Production and Characterization of α-Amylase from Indigenously Isolated *Streptomyces* sp.

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Streptomyces species have been exploited widely as microbial cell factories, especially for antibiotic production. However, their potential for alpha-amylase production has not been extensively studied. This study reports the isolation, molecular identification, and optimization of the physiological conditions for alpha-amylase production from *Streptomyces* sp., isolated from soil in Kotli Azad Kashmir. The maximum growth of *Streptomyces* MI-1 was observed at a neutral pH and a temperature of 35 °C. An amylase activity of 1.15 IU/mL was observed when 4% starch was added to the nutrient medium. During the submerged state fermentation, the maximum amylase activity of 2.136 IU/mL/min was observed after 144 h of incubation. The characterization of bacterial amylase revealed an optimum temperature of 40 °C, and its optimum pH was 7.0. Furthermore, during the study, examining the effect of different metal ions found that Mg^{2+} and Ca^{2+} ions had a positive effect, while Cu^{2+} and Fe^{2+} ions had an inhibitory effect compared to the control. This preliminary study provides basal line information for the discovery of novel microbes from the unexplored natural resources for amylase production, which will be used for many purposes.

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INTRODUCTION

Amylases are high-value biocatalysts that are used in the starch processing industry for the hydrolysis of starch. Amylases are found in all life domains, but microbial amylases are primarily used in industrial processes because of their easy culturing and economical enzyme production at a massive scale. Other advantages of microbial enzymes include the easy directed manipulation in terms of introducing/enhancing desired characteristics (Sidhu *et al.* 1997; Li *et al.* 2007). Amylases have found their applications in starch saccharification, clinical and analytical chemistry, and the food, beverage, and textile industries (Paneley *et al.* 2007). Because of their prodigious demand and application, interests are increasing in isolating new microbes with amylase production abilities.

Amylase may be generated by a variety of bacteria; however for commercial purposes, amylase is mostly sourced from the genus *Bacillus* (Pandey *et al.* 2000). The well-known thermostable amylase producers, *Bacillus subtilis, Bacillus licheniformis, Bacillus stearothermophilus,* and *Bacillus amyloliquefaciens* have been extensively used in the manufacture of the enzyme for a variety of commercial purposes (Prakash and Jaiwal

2009).

There has been a continued interest among scientists to discover and gain insight into new bacterial strains (actinobacteria) from diverse environments, such as from deserts and soils in temperate region, for producing this high demanding enzyme (Kurapova *et al.* 2012; Ding *et al.* 2013). Multi prospective demands and applications in different sectors pave the way for searching for more useful processes for native amylase production (Hoque *et al.* 2003). There have been a few reports on amylase production by *Streptomyces* species from various environments such as brick-kiln soil (Kar and Ray 2008), mushroom-compost (Singh *et al.* 2011), marine-soil and marine sponge of *Ircinia* sp. (Krishnan and Sampath Kumar 2015), desert (Nithya *et al.* 2017), and fertile soil (Al-Dhabi *et al.* 2020).

Streptomyces is the largest and very important genus from the actinobacteria family and has considerable economic importance. A majority of them are chemotrophic and ubiquitously found in soil and water. Approximately half of the discovered metabolites are produced by this group, *e.g.*, antibiotics, enzymes, enzyme inhibitors, immunosuppressant agents, vitamins, hormones, and many other secondary metabolites (Berdy 2005). However, there are few reports describing the starch hydrolyzing activities from the *Streptomyces* species (Hoque *et al.* 2006). Since there are no reports on isolation and characterization of *Streptomyces* species from the soil of Kotli, Azad Kashmir, Pakistan, the present study has been designed to isolate and screen actinomycetes and determine the amylase activity from *Streptomyces* found in soil.

EXPERIMENTAL

Isolation and Selection of Streptomyces

Streptomyces MI-1 was isolated from soil samples collected from different localities of the Kotli Azad Kashmir district. Casein KNO_3 agar media was used for the isolation of the strain. The isolated strain was monitored for amylase production on a starch agar plate containing starch as the carbon source. The strain was identified on the basis of 16S rDNA sequence analysis as well as its morphological and biochemical properties, *e.g.*, colony morphology, gram staining, acid fast staining, catalase production, and starch hydrolysis. The polymerase chain (PCR) product was sequenced using an automatic sequencer.

