in the production medium its concentration reached to a maximum at the late log or stationary phase of the growth.

Table 1 — Secondary screening of soil isolates showing enzyme activity (U/mL)

Strains	Chitinase	COD	Heparinase	Glutaminase
S1	0.16	0.453	-	-
N1	0.28	-	-	-
H1	0.24	-	-	-
R6	0.15	2.4	-	-
A(y)	-	1.82	-	-
RA	-	-	-	5.3
RN-B	-	-	-	7.2
RB	-	-	-	12.3
V1	0.03	-	0.4	-
V4	-		0.19	
YE21	0.23	0.85	-	6.3
YE23	0.26	1.6	-	2.9
HE32	0.35	-	-	-

Among seven COD producers, Ay and YE23 produced COD in higher concentration as compared to other strains. The COD production started at the exponential phase and reached to maximum on stationary phase (2.4 and 1.6 U/mL, respectively). Among glutaminase producers, four strains (RA, RB YE 21 and YE 22) were found to produce Gln in higher concentration. Finally, RB was selected which produced Gln in substantial quantities during the exponential phase of growth.

For the selection of strong Hep producer, submerged fermentation was performed in HSM, where heparin acted as an inducer and sole carbon and nitrogen source. Two cultures V1 and V4 were found to produce maximum heparinase concentration 0.4 U/mL after 28 h and 0.34 U/mL after 44 h of incubation, respectively. With further incubation, heparinase production decreased for both the cultures. As heparin is a costly substrate, HSM was employed as a screening medium only and for bulk production, X medium was used where maximum Hep activity was observed in the stationary phase of incubation (96 h) as 0.35 and 0.22 U/mL for V1 and V4, respectively.

Out of 15 strains, five potent enzyme producing strains (N1, S1, RB, Ay and V1) were characterized through morphological and biochemical characteristics (Table 2) and finally through 16 S rRNA homology studies from Microbial Type Culture Collection MTCC, Chandigarh, India as N1 (*Streptomyces violascens*), S1 (*Streptomyces xanthochromogenes*

Table 2 — Cultural characteristics of the soil isolates

Activity	Medium	Parameter observed	Producer strains N1	S1	Ay	RB	V1
Growth on ISP medium	ISP2	Growth	+	+++	++	++	++
		GP	Rough & granular	Filamentous	Rough & granular	Smooth sticky	Rough granular
		Sporulation	-	Cream	white	-	-
		DP	Brownish yellow (light)	Yellow	Yellow	Yellow	Yellow
	ISP3	Growth	+	+++	++	++	++
		GP	Smooth & granular	Filamentous	Rough & granular	Rough granular	Rough granular
		Sporulation	Whitish grey	Cream	Whitish grey	Grey	Grey
		DP	Brown	Yellow	Yellow	Yellow brown	Yellow
	ISP4	Growth	++	+++	+++	+++	+++
		GP	Rough & granular	Filamentous	Rough & granular	Rough granular	Rough granular
		Sporulation	Grey	White	Grey	Purple grey	Grey
		DP	Yellowish brown	Light yellow	Brownish yellow	Light yellow	Yellow brown
	ISP5	Growth	++	+++	++	+	++
		GP	Smooth & granular	Filamentous	Rough & granular	Rough granular	Rough granular
		Sporulation	Grey	Cream	Whitish grey	-	Grey white
		DP	Light yellow	Light yellow	Grey	-	Grey
	ISP6	Growth	++	++	+++	++	+++
		GP	Rough & granular	Filamentous	Slimy & sticky	Smooth	Slimy sticky
		Sporulation	-	Grey	white	-	-
		DP	Brownish yellow	Black	Brownish yellow	Yellow brown	Yellow brown
	ISP7	Growth	++	+	+	+	+
		GP	Rough & granular	Filamentous	Rough & granular	Rough granular	Rough granular
		Sporulation	Grey	Grey	Whitish grey	Grey white	Grey white
		DP	Greyish brown	Light grey		Brown black	-
NaCl tolerance	1%	Growth	+++	+++	++	+++	+++
		GP	Rough & granular	Smooth granular	Rough granular	Rough granular	Smooth granular
		Sporulation	Greyish white	Grey	Whitish grey	Grey white	Grey
		DP	Brownish yellow	Dark brown	yellow	Dark brown	Dark brown
	3%	Growth	++	+++	+	+	+++
		GP	Rough & granular	Smooth granular	Rough granular	Rough sticky	Smooth granular
		Sporulation	Greyish white	White	Whitish	-	White
		DP	Brownish yellow	Brown-black	Light yellow	Brown-black	Brown-black
	5%	Growth	-	++	-	-	++
		GP	-	Smooth	-	-	Smooth
		Sporulation	-	White	-	-	White
		DP	-	Yellow brown	-	-	Yellow brown
	7%	Growth	-	+	-	-	+
		GP	-	Smooth granular	-	-	Smooth, granular
		Sporulation	-	Light brown	-	-	Light brown
		DP	-	Dark yellow	-	-	Dark yellow
pH tolerance	pH 5	Growth	+++	+++	++	+++	+++
		GP	Rough granular	Rough granular	Rough granular	Rough granular	Rough granular
		Sporulation	white	Grey	Whitish grey	White	Grey
		DP	Brownish yellow (light	Chocolaty	Yellow	Brown black	Chocolaty

