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CHARACTERIZATION OF NOVEL ALKALINE PROTEASE PRODUCING *STREPTOMYCES* FROM ALKALINE SOIL OF LUCKNOW, (U.P.), INDIA.

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ABSTRACT

Three alkaline protease producing gram positive, spore forming *Streptomyces* (J1, J2 and J3) were isolated from alkaline soil (pH range: 9-10) samples collected from Central Soil Salinity Research Institute (C.S.S.R.I), Lucknow. On the basis of halo zone, morphology, texture and microscopic characteristics they were identified as *Streptomyces*. Further characterization both genetically and phenotyphically were done in order to establish their phylogeny. The results of the 16S rDNA sequences were analysed using BLASTn. On the basis of the homology results the isolates ((J1, J2 and J3) were identified as *S. macrosporeus, S. bacillaris,* and *S. rubrolavendulae,* respectively. Optimization studies under different carbon and nitrogen sources at pH 10 under 72 h of incubation revealed maximum protease production by *S. rubrolavendulae* (12.50 U/ml) and *S. bacillaris* (8.82U/ml) and the minimum by *S. macrosporeus* (0.95 U/ml). The crude alkaline protease thus revealed maximum activity at 37 ^{0}C at pH 10.

KEYWORDS: **Alkaline protease, Streptomyces, 16S rDNA**

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INTRODUCTION

Microbial proteases are the most important proteolytic enzymes and have been studied extensively since the advent of enzymology. The world enzyme market is currently at $5.1 billion and is expected to rise by 6.3% annually by 2013.Proteases share a major part of the global enzyme market. Alkaline proteases (EC.3.4.21–24, 99) are those enzymes that are active at alkaline pH with optimum pH in between 9 to 11(Singhal *et al*., 2012). Among various proteases, alkaline protease is the most important enzymatic group which play a vital role in industrial field such as leather, detergent, silk degumming, pharmaceutical, photography, meat tenderization, cosmetics, medicinal and peptide synthesis due to their robustness, catalytic activity, high degree of substrate specificity and production capacities (Kumar and Takagi, 1999). *Streptomyces* belongs to the order of Actinomycetales which are aerobic, gram positive bacteria of soil population and are widely distributed (Kuster, 1968). They could not be identified simply by using microscopic technique and at species level the best way of identifying actinomycetes is through biochemical tests but the process is time consuming and expensive. Hence a more advanced and time extensive technology has become more popular during the last decade which has been used for identifying actinomycetes at the molecular level and primers have accordingly been developed by researchers to target specifically 16S rRNA gene sequences (Wang *et al*., 1999). Recently many *Streptomyces* were investigated for protease production and biochemical characterization e.g., *Streptomyces pectum* for serine*, Streptomyces exfoliatus, Streptomyces rimosus* for metallo and serine proteases, respectively (Rifaat *et al*., 2007). Protease production by the microorganisms are known to be improved by studying the impact of the type of nitrogen and carbon source, fermentation period, growth temperature and initial pH of the culture medium in cultivation optimization experiments (Saleem *et al*., 2012). Production and optimization of alkaline protease from various actinomycetes such as *Streptomyces albidoflavus* (El-Shafie *et al*., 2010)*, Streptomyces puvereceus* (Jayasree *et al*., 2009), *Streptomyces gulbargensis* (Vishalakshi *et al*., 2009), Actinomycete strain, PS-18A (Vonothini *et al*., 2008) has been already determined. An alkaline protease producer strain NRC-15 was isolated from Egyptian soil and represents a novel species of the genus *Streptomyces,* hence the name *Strptomyces pseudogrisiolus* NRC-15. The culture conditions for higher protease production by NRC-15 were optimized with respect to carbon and nitrogen sources, metal ions, pH and temperature (Awad *et al*., 2012).The present study aims to screen isolates of *Streptomyces* from alkaline soil for protease production, characterization and optimization. In this paper we report studies on screening of actinomycetes isolates for protease production under different carbon and nitrogen sources and further identification by 16S rDNA sequencing. They are shown to have highly conserved region between them and the related strains from NCBI.

MATERIALS AND METHODS

The goal of the present investigation was to screen and characterize alkaline protease producing *Streptomyces* strains and establishing their phylogeny.