Physical Properties

The temperature tolerance and pH requirements of the selected strain were determined by growing the strain at different temperatures and a wide range of pHs.

Enzyme Production

For the production of amylase, a starch (1%) containing medium was used. The medium (100 mL) was inoculated with 1 mL of fresh overnight culture of *Streptomyces* MI-2, incubated for 4 d at a temperature of 37 °C in a shaking incubator at 150 rpm. Bacterial cells were removed *via* centrifuge and the supernatant was used for the amylase activity assay.

Amylase Activity Assay

The amylase activity of the supernatant was assayed by the detection of reducing sugars. The reaction mixture contained 800 µL of 1% soluble starch and 200 µL of enzyme

solution. The enzyme reaction was stopped with a dinitrosalicylic acid (DNS) solution. The absorbance was measured at 540 nm in a spectrophotometer, and the activity was calculated from the standard curve. One unit of amylase activity was defined as the amount of enzyme that released 1 µmol of reduced sugar per minute under assay conditions.

Optimization Conditions for Amylase Activity

In order to elucidate the optimum conditions for the alpha amylase activity, assays were performed at different temperature and pH ranges and different concentrations of substrates under standard conditions.

Effect of Metal Ions on the Amylase Activity

Salts of various metal ions, *e.g.*, Mg^{2+}, Ca^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} (10 mM each) were added to the reaction mixture and then incubated at a temperature of 40 °C for 25 min before performing the activity assay under standard conditions. The enzyme activity was determined as compared to the control without metal ions.

RESULTS AND DISCUSSION

Isolation and Identification of the Starch Hydrolyzing Isolate

In total, 12 isolates were selected based on their appearance on culture plates from the soil sample. All isolates were screened for amylase production. Among all of these, one was found to be the most promising starch degrader. This isolate was designated as MI-1 and was selected for further characterization. After the 3rd day of incubation, white colonies appeared (Fig. 1a) which produced brown pigmentation after the 5th day of incubation (Fig. 1b). Microscopic observation and staining properties of the selected strain (MI-1) revealed that the strain exhibited a filamentous structure (Fig. 1c). The present isolate was found to be gram positive and non-acid fast, which is the most important criteria of the *Streptomyces* species.

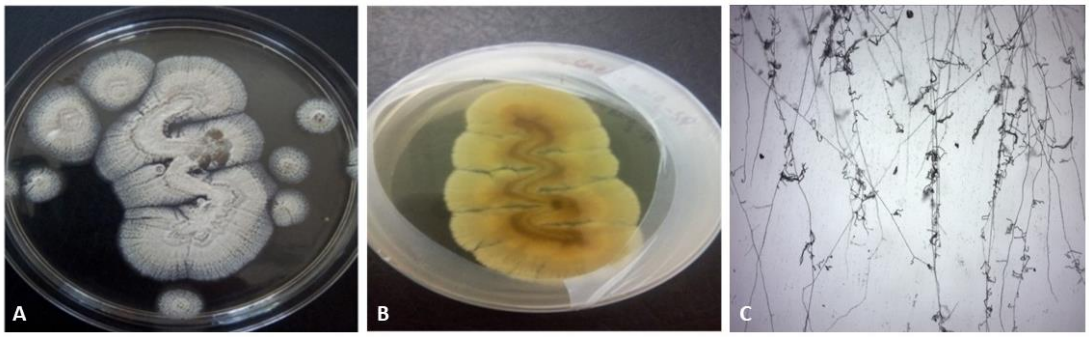

Fig. 1. Colony morphology of the MI-2 isolate on *casein*-KNO_3 agar medium at 28 °C: (A) after 3 d of incubation; (B) after 5 d of incubation; and (C) microscopic view of the MI-2 isolate at 10X magnification

The DNA extracted from *Streptomyces* MI-1 was run on 0.8% agarose gel for confirmation. The 16S rRNA was amplified through PCR using a *Streptomyces* specific forward and reverse primer. The amplified PCR product was run on 0.8% agarose gel, and the amplified product (1.5 Kb) was confirmed 16S rDNA amplification. To identify the strain up to the species level, sequencing of the 16S rRNA was performed. A phylogenetic tree was constructed. The length of the sequence was 1470 bp (as shown in Fig. 2).

