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Research Article

SCREENING AND OPTIMIZATION OF THERMO-TOLERANT *Bacillus sp.* FOR AMYLASE PRODUCTION AND ANTIFUNGAL ACTIVITY

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ABSTRACT

Amylases are starch degrading enzymes which are produced by plants, animals and microorganisms. Amylases produced by microorganisms have a wide range of industrial applications such as in pharmaceutical, food, textile and paper industries. However, there are still limitations in the isolation of amylase producing microorganisms. The objective of this study was to isolate the potent amylase producing *Bacillus* sp. from soil samples and evaluate their abilities for inhibiting the aflatoxin producing *Aspergillus flavus*. In this study, 30 soil samples were used. For the screening and identification of *Bacillus* strain, morphological and biochemical tests were performed. Iodine assay was done to screen the potent amylase producers. Two parameters (pH and temperature) were used to optimize the cultural conditions for the production of amylase. To determine the total reducing sugar, dinitrosalicylic acid (DNS) assay was used. Altogether 29 colonies were selected and identified as *Bacillus* spp out of which 16 were selected to determine enzyme activity by cup plate method. Four isolates (DK9, DK10, IM4 and KD7) showing highest amylolytic activities (16 mm, 12 mm, 14 mm and 14 mm zone of hydrolysis) were subjected for further study. Isolate KD7 showed the highest amylolytic activity (0.19 U/mL) compared to other isolates. Maximum amylase production was found at pH 6 and temperature 50° C (0.19 U/mL). Among these 4 isolates, DK9 and KD9 showed strong antagonistic activity against *Aspergillus flavus* while DK10 and IM4 showed moderate antifungal activities. Thus, the bacterial isolate KD7 was identified as the most potent strain for maximum amylase production.

Keywords: Soil, Thermotolerant *Bacillus* sp., Amylase producer, Aflatoxin producing *A. flavus*

INTRODUCTION

Geographical and climatic diversity in Nepal has unquestionably gifted to multiple biological diversities including thermotolerant soil microorganisms. Such microbial array is always a motivation for the scientific community to investigate for the remarkable microbial potency and of all; soil microbes showing amylolytic and antifungal activity are emphasized for their application in industrial and commericial purposes (Pokhrel *et al*. 2013). Amylases which degrade starch and related polymers to yield products characteristics of individual amylolytic enzymes, are the most significant industrial enzymes. Its great implication in biotechnology, constitutes a class of industrial enzymes having approximately 25-30 % of the world enzyme market (Azad *et al.* 2009, Aiyer 2005). Amylases can be obtained from several sources such as plant, animal and microbes amongst which microbial sources are the most preferred (Kathiresan & Manivannan 2006). In industrial scale, exploitation of microorganisms for enzyme production is increasing and amylase productions and applications have almost entirely replaced chemical method of hydrolyzing starch in starch processing industry (Pandey *et al.* 2000). The major advantage of using microorganisms for production of amylases is economical bulk production and easy manipulation to obtain enzymes of desired characteristics (Aiyer 2005).

A large diversity of extracellular enzymes such as amylases, proteases, and lipases, with industrial importance are produced by *Bacillus* species (Cordeiro *et al.* 2002). Different *Bacillus* species produce different categories of amylases which vary in their feature (saccharifying or liquefying) and range of pH and temperature they thrive (Fossi *et al.* 2014). Thermo-tolerant *Bacillus* species are widely used for the production of amylases in different part of this globe and researchers exploited this group of bacteria for industrial amylase production (Afrisham *et al.* 2016). Thermo-tolerant microorganisms are those that are capable of growing in high temperature but can exhibit optimal growth under moderate conditions. Optimum condition for growth of microorganisms is influenced by pH, temperature and other factors. At optimum pH and temperature microorganisms grow rapidly and produce large amount of enzymes.

Soil is one of the rich sources of starch degrading bacteria. Amylase stable to higher temperature are known to have commercial application in textile desizing, fermentation,

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food and paper industry (Lin *et al.* 1997). The capacity of *Bacillus* strains to produce large quantities of enzymes has placed them among the most important industrial enzyme producer. They produce about 60 % of commercially available enzymes (Burhan *et al.* 2003). Bacterial enzymes are found in acidophilic, alkalophilic and acidothermophilic bacteria (Boyer & Ingle 1972). Nowadays, amylase from bacterial sources is widely used in amylase production under extreme conditions of pH and temperature. There are various reports on starch degrading bacteria from different sources and respective amylase activity (Aiba *et al.* 1983).