Sample collection

Soil samples were collected from Central Soil Salinity Research Institute (C.S.S.R.I), Lucknow, situated at an elongation of 120m above mean sea level. It extends from 26˚47 feet 45 inch to 26 ˚46 feet 13 inch latitude and 18˚ 46 feet 7 inch to 80˚ 46 feet 32 inches longitude.

Isolation and Screening

10 g of soil samples (pH 9 and 10) was transferred into 90 ml of sterile water in 250 ml Erlenmeyer flask and the soil suspension was

diluted in serial dilution up to 10^{-1} to 10^{-7}. One ml of each dilution was poured onto Petri plates on the alkaline agar medium of the following constituents containing 1% glucose, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, 1% Na_2CO_3 and 1.5% agar, pH 10.5 (Horikoshi 1990). The inoculated plates were incubated at 37^0C for 24 h and the isolates were examined on the medium containing (g/l): Skimmed milk 100, yeast extract 10, Na_2CO_3 15 and agar 20. Skimmed milk, Na_2CO_3 and other constituents were autoclaved separately and mixed (Ibrahim *et al*., 2007). The plates were incubated for two days at 32^0 C. A zone of skim milk hydrolysis around the colonies gave a clear indication of protease producing organisms.

Strain Characterization

Isolated culture with a prominent zone of clearance was identified based on both the morphological and physiological characteristics.

Determination of Protease Activity

Protease activity was assayed by the modified procedure (Tsuchida *et al*., 1986) using 1% Casein in .05 M Sodium phosphate buffer (pH 8) as substrate. Casein solution (1.0 ml) with an equal volume of suitably diluted enzyme solution was incubated at 45^0C for 10 min followed by an addition of 4 ml of Trichloroacetic acid. The mixture was centrifuged and to the supernatant was added 5ml of 0.4M Na_2CO_3 and 1 ml of one fold diluted Folin ciocalteau reagent which was further incubated for 30 min. The absorbance was measured against an appropriate blank at 660 nm. One unit of alkaline protease activity was defined as the amount of the enzyme able to produce 1g tyrosine ml^{-1} min^{-1} and expressed as Unit/ml of enzyme.

Protein Determination

Protein concentration was determined according to the method described by (Lowry *et al*., 1951).

Determination of enzyme production at different pH and Temperature

Effect of pH on alkaline protease production was determined by assaying the enzyme activity at varying pH values ranging from 7 to 11 at 37^0C using 50 mM of suitable buffers and the influence of temperature on protease production was determined by measuring the enzyme activity at different temperatures ranging from 37^0C to 50^0C under the standard assay conditions.

Determination of enzyme production under different carbon and nitrogen sources

The effects of carbon and nitrogen sources on protease production in the isolated species were studied. The liquid broth was supplemented with various sugars viz., Glucose, galactose, maltose, lactose, sucrose and starch at 1% (w/v) as carbon source while glycine, casein, beef extract, yeast extract, peptone and tryptone at 1% (w/v) were used as nitrogen sources. After 72 h of growth at 37^0C under shaking condition the effect of the either sources were investigated for protease production.

Characterization of crude enzyme

Effect of temperature on enzyme activity

The optimum temperature for enzyme activity was determined by assaying activity at various temperatures from 37^0C to 50^0C. The thermo stability of enzyme was measured after preincubation of enzyme in the 50 mM sodium phosphate buffer (pH 8.0) for one h.

Effect of pH on enzyme activity

Effect of pH on the activity of the crude alkaline protease was determined by measuring the enzyme activity at varying pH values ranging from 7 to 11 using 50 mM of sodium phosphate buffer of desired pH. Activity of the enzyme was measured after 1 h of preincubation at 37^0C.

Effect of different Surfactants on enzyme activity

The effect of enzyme activity in the presence of various surfactants such as SDS, Tween 20 and

Triton X-100 was determined after preincubation of the enzyme solution for 30 min at 37^{0}C before the addition of substrate.