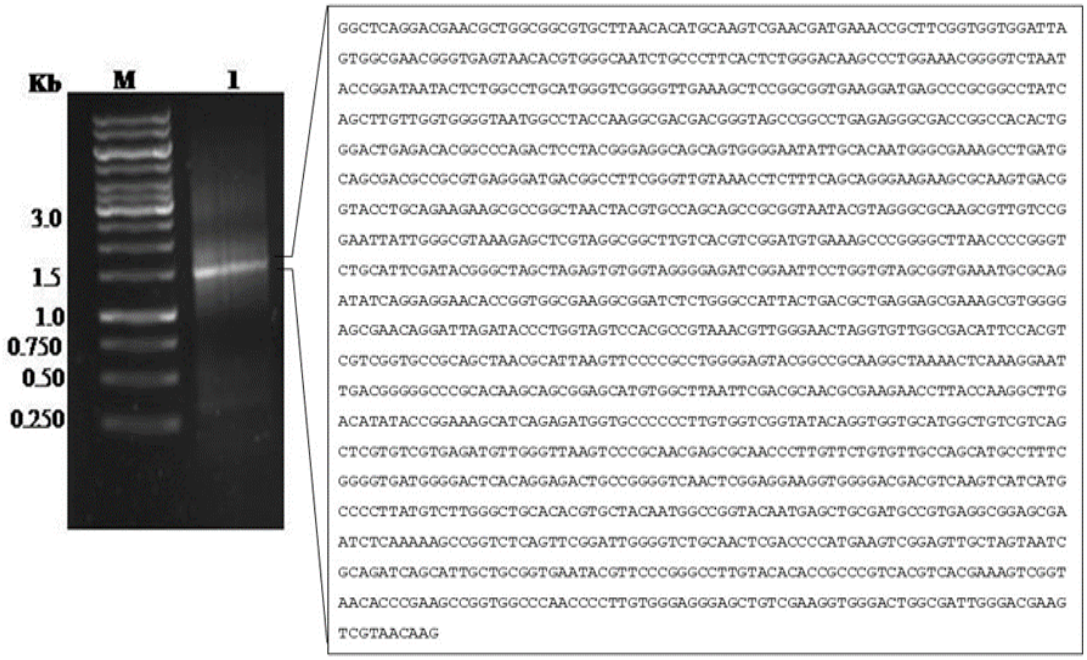

Fig. 2. The PCR amplified 16S rRNA fragment (1.4 Kb) and its nucleotide sequence

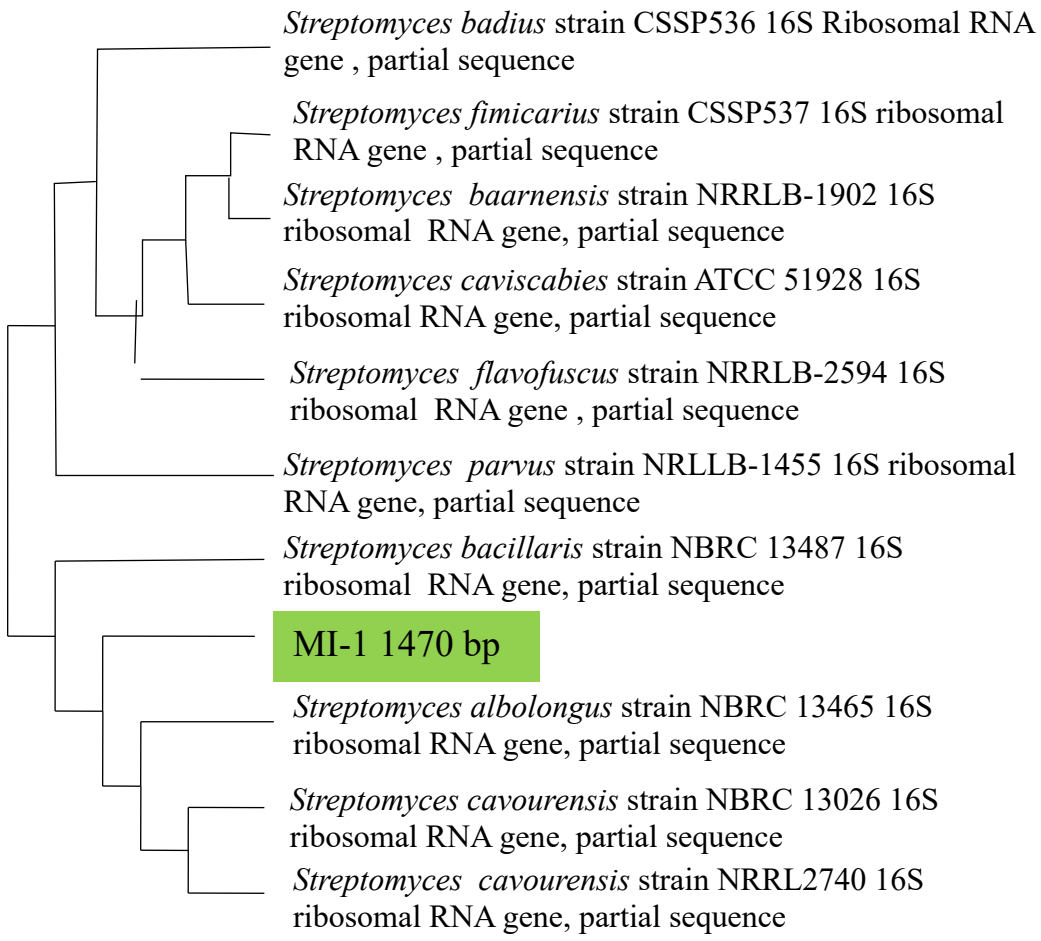

Fig. 3. Phylogenetic tree of the newly isolated *Streptomyces* strain MI-1

A tree based on the 16SrRNA gene sequences showed that the isolate occupied a new phylogenetic position within the sub cluster. Therefore, MI-1 was determined to belong to the *Streptomyces* species (Fig. 3).

Screening of the Amylase Activity

The screening of the isolated bacterial strain for amylase activity was performed on a blue color agar plate. Conversion of the blue color to white indicated the hydrolysis of starch in the media. Among the 12 isolates under study, MI-1 exhibited positive starch degrading activity on starch agar plate with a clearing zone of 10 mm ± 0.24 mm (Fig. 4).

Fig. 4. Starch hydrolysis *via* the MI-1 S*treptomyces* strain on an agar plate

Cultural Characteristics

The amylase activity positive strain MI-1 was studied for certain morphological parameters from its culture plates. The culture of the selected strain MI-1 on a caesin-KNO_3-agar medium and starch-casein-agar medium indicated it as strain of the *Streptomyces* species (Table 1).

Table 1. Cultural Characteristics of the Selected Isolate MI-1 on Different Media

Media Used	Growth	Colony Properties	Aerial Mycelia Color	Substrate Mycelia Color
Casein KNO_3 agar medium	Abundant	Brown, powdery Spreading	White	Brown
Starch casein agar medium	Abundant	Light brown, powdery	White	Light brown

Effect of the pH and Temperature on the Growth