Apart from the highly useful amylase *Bacillus* spp. are known for their antagonistic activity against potential harmful fungi and their mycotoxins which are secondary metabolites obtained from biosynthetic routes of certain fungi and are a group of toxic substances that are carcinogenic, neurotoxic, teratogenic and immunotoxic in nature (Zain 2011, FAO 2001, Petzinger & Ziegler 2000). Several promising characteristics including low nutritional requirements, resistance to adverse environmental conditions, low or none toxicity to environment and the production of a large number of antifungal compounds are exhibited by *Bacillus* sp. isolated from soil (Ongena & Jacques 2007, Pérez-García *et al.* 2011).

Various enzymes such as chitinases, glucanases and proteases are synthesized by *Bacillus* spp. which showed antagonistic activity against fungi and detoxify aflatoxins, the most toxic and potent hepatocarcinogenic mycotoxins produced by *A. flavus*; consisting several toxic compounds like sterigmatocystin, cyclopiazonic acid, kojic acid, nitropropionic acid, aspertoxin, aflatrem, gliotoxin, and aspergillic acid (Hedayati *et al.* 2007). Some research reported that Aflatoxin B1 could be degraded by *Bacillus* sp. isolated from fermented food (Petchkongkaew *et al.* 2008).

One hundred twenty confirmed deaths with 39.0 % mortality rates were reported from Kenya due to aflatoxins (CDC, 2004). Researchers in Nepal have shown a presence of aflatoxins in foods and feeds (Kumar & Yadav 2005, Desjardins *et al*. 2000). Furthermore, several neonatal exposures to aflatoxins have reported in the country. Therefore, finding an efficient and economical solution to deal with deleterious *A. flavus* has been a challenge to all the scientific communities of the world. Few researches regarding the *Bacillus* spp. producing amylase and exerting antifungal property have been done elsewhere in the country. In the light of this background, this study was aimed to isolate, screen and optimize thermo-tolerant *Bacillus* sp. showing high amylolytic and antifungal activity.

MATERIALS AND METHODS

Collection of soil samples

Thirty soil samples were collected aseptically during day time from different parts of Nepal including Janakpur, Khusibun, Illam, Sandakpur, Dhankuta and Begnas taal. About 100 gram of each soil samples was taken in polythene zip-lock bags with spatula and transported to the laboratory.

Screening of amylolytic bacteria

One gram of each soil samples was transferred to 50 mL sterile GYP broth in a conical flask for enrichment and incubated at 50° C for 3 h. After enrichment, 1 mL solution was transferred into tube containing 9 ml of sterile water, labeled and was mixed thoroughly. Serial dilution was performed up to 10^{-6} dilution. After each dilution, the tube was shaken well to make a homogenate sample. From the dilution 10^{-2}, 10^{-4} and 10^{-6} prepared earlier 0.1 ml of dilution was surface plated on pre-poured starch yeast extract peptone (SYP) agar (0.5 % soluble starch, 0.5 % yeast extract, 0.3 % peptone, 0.5 % NaCl, 1.5 % agar). Spread plate technique was performed and plates were incubated at 50° C for 24 h. The isolated colonies showing clear zone in the media were sub-cultured in SYP media in duplicate plate and incubated at 50° C for 24 h. In one set of plates, iodine solution was flooded and zone of hydrolysis was observed.

Identification of isolated microorganism

The strain showing the maximum zone of clearance was then subjected to morphological (Gram and endospore staining) and biochemical test. The isolates were identified by performing catalase test, citrate test and Voges Proskaur test.

Bacterial culture and enzyme fermentation

Starter culture was prepared by adding a loopful of the strain into 5 mL of sterile SYP broth. It was mixed properly and incubated at 50° C for 24 h. From this starter culture, 1 mL of suspension was aseptically transferred to 100 mL Erlenmeyer flask containing one gram of soluble starch in 100 mL Yeast Peptone broth. The flasks were then incubated at 50° C for 5 days.