Isolation of Genomic DNA

For their molecular characterization, Genomic DNA was isolated by modifying the protocol of Boudjella, (2006). Cultures were prepared by inoculating a single colony from a freshly streaked plate into 20ml GYP (Glucose yeast extract peptone broth) and incubated at 37^{0}C at 200 rpm for 24-72 h. After incubation, the broth was centrifuged and the supernatant discarded and the pellet suspended in 50 mM Tris EDTA and kept at -20^{0}C for 30 min. The cell suspension was incubated with 15 µl (50 mg/ml) lysozyme solution and kept at 37^{0}C for 1 h. After that 10 µl of SDS (10%) and 10 µl of proteinase K (10 mg/ml) was added and incubated at 55^{0}C for 3 h to lyse the cells. To the mixture 150 µl NaCl (5M), equal volume of water saturated phenol and chloroform: isoamyl alcohol (24:1) were added and centrifuged at 10,000 rpm for 10 min. The aqueous layer was transferred into new tube and added 0.1 ml of 3M sodium acetate and 2 ml of 95% alcohol. The tube was kept overnight at -20^{0}C. Centrifuged the precipitated DNA at 10,000 rpm for 15 min and washed with 70% ethanol and DNA pellet was dissolved in 50 µl milli Q water for further use.

Phylogenetic Analysis of 16S rDNA sequence

PCR amplification of 16S rDNA was done by using universal primers pA (5'AGAGTTTGATCCTGGCTAG3') and pH (5'AGGAGGTGATCCAGCCGCA3') (Edward *et al*., 1989). Amplification was carried out in a 100 µl volume containing: 1X PCR Buffer, 2 M dNTPs, 100ng of each primer, 50ng of template DNA, 1unit Taq DNA polymerase and the final volume adjust by mili Q water. Phylogenetic analysis of *Streptomyces* was determined by BLASTn and the result was aligned with those in the Genebank Database (NCBI) by ClustalW. The phylogenetic dendrogram was derived from Mega 4.0 software package (Tamura *et al*., 2007) using neighbour joining (NJ) methods (Saitou and Nei, 1987). Bootstrap analysis (Felsenstein, 1985) was constructed using 1000 resampling of data and sequences were submitted to NCBI Gene bank.

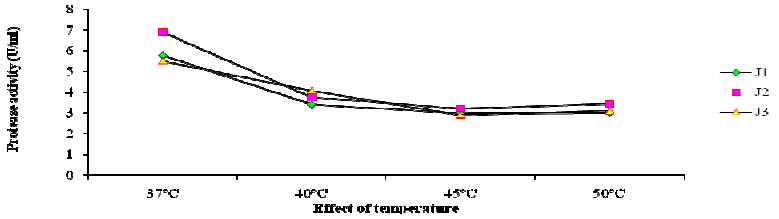
RESULTS AND DISCUSSION

In the present study, out of the 45 alkaline protease producing bacterial isolates, only 3 strains resembling *Streptomyces* were identified following 16S rDNA sequence analysis and were subsequently designated as J1 (*S. macrosporeus),* J2 (*S. bacillaris*) and J3 (*S. rubrolavendulae*). These 3 strains were submitted to NCBI under accession numbers JX041524, JX041525, JX041526, respective to their identification. These isolates showed a translucent zone upon qualitative screening on skimmed milk agar medium. The cultural characterization revealed that the optimum growth was centred at 37^{0}C and pH 8 & 9. Strains J1, J2, J3 were able to grow up to 40^{0}C. Their ability to grow over a wide range of initial pH (7 to 10) makes them ideal candidate for wide range of commercial applications. The J2 strain was able to grow in the presence of 10% (w/v) NaCl indicating that it was moderate halophilic actinomycetes while J1 was able to grow in the presence of 2-7% (w/v) NaCl and J3 was able to grow in presence of 2-5% (w/v) NaCl indicating that they were slightly halophilic actinomycetes. Qualitative analysis for starch hydrolysis & citrate utilization was carried out for all the three strains. Results revealed that the strains J1and J3 could positively hydrolyse starch but strain J2 was negatively reported for the test. On the contrary, the strain J2 revealed citrate utilization which was otherwise absent in the strains J1 & J3. Studies on optimization of alkaline protease production by the test strains revealed that the rate of protease production varies with the physicochemical conditions as well as each carbon and nitrogen sources. Supplementation of the culture medium with carbon and nitrogen sources is known to play an important role in microbial growth and enzyme production (Ningthojam *et al*., 2009).

Effect of pH and Temperature on enzyme production

In order to optimize the protease production under different physicochemical conditions an attempt was made to study the effect of different pH (7-11) and temperature (37^0C-50^0C) on enzyme production following an incubation of 72 h. The results as illustrated in Graph1 indicated that the crude alkaline protease showed reasonable activity within temperature range of 37^0C to 50^0C with maximum activity at 37^0C in all the three strains (J1: 3.86U/ml; J2: 3.91 U/ml; and J3: 4.21 U/ml). The protease production was found to decline with an increase in temperature beyond 37^0C in all the strains. The strain J2 was found potentially stable at all the temperatures as compared to the other two strains (Rana *et al*., 2010).