The effect of changing the media pH and temperature on the growth of the bacterial strain was checked by monitoring the OD of culture at 540 nm. The results showed that the MI-1 strain had a growth pattern between 25 and 40 °C, with maximum growth at a temperature of 35 °C (Fig. 5a). It also exhibited good growth under a wide range of pHs, from 3 to 9, with the maximum growth at a pH of 7 (Fig. 5b). A wide growth range of pHs and temperatures make the bacterial strain and its extracellular enzyme suitable for various industrial processes taking place under these conditions.

Characterization for the Hydrolysis of the Carbon Sources

Different carbon sources, *e.g.*, starch, maltose, glucose, and fructose were added to the nutrient medium from 1% to 5% for the growth optimization of *Streptomyces* MI-1. The maximum cell density was recorded when 3% starch (Fig. 5c) and glucose (Fig. 5d) were added to the nutrient media. Maltose and fructose were also used as carbon sources in the nutrient media. The maximum growth of MI-1 was recorded with maltose and fructose concentrations of 4% (Fig. 5e) and 5% (Fig. 5f), respectively. These findings indicated that the isolated strain has the ability to hydrolyze a wide range of carbon sources *via* its hydrolytic enzymes secreted in the media.

Such a bacterial strain could be desirable as a microbial factory for various hydrolytic enzymes, including alpha amylase.

Alpha Amylase Production in the Culture Supernatant

In the culture supernatant, the α-amylase production was measured after every 24 h until 192 h was reached. After 24 h of growth, the α-amylase production was found to be 0.202 IU/mL, which gradually increased, and the maximum production was found to be 2.136 IU/mL after 144 h (Fig. 6).