Contd…

Table 2 — Cultural characteristics of the soil isolates (*Contd*.)

Activity	Medium	Parameter observed	Producer strains				
			N1	S1	Ay	RB	V1
	pH 7	Growth	+++	+++	++	+++	+++
		GP	Rough & granular	Rough granular	Rough granular	Rough granular	Rough granular
		Sporulation	Greyish white	Grey	Whitish grey	Grey	Grey
		DP	Brownish yellow	Yellow brown	Yellow	Yellow brown	Yellow brown
	pH 9	Growth	+++	+++	++	-	+++
		GP	Rough & granular	Rough granular	Rough granular	-	Rough granular
		Sporulation	Greyish white	Grey	Whitish grey	-	Grey
		DP	Brownish yellow	Dark brown	Yellow	-	Dark brown
	pH 11	Growth	++	+++	++	_	+++
		GP	Rough & granular	Rough, granular	Rough granular	Rough granular	Rough, granular
		Sporulation	Grey	Grey	Whitish grey	Grey	Grey
		DP	Brown	Dark brown	Yellow	Dark brown	Dark brown
Temp. toler-ance	28°C	Growth	++	+++	++	+++	+++
		GP	Rough & granular	Rough, granular	Rough granular	Rough granular	Rough, granular
		Sporulation	Greyish white	White	Brown pink	Grey	White
		DP	Brownish yellow	Dark brown	Yellow	Yellow brown	Dark brown
	37°C	Growth	+++	+++	+	+++	+++
		GP	Rough & granular	Rough granular	Rough granular	Smooth granular	Rough granular
		Sporulation	Greyish white	Grey	Brown pink	Grey white	Grey
		DP	Brownish yellow	Yellow brown	Yellow	Brown	Yellow brown

[where, (-) = No growth, (+) = Poor growth, (++) = Moderate growth and (+++) = Heavy growth; GP: Growth pattern; DP: Diffusible pigment]

Table 3 — Soil isolates and enzyme production conditions

strain	Name	Enzyme	pH	Temp (^{O}C)	Rpm	Inoculums size (%)	Harvesting time (h)
S1	*Streptomyces xanthochromogenes*	Chi	7	28	180	2	96
H1	*Streptomyces xanthophaeus*	Chi	7	30	180	1	72
Ay	*Streptomyces rimosus*	COD	7	30	200	3	48
RB	Actinomycetes	Gln	7	28	180	2	96
V1	Actinomycetes	Hep	7	32	180	2	96

MTCC 11937), H1 (*Streptomyces xanthopheus* MTCC 11938), Ay (*Streptomyces rimosus* MTCC 10792). Rest of the cultures were identified as actinomycetes through cultural and morphological characterization.