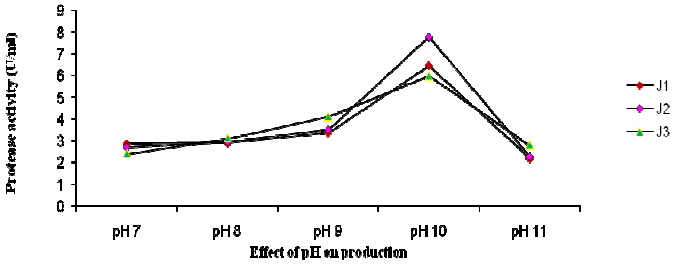
Graph 1



Effect of temperature on protease production by Streptomyces spp. An average of three independent observations. Standard error ±2.70 % p<0.002. The p value refers to the comparison of protease activity at different pH. All the comparisons are statistically significant.

The enzyme activity is greatly influenced by hydrogen ion concentration of the culture media. A pH range from 7-11 were chosen to study the effect on protease production by the three isolates (J1, J2, J3). The maximum enzyme production (Graph 2) as revealed by strain J2 was 3.77 U/ml and J3 was 4.11 U/ml after 72 h of incubation at pH 10 as reported by *Helal et al*. (2012) followed by J1 (3.45U/ml). The maximum alkaline protease production was also reported by Gurielidze *et al*. (2010) in *Streptomyces globisporus* 203A, *Streptomyces* spp. 387H (1.2 U/ml) at pH 10 and some mesophilic actinomycetes showed maximum activity at pH 11 in *Streptomyces glaucus71D* (2.5 U/ml), *Streptomyces levoris 114D* (1.3 U/ml) *and Streptomyces coelicolor* (2.3 U/ml).

Graph 2

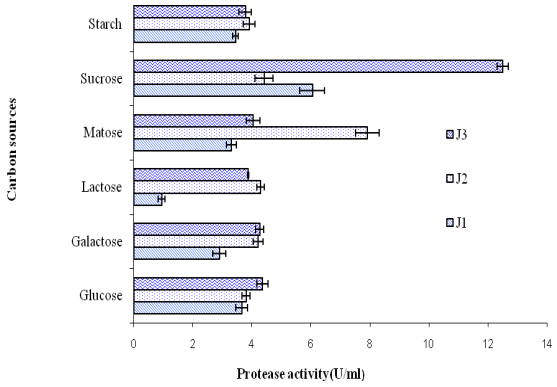


Effect of pH on protease production by Streptomyces spp. An average of three independent observations. Standard error±2.85 % p<0.002. The p value refers to the comparison of protease activity at different pH. All the comparisons are statistically significant.

Effect of different carbon and nitrogen sources on enzyme production

Efficiency of protease production was checked under different carbon and nitrogen sources in which J1 and J3 isolates showed maximum activity (6.05 & 12.50 U/ml, respectively) in sucrose while J2 showed maximum activity (0.95 U/ml) in maltose at 37^0C after 72 h incubation (Graph 3).Jayashree *et al*. (2010) reported maximum protease production in the presence of starch and casein by *Streptomyces pulveraceus* after 72 h of cultivation. A gradual increase in biomass as well as protease production during stationary phase reported in the study also coincides with our results. Sucrose proved to be the most favourable carbon sources for the production of active alkaline protease by *Streptomyces avermectinus* NRRL B-8165 (2.61 U/ml) as reported by *Ahmed et al*. (2008) . On the other hand maltose, glucose, galactose, starch and lactose showed lower level of enzyme production as compared to sucrose as reported earlier by *Chi and Zhao* (2003). Starch and sucrose have also been reported as the best C-source for *Streptomyces gulbargensis* and *Streptomyces clavuligerus* MIT-1, respectively, as reported by Jignasha and Satya (2007).