Determination of crude enzyme activity by augur cup plate method

The activity of the extracellular enzyme was analyzed at an interval of every 24 hours up to 5 days of fermentation. About 1 mL of broth was pipetted into a sterile Eppendorf tube and refrigerated. After 5 days of fermentation, analysis of crude enzyme was done by augur cup plate method.

About 20 mL of sterile SYP media was poured on sterile petriplates. The media were allowed to cool. Four holes were made on each plate with sterile cork borer of 6 mm diameter. Then 50 µL of the crude enzyme was added in each hole and the plates were incubated at 50° C for 24 h.

After incubation, iodine solution was poured on each plate. The zone of hydrolysis was observed and the diameter of each hydrolysis zone was noted for up to 5 days of fermentation. The strains showing highest zone of hydrolysis were selected for the optimization of the condition for enzyme production.

Optimization of culture conditions for amylase production

Bacillus isolates showing highest zone of hydrolysis were selected and the optimum growth characteristics of the isolate for the production of amylase were studied. The effect of temperature and pH on amylase production was studied.

Effect of pH and temperature on amylase production

The effect of pH on the enzyme activity was determined by preparing the media of different pH (5, 5.5 and 6) in the Erlenmeyer flask. The pH of media was maintained by using 0.1 N HCl. The loopful of freshly culture (50º C, 24 h) organism was inoculated in the SYP broth. The flask was incubated at two different temperatures (35° C and 50° C). About 2.5 ml of the suspension was pipetted out at every interval of 2 h and the process was repeated up to 6 h. The tube was centrifuged at 3000 rpm for 15 minutes. The supernatant was transferred to new sterile tube. Acetone was then added till the volume becomes double. It was kept at 4° C in the refrigerator overnight. The content was centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded and the enzyme pellet was dissolved in small amount of phosphate buffer (pH 7). Thus obtained solution was taken as partially purified.

DNS assay

One mL of partially purified enzyme was added with 1 mL of 1 % starch solution. The tubes were incubated at 50° C for 15 minutes then 1 mL of DNS reagent was added to stop the reaction. The tubes were kept in boiling water bath for 15-20 minutes or until red brown color developed. The solution was cooled and 10 mL distilled water was added. The blank solution was prepared by taking mixture of 2 mL distilled water and 1 mL DNS reagent. Ninety six well plates reader was used for DNS assay. The blank solution was used to calibrate the spectrophotometer. The absorbance of the mixture was observed at 540 nm. The absorbance was used to determine the reducing sugar concentration and enzyme activity (Miller 1959, Bernfeld 1955).

Isolation and screening of *Aspergillus flavus*

Five different corn, maize and peanut samples were collected from the market randomly for the isolation of *Aspergillus flavus* using Potato dextrose agar. Phenotypic characteristics of isolates were confirmed by colony morphology. *Aspergillus flavus* differentiating medium (ADM) was used to screen toxigenic *A. flavus* (orange color at the bases) (Bothast & Fennell 1974).

Test for antifungal activities

An antifungal activity of the four isolates was performed by dual streaking method. Briefly, isolates DK9, DK10, IM4 and KD7 were inoculated perpendicularly on potato dextrose agar and a loopful inoculum of *A. flavus* was point inoculated at both sides of line 3 cm apart from *Bacillus* isolate. The Petri dishes were incubated at 30° C for 8 days, and then the areas of inhibition were measured. The antagonistic activity was evaluated according to the scale proposed by (Bacon & Hinton 2002): − for no antagonism; + for weak antagonism (clear zones of inhibition < 3 mm); ++ for moderate antagonism (clear zones of inhibition ≥ 3–9 mm) and +++ for strong antagonism (clear zones of inhibition > 9 mm).

RESULTS

Isolation, screening and identification of isolates

This study was carried out to isolate potent amylase producing *Bacillus* spp. from different soil samples. From 30 different soil samples, 29 isolates were obtained. On SYP agar, the characteristics of many isolates were moist, flat, irregular and slightly convex colonies. Most of the isolates were motile and catalase positive. All these isolates were amylase producers, Gram positive rods and spore formers (Table 1).