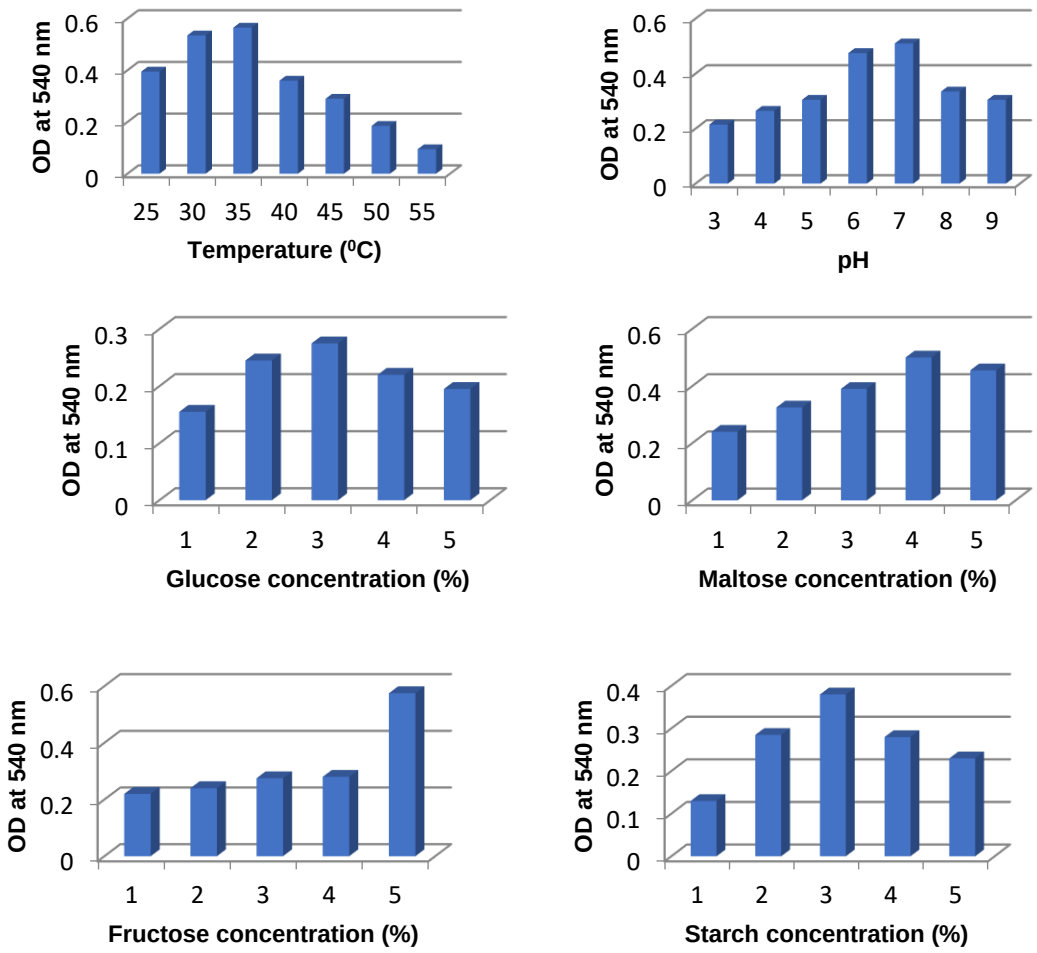

Fig. 5. Effect of the physical and nutritional parameters on the growth of *Streptomyces* MI-1

Enzymatic activity decreased after 144 h (day 6), which was possibly due to the depletion of nutrients and the accumulation of wastes as a result of metabolic activities.