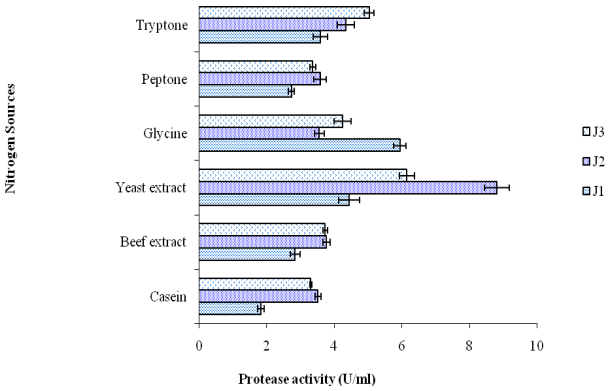
Graph 3



Effect of carbon sources on protease production by the Streptomyces spp. at pH 10 and temperature 37^0C. Average of three independent experiments p0.002.The p value refers to the comparison of protease activity at 1 % glucose with other carbon sources. All the comparisons are statistically significant.

The studies on the effect of different organic nitrogen sources on enzyme production revealed an optimum enzyme yield with yeast extract in the isolates J2 and J3 (8.82 U/ml; 6.16 U/ml, respectively) in Graph 4. Our results are in agreement with Kathiresan and Manivannan, (2007) and Narayana and Vijayalakshi, (2008) who have shown that addition of peptone and yeast extract were found optimum for alkaline protease production from the coastal mangrove *Streptomyces sp.* and *Streptomyces albidoflavus,* respectively. However, maximum protease yield as figured out with strain J1 happened to be Glycine giving 5.95 U/ml activity. Although addition of certain amino compounds was shown to be effective in the production of extracellular enzymes by alkalophilic *Bacillus* sp. (Ikura and Horikoshi, 1987) glycine appeared to have inhibitory effects on both amylase and protease production (Sen and Satyanarayana, 1993). Our results on the contrary revealed almost 1.3 fold greater production of the enzyme as compared to the basal medium (containing yeast extract).

Graph 4



Effect of nitrogen sources on protease production by the Streptomyces sps. at pH 10 and temperature 37^{0}C. Average of three independent experiments. p<0.002.The p value refers to the comparison of protease activity at 1 % casein with other nitrogen sources. All the comparisons are statistically significant.

The overall studies thus indicated that the best carbon source for protease production was obtained using sucrose while the Yeast extract was better as an organic nitrogen source for growth and enzyme production for the test isolates.

Characterization of enzyme activity

Effect of pH

Effect of pH was observed in all strains of *Streptomyces* and optimum activity was found at pH 10 indicating that enzyme was alkaline in nature and has a potential application for detergent formulation as reported by Gupta *et al*., (2002). All strains were stable at pH 10 for optimum growth in culture media. Activity profiles from pH range 7-11 showed a similar pattern in J1 strain (Table: 1). Maximum enzyme activity was observed in J2 (16.89 U/ml) followed by J3 (15.44U/ml) and J1 (14.57U/ml) at pH 10.

Table 1
Effect of pH on enzyme activity (U/ml)

pH	J1	J2	J3
7	14.24	14.27	14.51
8	14.29	14.77	15.44
9	14.57	14.95	13.90
10	14.02	16.89	13.95
11	14.07	15.47	13.50

Effect of Temperature

The crude enzyme activity revealed a temperature dependent catalytic nature of enzyme. A remarkable crude alkaline protease activity was found at 37^{0}C for all isolates of *Streptomyces* but slightly increased activity was found in J2 at 40^{0}C (Table: 2).

Table 2
Effect of Temperature on protease activity (U/ml)

Temperature	J1	J2	J3
37^{0}C	18.42	17.70	17.50
40^{0}C	16.29	20.12	17.20
45^{0}C	15.23	15.50	18.99
50^{0}C	13.18	13.90	13.82

Effect of Surfactants

The protease produced by *Streptomyces* sp. were stable towards both non-ionic and ionic surfactants, such as SDS and Tween 20, and Triton X-100, respectively (Table:3). Strain J2 & J1 revealed 40% and 1% increase in the protease activity, respectively, as compared to control (without surfactants). On the contrary, J3 revealed an 18% inhibition in the enzyme activity as compared to control. Many researchers reported that anionic surfactant SDS inhibited the alkaline protease activity such as *B. pumilus* (Kumar *et al*., 2002) while *B. clausii* retained 75% enzyme activity on treatment with 5% SDS (Joo *et al*., 2003, Uyar *et al*., 2011). The stability of the enzyme in the presence of SDS shows the potential of the isolates to be considered for industrial application particularly in detergent industry. Tween 20 decreased the activities in all strains of *Streptomyces* while Triton X 100 showed considerable residual activity in the strain J1 (67.1%), J2 (81.12%) and J3 (78.43%). Influence of different factors viz temperature, pH, surfactants, inhibitors on protease activity was also achieved by *Bacillus subtilis* DKMNR and defined their stability in detergent formulation (Kezia *et al*., 2011).