Optimization of physical parameters for enzymes production

Studies of the various physical parameters influencing the production of selected enzymes by the isolated strains revealed that the conditions for maximum enzyme production at flask level varied from species to species as summarized in Table 3. *Streptomyces* strains isolated from the soil sample of middle India was found to grow at pH between 7-8, temp 28-35°C and rpm 200-300. However, above or below these ranges resulted in decreased enzyme activity.

Purification of enzymes

The culture supernatant was initially precipitated and dialyzed to produce a concentrated protein solution enriched with enzymes. For precipitation, various concentrations of ammonium sulphate (60-90%) were tried. For culture supernatant of the Ay and RB strains 60% saturation was required for COD and Gln precipitation, respectively. 70% ammonium sulphate saturation is required for Hep precipitation from V1 strain. 75 and 80% of ammonium sulphate saturation were optimum concentration to precipitate Chi from H1 and S1 strain. DEAE cellulose ion exchange chromatography was used in the first step of purification. pH and temperature stability of the enzyme was studied using an active fraction of the above column and it was found all the selected

Table 4 — Soil isolates and enzyme characterization

strain	Name	Enzyme	$(NH_4)_2SO_4$ % saturation for precipitation	Optimum condition for enzyme activity		condition for Enzyme stability	
				pH	Temp °C	pH	Temp. °C
S1	*Streptomyces xanthochromogenes*	Chi	80	7.0	37	5-8	25-50
H1	*Streptomyces xanthophaeus*	Chi	75	7.0	37	5-8	28-55
Ay	*Streptomyces rimosus*	COD	70	7.0	37	6-9	28-40
RB	Actinomycetes	Gln	60	7.5	37	6-9	25-45
V1	Actinomycetes	Hep	70	7.0	37	6-8	30-45

enzymes were stable near 7-7.5 pH and show maximum activity at 37°C (Table 4).

Discussion

Enzymes catalyze several complex chemical reactions under appropriate physiological conditions in humans. Therefore, these can be used as therapeutic agents. Plenty of literature is available regarding the use of enzymes in diseases related to heart, kidney, pancreatic and respiratory system, including cancer[16,-18]. Glutaminase free L-asparaginase has gained more importance as an effective therapeutic agent for the treatment of acute lymphoblastic leukemia and lymphosarcoma[18]. Cholesterol oxidase has received great attention due to its clinical applications (determination of serum cholesterol). Besides this COD possess potent insecticidal activity and is also implicated in the microbial diseases such as tuberculosis (bacterial), HIV (viral) and Alzheimer's (non-viral prion origin)[19]. Similarly, chitinases are used for the production of chito-oligosaccharides and N-acetyl glucosamine that can be used as an anti-inflammatory drug[20]. Applications of the enzymes in the treatment of various diseases warrant the search for novel and potent producers of therapeutic enzymes.

Screening of desired enzyme producer is generally based on some colour changing analysis which can be easily visualized by necked eye. Like Chi producers have been further confirmed by the appearance of reddish purple colour on the indicator plate[21,], which is due to the hydrolytic conversion of chitin to N-acetyl glucosamine which is basic in nature that shifts the medium pH towards alkaline side. As the pH changes, bromocresol purple dye present in the indicator plate transforms its yellow colour (acidic pH 4.7) to purple[22].

Gln producers, among the microbial population, can be identified by observing colour change of indicator plate from yellow (pH <6.8) to pink (pH >8.2)[18]. This colour change has due to the breakdown of glutamine to glutamic acid and ammonia, indicating extracellular L-glutaminase production. Similarly, strains capable of producing extracellular COD that converts cholesterol into 4-cholesten-3-one and hydrogen peroxide (H_2O_2) which in turn form azo compound with schiff's base present in the indicator plates and change of the medium colour to intense brown colour[23].

During the screening of Hep producers, Protamine sulphate is used which is a polycationic polypeptide with high arginine content. The Protamine sulphate and heparin reaction largely occur due to their opposite surface charges; protamine as a polycation readily reacts with the polyanion heparin to form protamine-heparin complexes or aggregates (PHA)[24]. Incubation with 2% protamine sulphate solution resulted in the electrostatic interaction of protamine sulphate with unconsumed heparin in the plate resulting in the formation of white colour precipitate and formation of a clearance zone around the microbial culture indicating heparin breakdown by heparinase produced by the microorganism.