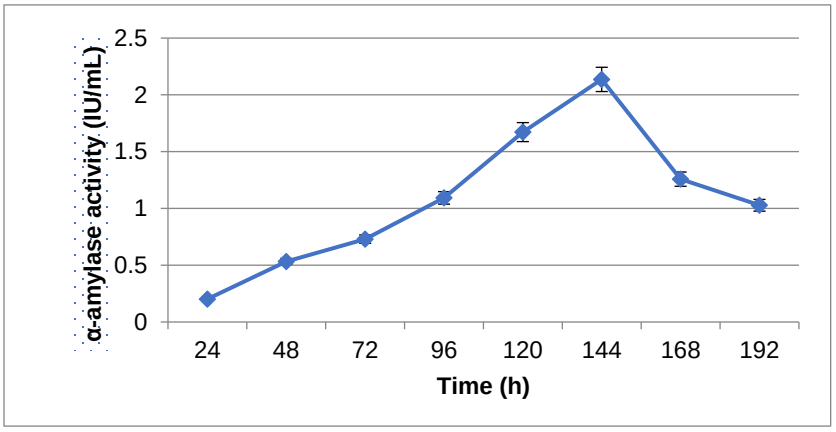

Fig. 6. Effect of the fermentation time on the amylase activity of the culture supernatant

Optimization Conditions for Amylase Activity

To find the effect of different physical parameters, *e.g.*, assay temperature, pH, substrate concentration, and metals ions, on the alpha amylase activity, enzyme assays were performed at different values (as shown in Fig. 7).

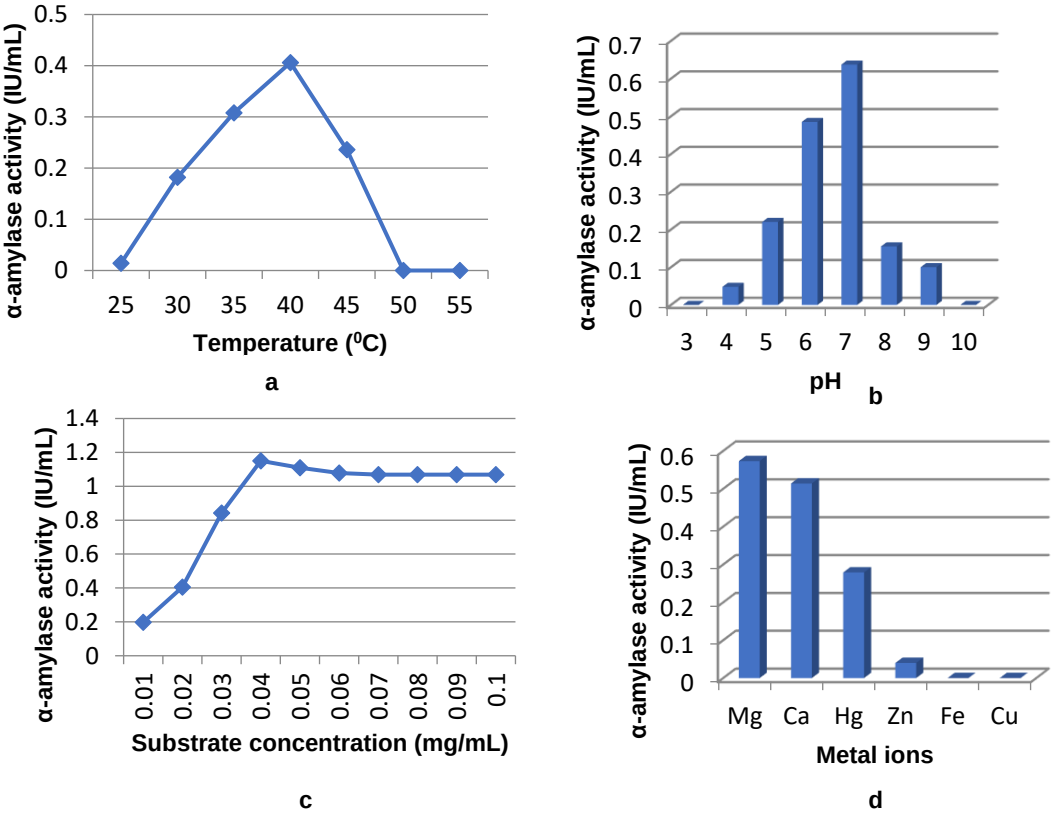

Fig. 7. Study of the effect of different parameters on the amylase activity for determination of the (a) optimum temperature; (b) optimum pH; (c) effect of substrate concentration; and (d) effect of metal ions

The results showed that the enzyme had good activity in the temperature range of 30 °C to 45 °C, with optimum activity at 40 °C (Fig. 7a). Alpha amylase activity reached zero as the temperature rose from 45 to 50 °C. The optimum pH for the production of alpha amylase by *Streptomyces* MI-1 was 7 (Fig. 7b). There was zero activity at a pH of 3 and at pH 10, indicating denaturation of the enzyme under extreme acidic and alkaline conditions.