Analysis of crude enzyme

Figure 1 shows the amylase activity of 16 different isolates at different time interval. For isolates KA5, KD8 and SD11, the zone of hydrolysis obtained was found to be 6 mm, 10 mm and 8 mm after 24 h, respectively. Similarly, the maximum amylase activity for KA1, SD12 and NC6 was 8 mm, 10 mm and 10 mm after 48 h, respectively. Isolates IM3 and DK13 showed the maximum amylase activity after 72 h. After 96 h, isolates KD6, IM4, DK3 and DK10 showed 10 mm, 14 mm, 12 mm and 12 mm zone of hydrolysis, respectively. Isolates KA2, KD7 and DK 9 showed the maximum amylase activity of 10 mm, 14 mm and 16 mm, respectively, after 120 h of fermentation.

Physiological and biochemical features of selected isolates

From 16 different isolates, 4 isolates (DK9, DK10, IM4 and KD7) were selected that showed the highest amylolytic activity during augur cup plate method (Figs 1 and 2) and these isolates were subjected to physiological and biochemical tests (Table 2).

Effect of pH and temperature on amylase production

Total sugar concentration was estimated using standard calibration curve of glucose. As shown in Fig. 3(a), the

reducing sugar concentration for isolates DK10, DK9, IM4 and KD7 were 93, 121, 107 and 121 mg/L, respectively, after 6 h at pH 5 and 35° C. Figure 3(b) shows the reducing sugar concentration for isolates DK10, DK9, IM4 and KD7 as 53, 182, 159 and 295 mg/L, respectively, after 6 h at pH 5 and 50° C. Similarly, Fig. 3(c) illustrates the reducing sugar concentration for isolates DK10, DK9, IM4 and KD7 as 136, 104, 132 and 127 mg/L, respectively, after 6 h at pH 5.5 and 35° C. Figure 3(d) describes the reducing sugar concentration for isolates DK10, DK9, IM4 and KD7 as 176, 213, 187 and 211 mg/L, respectively, after 6 h at pH 5.5 and 50° C. Figure 3(e) illustrates the reducing sugar concentration for isolates DK10, DK9, IM4 and KD7 as 226, 213, 172 and 228 mg/L, respectively, after 6 h at pH 6 and 35° C. Figure 4(f) shows the reducing sugar concentration for isolates DK10, DK9, IM4 and KD7 as 312, 467, 323 and 523 mg/L, respectively, after 6 h at pH 6 and 50° C.

Table 1. Morphological characteristics of isolates

Soil sample location	Isolate	Gram staining	Endospore staining	Catalase test	Tentatively identified genus
Khushibu	KA1	Gram Positive	Central	Positive	*Bacillus*
	KA2	Gram Positive	Central	Positive	*Bacillus*
	KA5	Gram Positive	Sub-terminal	Positive	*Bacillus*
	KD6	Gram Positive	Central	Positive	*Bacillus*
	KD7	Gram Positive	Sub-terminal	Positive	*Bacillus*
	KD8	Gram Positive	Central	Positive	*Bacillus*
Illam	IM3	Gram Positive	Central	Positive	*Bacillus*
	IM4	Gram Positive	Sub-terminal	Positive	*Bacillus*
	IM8	Gram Positive	Central	Positive	*Bacillus*
Sandakpur	SD9	Gram Positive	Sub-terminal	Positive	*Bacillus*
	SD11	Gram Positive	Sub-terminal	Positive	*Bacillus*
	SD12	Gram Positive	Central	Positive	*Bacillus*
Dhankuta	DK3	Gram Positive	Central	Positive	*Bacillus*
	DK9	Gram Positive	Central	Positive	*Bacillus*
	DK10	Gram Positive	Sub-terminal	Positive	*Bacillus*
	DK13	Gram Positive	Sub-terminal	Positive	*Bacillus*
Nuwakot	NC4	Gram Positive	Sub-terminal	Positive	*Bacillus*
	NC6	Gram Positive	Central	Positive	*Bacillus*
Janakpur	J1	Gram Positive	Central	Positive	*Bacillus*
	J2	Gram Positive	Central	Positive	*Bacillus*
	J3	Gram Positive	Sub-terminal	Positive	*Bacillus*
	J4	Gram Positive	Central	Positive	*Bacillus*
	J5	Gram Positive	Central	Positive	*Bacillus*
	J7	Gram Positive	Sub-terminal	Positive	*Bacillus*
	J8	Gram Positive	Sub-terminal	Positive	*Bacillus*
	J11	Gram Positive	Sub-terminal	Positive	*Bacillus*
	J12	Gram Positive	Central	Positive	*Bacillus*
	J14	Gram Positive	Central	Positive	*Bacillus*
	J16	Gram Positive	Sub-terminal	Positive	*Bacillus*