Literature search about the producer strains was performed using scifinder software and it was found that *S. xanthochromogenes* is known to produce a diverse array of chemical compounds such as diastereoisomeric I-Na, nitropeptin, xanthicin (I), pravastatin, reductiomycin, alkaloid AM-6201[25-30]. *Streptomyces xanthophaeus* is known to produce postproline endopeptidase, benarthin, β-galactosidase inhibiting isoflavonoids and geomycins[31-34]. *Streptomyces rimosus* is known to produce oxytetracycline and other tetracycline antibiotics[35]. The enzymes purified from our strain are different from the above-reported ones suggest the significance of the work.

Results of the performed screening study suggest that the *Streptomyces* strains isolated from diverse soil samples are capable of producing a high concentration of the desired enzymes of clinical and commercial importance. The preliminary work of enzyme characterization led that isolated enzyme possesses broad range of pH and temperature stability indicating their capability to be used in clinical and pharmaceutical fields.

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References

1 Cragg GM, Newman DJ & Snader KM, Natural products in drug discovery and development. *J Nat Prod*, 60 (1997) 52.

2 Nandakumar R, Yoshimune K, Wakayama M & Moriguchi M, Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. *J Mol Cat B: Enzymatic*, 23 (2003) 87.

3 Singh V, Verma N, Banerjee B, Vibha K, Haque S & Tripathi CKM, Enzymatic degradation of bacterial biofilms using *Aspergillus clavatus* MTCC 1323. *Microbiol*, 84 (2015) 59.

4 Schmid I, Sattler I, Grabley S & Thiericke R, Natural products in high throughput screening: automated high-quality sample preparation. *J Biomol Screening*, 4 (1999) 15.

5 Lonsane B & Ramakrishna M, Microbial enzymes in food processing industry: present status and future prospects in India. *Indian Food Industr*, 8 (1989) 15.

6 Wagner G & Schmid RD, Biosensors for food analysis. *Food Biotechnol*, 4 (1990) 215.

7 Zimmermann J, Langer R & Cooney C, Specific plate assay for bacterial heparinase. *Appl Environ Microbiol*, 56 (1990) 3593.

8 Miller GL, Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem*, 31 (1959) 426.

9 Mathivanan N, Kabilan V & Murugesan K, Purification, characterization and anti-fungal activity from *Fusarium chlamydosporum*, a mycoparasite to groundnut rust, *Puccinia arachidis*. *Can J Microbiol*, 44 (1998) 646.

10 Allain CC, Poon LS, Chan CS, Richmond W & Fu PC, Enzymatic determination of total serum cholesterol. *Clin Chem*, 20 (1974) 470.

11 Imada A, Igarasi S, Nakahama K & Isono M, Asparaginase and glutaminase activities of micro-organisms. *J Gen Microbiol*, 76 (1973) 85.

12 Abirami P, Arumugam M, Ajithkumar T & Balasubramaniam T, Isolation and characterization of 37KDa heparinase from the purple fluid of *Dolabella auricularia*. *Indian J Mar Sci*, 40(2011) 112.

13 Grant AC, Linhardt RJ, Fitzgerald GL, Park JJ & Langer R, Metachromatic activity of heparin and heparin fragments. *Analytical Biochem*, 137(1984) 25.

14 Lowry OH, Rosebrough NJ, Farr AL & Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem*, 193 (1951) 265.

15 Veda M & Arai M, Purification and some properties of chitinases from *Aeromonas* sp. No. 10S-24. *Biosci Biotechnol Biochem*, 56 (1992) 460.

16 Uma Maheswari T, Hemalatha T, Sankaranarayanan P & Puvanakrishnan R, Enzyme Therapy: Current Perspectives. *Indian J Exp Biol*, 54 (2016) 7.

17 Jose D, Jayesh P, Gopinath P, Mohandas A, & Singh IS, Potential application of β-1, 3 glucanase from an environmental isolate of *Pseudomonas aeruginosa* MCCB 123 in fungal DNA extraction. *Indian J Exp Biol*, 52 (2014) 89.