To study the effect of substrate concentration, the enzyme activity was evaluated with 0.01 to 0.1 mg/mL starch solution as substrate. The results showed that there was gradual increase in activity up to 0.04 mg/mL starch, and the graphed line reached a plateau in the range 0.04 to 0.1 mg/mL, indicating saturation of active sites by substrate molecules (Fig. 7c). There was positive effect of magnesium (Mg), calcium (Ca), and no effect of mercury (Hg) metal ions (10 mM conc. of each). There were inhibitory effects shown by zinc (Zn), copper (Cu), and iron (Fe) metals ions on amylase by MI-1 strain compared to the control, *i.e.* without metal ions (0.29 IU/mL) (Fig. 7d).

Discussion

As an unexplored ecological niche that may produce novel and unique metabolites, *Streptomyces* species are found under different environmental conditions (Ball *et al.* 1989; Sarrouh *et al.* 2012). The *Streptomyces* strain was isolated on selective media by applying physical and chemical treatments to soil samples and suitable culture conditions, as suggested by Wachinger *et al.* (1989) and Hayakawa *et al.* (2004). The *Streptomyces* strains can be easily distinguished from other bacteria due to their unique growth pattern, rough and embedded colonies, and color of the substrate, as well as aerial mycelia and diffused pigments on the media (Hayakawa *et al.* 2004).

The selected isolate was characterized on the basis of the colony morphology on starch casein agar plates and microscopic morphology *via* the cover slip and slide culture technique (Anderson and Wellington 2001). The criteria for the physiological characterization and utilization of different carbon sources and results of other physiological characteristics, *e.g.*, the effect of temperature and the effect of pH on the growth of *Streptomyces*, give further confirmation and identification of the selected isolate MI-2 up to the genus level (Shirling and Gottlib 1996; Vijayakumar *et al.* 2007). After 7 d of incubation, the selected isolate MI-1 produced a brownish colour on the reverse side of the starch casein agar media at a temperature of 28 °C. Similar results have been revealed by Vijayakumar *et al*. (2007). The selected colony of the MI-1 strain exhibited a leathery and chalky texture, a branching and filamentous substrate, as well as aerial mycelia (Lechevalier 1989). As such, the aerial spore colour and reverse side of the *Streptomyces*, which are grown on culture media, could vary depending on the nutrients that are provided to the medium.

Microscopic studies and staining properties of the selected isolate MI-1 showed that the species was Gram positive and non-acid fast, which is an important criterion of the *Streptomyces* species (Oskay *et al.* 2004). Different biochemical characteristics of *Streptomyces* bacteria are used for identification (Islam *et al.* 2014). In the present study, some biochemical tests were performed. Positive results were observed for catalase, casein, and starch hydrolysis in the case of the *Streptomyces* MI-1 in the present study. Apart from a few, most of the isolates of actinobacteria are efficient in casein and starch hydrolyzing, and all the isolates were found to be positive in the catalase test. Comparatively, Stack *et al*. (1969) characterized many strains of actinobacteria and none of them were found to be positive for the catalase test. Based on the present and previous studies, it was concluded that the biochemical characteristics of actinobacteria varied and depend on the nutrients

that are supplemented into the biochemical media and biochemistry of the microbes. Therefore, this aspect can be potentially used as taxonomic criteria for identification at a genus level.

The present investigation revealed that *Streptomyces* MI-1 showed its optimum growth at a temperature of 35 °C. Moderate growth of the isolate was recorded from a temperature of 25 to 40 °C, which means that the isolates are mesophilic. Similarly selected *Streptomyces* species grew well at a pH of 6 to 7 and behaved neutrophilic in the culture. There are different reports categories for amylases. Not all of them are thermotolerant; rather, some are very sensitive, and some bacteria produce thermotolerant amylase which could tolerate higher range of temperature. The one that was isolated in this work did not belong within this class and would potentially fall into a different category, which is thermosensitive. It became denatured at higher temperatures, as can be seen in Fig 7, where the optimum temperature appears to be 40 °C. In the case of pH, the best performance was at 7. Similar findings have been reported by Islam *et al.* (2014). Growth of *Streptomyces* MI-2 occurred in all the carbon sources tested in the present study. These findings enhanced the applicability of the isolated strain in various industrial processes. According to the morphological, cultural, physiological, biochemical, and molecular level characterization, the present *Streptomyces* species, MI-1 is closely related to the better-known species *Streptomyces bacillaris.*