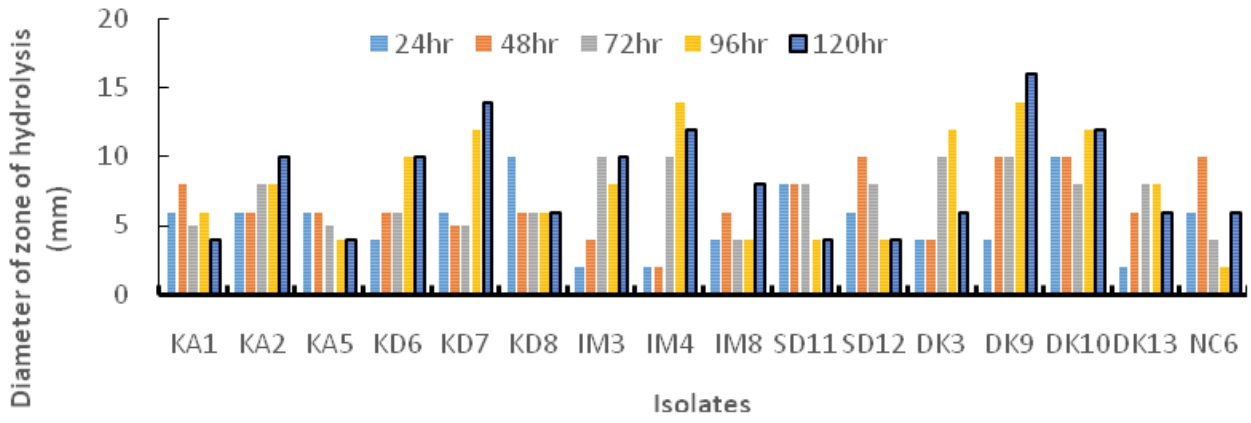

Fig. 1. Diagram showing zone of hydrolysis by the isolates

Table 2. Physiological and biochemical test of isolates

Strain code	Gram reaction	Endospore	Catalase	Voges Proskauer	Citrate utilization	Growth at 60° C	Probable identified
DK9	+	+	+	+	−	−	*Bacillus* spp.
DK10	+	+	+	+	+	+	*Bacillus* spp.
IM4	+	+	+	+	+	+	*Bacillus* spp.
KD7	+	+	+	+	+	+	*Bacillus* spp.

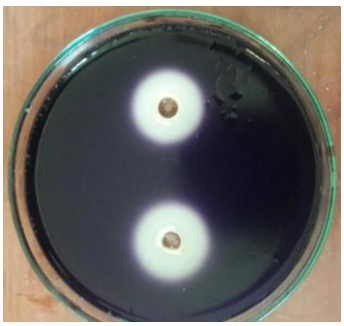

Fig. 2. Amylolytic activity of an isolate KD7 (volume 50 µL, incubation time and temperature 24 h and 50° C)

Determination of amylase activity

Table 3 shows enzyme activity of different isolates at 35° C and different incubation time (2 h, 4 h and 6 h). The amylase activity of isolates DK10, DK9, IM4 and KD7 was maximum (0.08, 0.07, 0.06 and 0.08 U/mL) at 6 h of fermentation and at pH 6. Similarly, Table 4 shows enzyme activity of different isolates at 50° C and different incubation time (2 h, 4 h and 6 h). The amylase activity of isolates DK10, DK9, IM4 and KD7 was maximum (0.11, 0.17, 0.12 and 0.19 U/mL) at 6 h of fermentation and at pH 6. DK 9 and KD7 exhibited high antagonistic activity while both INP4 and DK10 showed moderate antagonistic activity against *A. flavus* as shown in Figs 4(a) and 4(b).