18 Saxena A, Upadhyay R & Kango N, Isolation and identification of Actinomycetes for production of novel extracellular glutaminase free L-asparaginase. *Indian J Exp Biol*, 53 (2015) 786.

19 Srivastava A, Niwas R, Singh V, Khan A, Tripathi CKM, Microbial Cholesterol Oxidases. *Int J Scient Innovative Res*, 3 (2015) 1.

20 Gangwar M, Singh V, Pandey AK, Tripathi CKM & Mishra BN, Purification and characterization of chitinase from *Streptomyces violascens* NRRL B2700. *Indian J Exp Biol*, 54 (2016) 64.

21 Lunge AG & Patil AS, Characterization of efficient chitinolytic enzyme producing Trichoderma species: A tool for better antagonistic approach. *Intl J Sci Environ Technol*, 1 (2012) 377.

22 Agrawal T & Kotasthane AS, Chitinolytic assay of indigenous Trichoderma isolates collected from different geographical locations of Chhattisgarh in Central India. *Springer Plus*, 1 (2012) 73.

23 Nishiya Y, Kawamura Y & Imanaka T, Enzymatic assay for chloride ion with chloride-dependent sarcosine oxidase created by site-directed mutagenesis. *Anal Biochem*, 245 (1997) 127.

24 Rossmann P, Matoušovic K & Horáček V, Protamine-heparin aggregates. *Virchows Archiv B*, 40 (1982) 81.

25 Otake N, Seto H, Nakayama H, Endo T, Oba K, Iwata M & Watanabe T, Antibiotic 6257 manufacture with *Streptomyces* for *Pyricularia oryzae* infection control in rice. *Jpn. Kokai Tokkyo Koho*, (1988) JP 63126495 (Accessed May 30, 1988).

26 Ohba K, Nakayama H, Furihata K, Shimazu A, Endo T, Seto H & Otake N, Nitropeptin, a new dipeptide antibiotic possessing a nitro group. *J Antibiot*, 40 (1987) 709. |

27 Zhang L, Zhang J, Yang W & Bai G, Classification of *Streptomyces* strain Z314 and purification of its product pravastatin. *Weishengwu Xuebao*, 48 (2008) 33.

28 Cho H, Beale JM, Graff C, Mocek U, Nakagawa A, Omura S Floss & Heinz G, Studies on the biosynthesis of the antibiotic reductiomycin in *Streptomyces xanthochromogenus*. *J Am Chem Soc*, 115 (1993) 12296.

29 Onda M, Konda Y, Hinotozawa K & Omura S, The alkaloid AM-6201 from *Streptomyces xanthochromogenus*. *Chem Pharm Bull*, 30 (1982) 1210.

30 Arishima M, Sakamoto J & Sato T, An antibiotic *Streptomyces* No. 689 strain. I. Taxonomic studies. *Nippon Nogei Kagaku Kaishi*, 30 (1956) 469.

31 Shibamoto N, Terasawa T, Okamoto R, Shin T & Murao S, Novel postproline endopeptidase and its manufacture with *Streptomyces* species. *Jpn Kokai Tokkyo Koho*, (1993) JP 05244947 A 19930924.

32 Aoyagi T, Hatsu M, Kojima F, Hayashi C, Hamada M &Takeuchi T, Benarthin: a new inhibitor of pyroglutamyl peptidase. I. Taxonomy, fermentation, isolation and biological activities. *J Antibiot*, 45 (1992) 1079.

33 Hazato T, Naganawa H, Kumagai M, Aoyagi T & Umezawa H, β-Galactosidase-inhibiting new isoflavonoids produced by actinomycetes. *J Antibiot*, 32 (1979) 217.

34 Brockmann H & Musso H, Antibiotics from actinomycetes. XXIX Geomycin 2. *Chemische Berichte*, 87 (1954) 1779.

35 Singh N, Rai V & Tripathi CKM, Oxytetracycline production by immobilized cells of a new isolate of *Streptomyces rimosus* MTCC 10792. *J Pharm Res*, 5 (2012) 2477.