The plate agar results of *Streptomyces* MI-1 showed that the strain MI-1 could produce amylolytic enzymes as a transparent zone formed around the bacterial colony (as shown in Fig. 4). This simple method is useful in the preliminary screening of amylase producing bacteria and agreed with previous reports. The maximum amylase activity of *Streptomyces* MI-1 after the 6^{th} day (144 h) of growth was 2.136 U/mL, which was higher than the amylase activity of *Streptomyces griseoflavus P4* on the 7^{th} day (168 h) of growth, which was 1.66 IU/mL, as reported by Julaluck and Hataichanoke (2012) and Kim and Goodfellow (2002). Furthermore, Mahon *et al*. (1997) and Julaluck and Hataichanoke (2012) stated that the maximum amylase production occurred after the 3^{rd} day (72 h) of incubation, while Wang *et al*. (2015) similarly reported the highest α-amylase yield occurred after 48 h of incubation. The differences in the production time were attributable to strain variation or a difference in the growth parameters of that strain.

The *Streptomyces* MI-1 exhibited maximum amylase activity at a temperature of 40 °C temperature. Similar findings have been reported by Julaluck and Hataichanoke (2012) for *Streptomyces griseoflavus* P4, which showed a maximum amylase activity at a temperature of 40 °C. In the same way, the α-amylase activity of *Streptomyces PDS1* reached its maximum at a temperature of 40 °C (Ragunathan and Padhmadas 2013). Many researchers reported the maximum alpha amylase activity in *Streptomyces* at a temperature range of 40 °C to 60 °C (Fogarty 1983; Ammar *et al.* 2002; Deutch 2002; Hogue *et al.* 2006). In the present study, among the substrate concentrations tested, the highest amylase activity was observed at a 4% starch concentration. Beyond 4% starch, the amylase activity did not increase and a further increase in the substrate concentration does not increase the enzyme activity. These results were also in agreement with the previous reports of Oyeleke and Oduwole (2009), who determined the highest amylase activity at 4% starch concentration for *Bacillus* species.

In the present study, the effects of different metal ions on the enzyme activity were evaluated. The maximum amylase activity in the case of *Streptomyces* MI-1 was recorded in the presence of Mg^{2+} followed by Ca^{2+}. These results were in confirmation with the results of Lonsane and Ramesh (1990), who reported the maximum amylase activity with

Mg^{2+}, followed by Ca^{2+}. In the present finding, the lowest alpha amylase activity was recorded with Hg^{2+} and Zn^{2+}, while Cu^{2+} and Fe^{2+} showed an inhibitory effect. These results agreed with the report by Gupta *et al.* (2008), who reported enzyme inactivation with Cu^{2+}. However, they were contrary to the results of Mayzaud (1980), who reported enzyme inactivation with Mg^{2+} and enhanced activity with Cu^{2+}. The inhibitory effect of some metal may be related to some pH changes, which are associated with their use in the reaction mixture. In the present study, the effect of chelating agent EDTA on the amylase activity was also evaluated. The chelating agent EDTA inhibits the α-amylase activity of *Streptomyces* MI-1. These results were corroborated with the results of Chakraborty *et al.* (2009). Thus, the present study has unveiled a locally isolated strain that is capable of producing enzyme and that could potentially find many uses in industrial applications. In principle, protein engineering could open new vistas, including the discovery of interesting domains in the enzyme from the soil-borne bacteria.

CONCLUSIONS

1. The current investigation showed promise for exploitation of soil for the discovery of new species that could potentially be major source for new enzymes to be used in industry.
2. Under optimized conditions, *Streptomyces* MI-1 yielded a considerable amount of catalytically active α-amylase and that could provide option of a relatively cheaper indigenous source for amylase production.
3. For the first time, the present work here reports on the alpha amylase production of a *Streptomyces* strain from the lands of Kotli Azad Kashmir.
4. Moreover, this study also revealed the values as well as the microbial wealth of amylase producing bacteria which can be helpful for the development of biotechnological processes, especially at industrial level.

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