Table 3. Enzyme activity at 35° C

pH	Isolates											
	DK10			DK9			IM4			KD7		
	Enzyme activity (U/mL) after different time intervals											
	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h
5.0	0.03	0.02	0.03	0.03	0.03	0.04	0.02	0.03	0.04	0.04	0.04	0.04
5.5	0.06	0.05	0.05	0.04	0.06	0.04	0.03	0.04	0.05	0.06	0.04	0.04
6.0	0.07	0.06	0.08	0.06	0.03	0.07	0.05	0.05	0.06	0.04	0.05	0.08

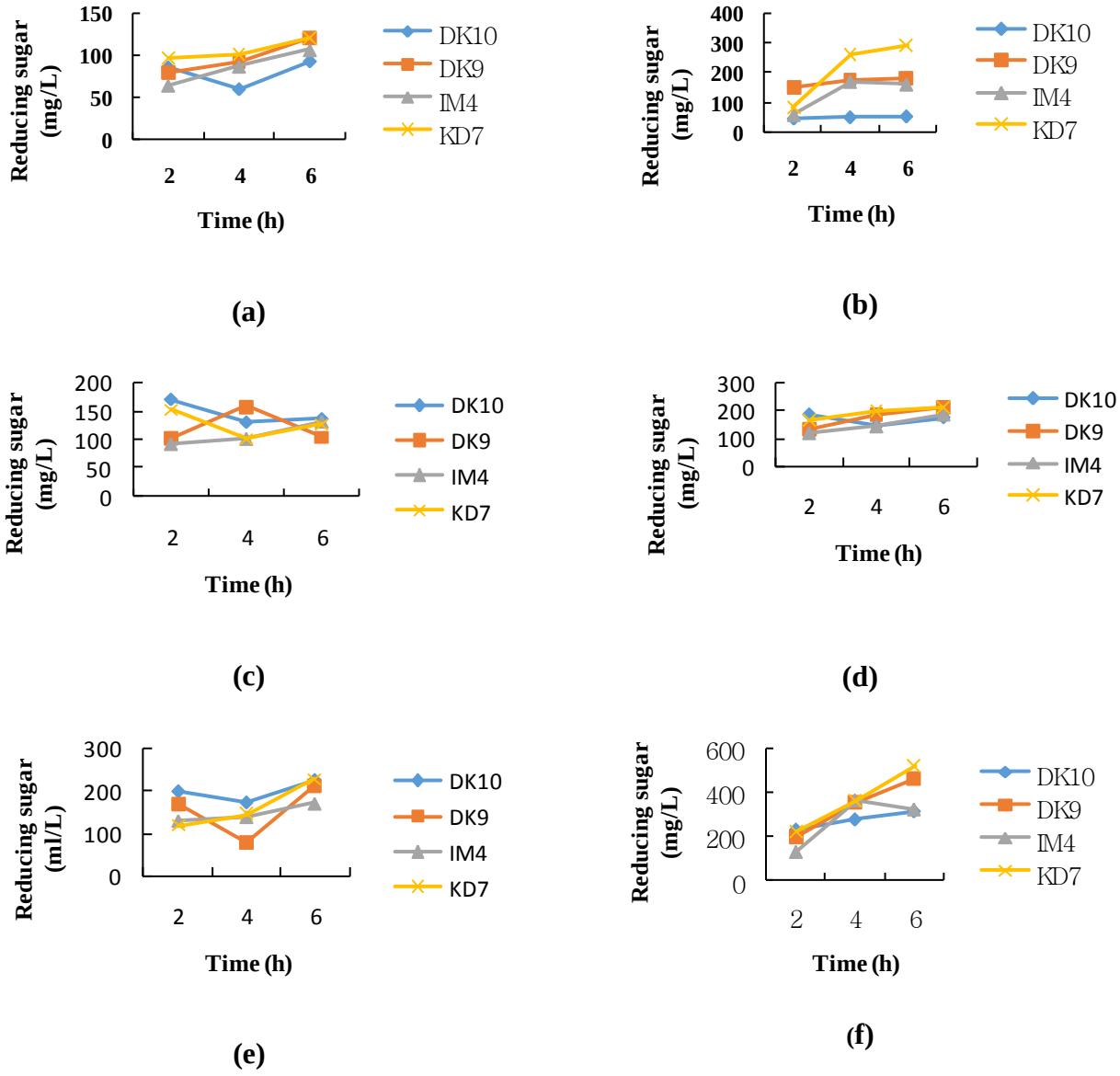

Fig. 3. Reducing sugar concentration at (A) pH 5.0 and 35° C, (B) pH 5.0 and 50° C, (C) pH 5.5 and 35° C, (D) pH 5.5 and 50° C, (E) pH 6.0 and 35° C and (F) pH 6.0 and 50° C

Table 4. Enzyme activity at 50° C

pH	Isolates											
	DK10			DK9			IM4			KD7		
	Enzyme activity (U/mL) after different time intervals											
	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h	2 h	4 h	6 h
5.0	0.02	0.02	0.02	0.05	0.06	0.07	0.02	0.06	0.06	0.03	0.09	0.10
5.5	0.07	0.05	0.05	0.06	0.07	0.08	0.04	0.05	0.07	0.06	0.07	0.08
6.0	0.08	0.10	0.11	0.07	0.13	0.17	0.05	0.09	0.12	0.08	0.13	0.19

Table 5. Primary screening of *Bacillus* sp. for the growth inhibition of *Aspergillus flavus*

Bacterial isolates	Antagonism (After 8 days incubation)	Inference
DK9	++++	Strong antagonism
DK10	++	Moderate antagonism