Table 3
Effect of surfactants on protease activity

Surfactants (5%)	J1	J2	J3
Control (without surfactants)	18.42 (100%)*	17.70 (100%)*	17.50 (100%)*
SDS	18.73 (101%)	24.95 (140%)	14.67 (83.82%)
Tween 20	12.67 (68.7%)	11.24 (63.50%)	9.67 (55.25%)
Triton X-100	12.37 (67.1%)	14.36 (81.12%)	13.71 (78.43%)

Values in parenthesis indicate percentage activity in the presence of individual surfactants incubated at 37^{0}C and pH 10.

Phylogenetic analysis

The amplified PCR product of representative isolates was identified sequenced. A BLASTn analysis carried out through GenBank (http://www.ncbi.nlm.nih.gov) revealed that all the isolates were members of the Genus *Streptomyces*. The identified sequences of isolated strains J1, J2 and J3 were submitted to NCBI (National Centre for Biotechnology Information) to which accession numbers JX041524, JX041525 and JX041526 were given. The 16S rDNA based phylogenetic analysis of *Streptomyces* sp. (Strains J1, J2 and J3) demonstrated 99.00% sequence similarity with the other *Streptomyces*. The phylogenetic tree was constructed from the sequence data using neighbourhood joining method (Fig 1). The evolutionary relationship between strain J1, J2, J3 and other closely related sp. of *Streptomyces* confirmed a distinct phylogenetic relationship within the genus. It was found that J2 is most close to EU119184, FJ486454, FJ486429 and J3 to GU227350, EU841659, EU570710 but J1 was observed as a separate branch in phylogenetic tree. The isolate J1 identified on the basis of multiple sequence alignment (MSA) as *S. macrosporeus* is reported for alkaline protease production for the first time. There are no reports on the alkaline protease production by the aforesaid isolate in the existing literature. However, the species has been reported for the production of a peptide

antibiotic Janiemycin, known for its bactericidal activity primarily against gram-positive bacteria (Meyers *et al*., 1970).

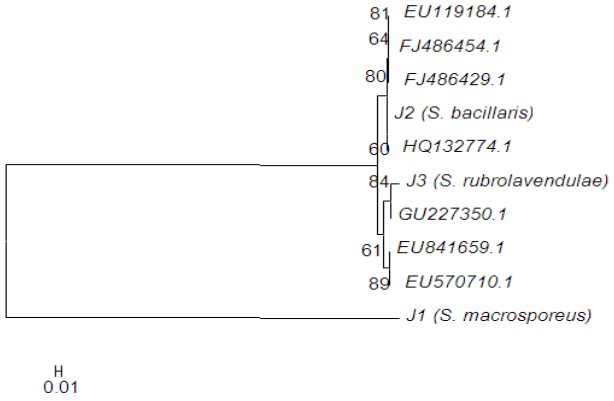


Figure 1

Phylogenetic analysis of Streptomyces sps from northern region of india based on partial nucleotide sequences (1.5 kb and 540 bp) of 16S rDNA. The tree was constructed using the neighbor-joining method. Percentages at nodes represent levels of bootstrap support from 1000 resampled datasets.Bootstrap values less then 50% are not shown.

CONCLUSION

Alkaline protease are mostly produced by *Bacillus* strains and considerably used in industrial applications. Besides *Bacillus* strains other groups also have a great potential for protease production. Therefore, there is always a chance of finding new organism producing novel enzyme with better properties in enzyme technology. Actinomycetes predominantly found in alkaline rich soil can be an alternative source of alkaline proteases. We isolated 3 strains of actinomycetes having potential of producing alkaline protease which were established to resemble *Streptomyces* by 16S rDNA sequence analysis and identified as J1 (*S. macrosporeus),* J2 (*S. bacillaris*), J3 (*S. rubrolavendulae*). *S macrosporeus* known for Janiemycin production has not been reported for alkaline protease production as yet. Moreover, the isolates function at high pH with their stability under wide pH range, temperature and surfactants, elaborates their potential for different industrial applications and further investigation at biochemical and molecular level can further reveal the versatility of the isolates for novel industrial applications.

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