IMP4	++	Moderate antagonism
KD7	++++	Strong antagonism

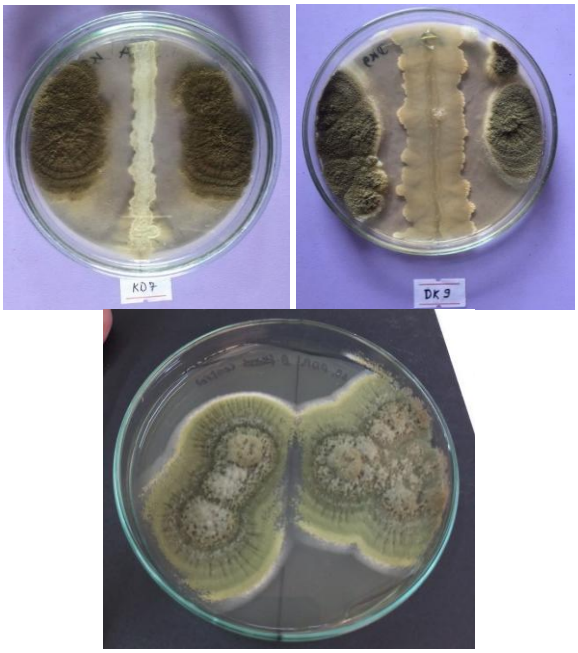

Fig. 4. KD7 inhibiting the growth of A. flavus, DK9 inhibiting the growth of A. flavus

DISCUSSION

In this study, 30 soil samples were collected from different locations. From these soil samples, 29 isolates were identified as *Bacillus* spp. from cultural and morphological characteristics and they were found to be Gram positive spore forming rod shaped bacteria (Singh *et al.* 2015). Among different bacterial isolates, best 16 isolates were selected and screened for the production of amylase. SYP agar was used and iodine solution was flooded to observe the clear zone of hydrolysis. The formation of clear zone was due to degradation of starch by amylase produced by *Bacillus* isolates. Starch in the medium turned blue as the iodine was trapped in the helical structure of starch but the monosaccharide cannot bind the iodine molecule and became colorless. Forgarty and Kelly (1979) and Iverson and Millis (1974) reported that the starch nutrient agar and iodine can be used for detecting amylase (hydrolytic enzyme) producing microorganisms that can hydrolyze starch forming clear zone surrounding an area of colony.

On the basis of maximum diameter of clear zone formation, 4 isolates (DK9, DK10, IM4 and KD7) were selected for further study. These isolates were purified by sub culturing the colonies on SYP agar for 3 to 4 times. These colonies were further incubated at 60° C. Three isolates (DK9, IM4 and KD7) were found to grow at this temperature. This indicates that these isolates might elicit the amylolytic activity even at 60° C, which requires further studies. Al-Johani *et al.* (2017) reported a thermo-tolerant *B. subtilis* isolated from hot-spring water with potent amylase producing capability.

Temperature and pH are the two factors that greatly affect the amylase activity (Poudel *et al.* 2015, Saha & Mazumdhar 2019, Swain & Ray 2007). Partially purified enzymes produced by these isolates were assayed at varying pH (5.0, 5.5 and 6.0) and temperature (35° C and 50° C). The reducing sugar concentration was determined at 2, 4 and 6 h of incubation according to the dinitrosalycyclic method (Miller 1959, Bernfeld 1955). The concentration of reducing sugar was found to be maximum after 6h of incubation. The concentration of reducing sugar was augmented with the increase in fermentation time. A high concentration of reducing sugar indicates high production of amylase by the isolates at that time period. The amylase activity was relatively greater at pH 6.0 (0.19 U/mL) given by isolate KD7. The enzyme activity was found to decrease with decreased in pH of the media. This indicated that high acidic condition is not suitable for the amylase production. This finding was supported by Singh *et al.* (2015) who verified that acidic environment is not appropriate for higher yield of enzyme.

In this study, 16 *Bacillus* spp. showing amylolytic activity were isolated from soil samples. All these isolates were able to grow at 50° C, showing thermo-tolerant in nature. Among these isolates, KD7, DK9, DK10 and IM4 (14 mm, 16 mm, 12 mm and 14 mm zone of hydrolysis) were found to be potent amylase producer. KD7 showed the highest amylolytic activity (0.19 U/mL) compared to other isolates. The amylase activity was relatively greater at 50° C than 35° C which indicates that amylases are active at high temperature and thermophilic temperature is suitable for amylase production. Hence, the optimum pH was 6.0 and optimum temperature was 50° C for amylase activity. Our research is consistent with work done by Femi-Ola and Olowe (2011) who reported that amylase produced by *Bacillus subtilis* BS5 had a molecular weight of 63 kDa and was optimally active at pH 6.0 and 50° C. However, Singh *et al.* (2015) showed that pH 7 and 40° C temperature were optimum values for growth of isolates and maximum amylase production by *Bacillus* spp. These

differences in result may have occurred due to the difference in media composition used, site of sample collection and incubation period. Furthermore, the size of inoculum used also affects enzyme activity. Nadia *et al.* (2003) reported that the production of the amylases slowdown as the inoculum size was increased because the growth of the organism was considerably amplified and the nutrients present in the medium were insufficient to overcome the growth of organisms. Similarly, growth of microorganism also decreased at low inoculum level as the time to reach stationary phase by organisms increased during their growth.

Our study revealed that DK 9 and KD7 exhibited high antagonistic activity while both IM4 and DK10 showed moderate antagonistic activity against *A. flavus.* Some studies showed that *Bacillus* spp. can produce lipopeptides with capability to impede fungal growth (Chen *et al.* 2009, Zhao *et al.* 2010). Similarly, a research conducted by Veras *et al.* 2016 showed that some *Bacillus* spp. isolated from fish intestine inhibited *A. flavus* and other mycotoxin producing fungi along with the detoxification of aflatoxins and other mycotoxins. Thakaew and Niamsup (2013) and Mnif and Ghribi (2015) showed that *Bacillus* spp. are potent producers of antifungal proteins, including enzymes such as chitinases, glucanases and proteases that can effectively inhibit the detrimental fungi and their mycotoxins.

CONCLUSION

Our study revealed, thermotolerant *Bacillus* spp. KD7, DK9, DK10 and IM4 were found to be potent amylase producer amongst which KD7 showed highest amylolytic activity compared to other isolates. The optimum pH and temperature for amylase production was found to be 6.0 and 50°C, respectively. Furthermore, antifungal activities shown by KD7 and DK9 indicated that these bacteria can be used as effective bio-control agents against toxigenic *A. flavus*. Hence, further strain selection and identification would pave the way for commercial enzyme production as well as search for a potent antifungal agent.

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