Isolation and characterization of different strains of *Bacillus licheniformis* for the production of commercially significant enzymes

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Abstract: Utilization of highly specific enzymes for various industrial processes and applications has gained huge momentum in the field of white biotechnology. Selection of a strain by efficient plate-screening method for a specific purpose has also favored and boosted the isolation of several industrially feasible microorganisms and screening of a large number of microorganisms is an important step in selecting a potent culture for multipurpose usage. Five new bacterial isolates of *Bacillus licheniformis* were discovered from indigenous sources and characterized on the basis of phylogeny using 16S rDNA gene analysis. Studies on morphological and physiological characteristics showed that these isolates can easily be cultivated at different temperatures ranging from 30ºC to 55ºC with a wide pH values from 3.0 to 11.0 All these 05 isolates are salt tolerant and can grow even in the presences of high salt concentration ranging from 7.0 to 12.0%. All these predominant isolates of *B. licheniformis* strains showed significant capability of producing some of the major industrially important extracellular hydrolytic enzymes including α-amylase, glucoamylase, protease, pectinase and cellulase in varying titers. All these isolates hold great potential as commercial strains when provided with optimum fermentation conditions.

Keywords: *B. licheniformis*, commercial enzymes, amylolytic enzyme, protease, pectinase.

INTRODUCTION

Utilization of bacterial strains specifically from genus *Bacillus* is gaining momentum because of their ability to resist and survive under harsh industrial conditions. *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus pumilus* are some of the strains that are associated with food spoilage and with a wide range of different clinical conditions (Turnbull, 1997).

B. licheniformis has proved itself as a multipurpose organism and has gained popularity along with *B. subtilis*. *B. licheniformis* is most commonly found in soil and as well as on the feathers of ground dwelling birds (Burtt and Ichida, 1999). With the rapid advancement in enzymology and fermentation technology, now commercially feasible quantities of different industrial enzymes are being produced by *B. licheniformis* (De-Boer *et al*., 1994). This strain is also used to produce a polypeptide antibiotic known as bacitracin (Vitkovic and Sadoff, 1977). *B. licheniformis* is capable of producing bacteriocin under aerobic conditions (Kayalvizhi and Gunasekaran, 2008; Anthony *et al*., 2009) as well as under anaerobic conditions against anaerobic microorganisms (Pattnaik *et al*., 2001). *B. licheniformis* in addition is a frequent contaminant of various industrial processes (Pirttijarvi *et al*., 2000).

Although this organism is exploited industrially for the large-scale production of various enzymes, but it's generally regarded as safe (GRAS) status is still a subject of debate (Salkinoja *et al*., 1999). This organism has been reported to produce some of the most important commercial enzymes including α-amylase, alkaline protease, keratinase and β-mannanase (Zhang, *et al.*, 2002; Feng *et al*., 2003; Hmidet *et al*., 2009). It has been reported that *B. licheniformis* 749/C is a producer of multiple forms of β- lactamase (Lampen *et al*., 1980) and similarly another isolate BAS50 which was previously isolated from a petroleum reservoir at a depth of 1,500 meters, produces a lipopeptide surfactant (Yakimov*et al*., 1995). Most recently this organism has shown the ability to synthesize gold nanocubes and in broad prospect this isolate can be manipulated genetically for the synthesis of nanoparticles with tailored properties (Kalishwaralal *et al*., 2009).

Keeping the significance of multiple uses of *Bacillus licheniformis* in view, present study was designed to isolate and screen various strains from indigenous sources for the production of commercially important enzymes.

MATERIALS AND METHODS

Isolation of bacteria

Soil samples (1.0g) from vegetative field were collected aseptically and suspended in 100 ml of sterile water. Diluted sample was transferred in multiple flasks containing nutrient broth medium and incubated at 37ºC for 24 to 72 hours. After appearance of growth, a loop full of bacterial culture was then streaked onto nutrient agar medium and again incubated at 37ºC for 24 to 48 hours.

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From different agar plates, isolated colonies were serially diluted and sample (0.1 ml) from last three dilutions were transferred into homologous medium and incubated at 37ºC for 24 hours. Some of the sterile nutrient agar plates were exposed to air for 30 minutes in order to isolate microbial cultures from environment. The above mentioned procedure for isolation was followed. All morphologically contrasting colonies were purified by repeated streaking. Identification of the genus was based on morphological and biochemical characteristics for *Bacillus* species. All the tests were performed on liquid cultures in late-logarithmic phase.

16S rDNA gene analysis and sequencing

Extraction of DNA and sequencing of 16S RDNA gene was performed by the method as described by Asma *et al.* (2012). Primers and PCR conditions were also same as described previously.

Screening of isolates for the production of alpha-amylase, protease, pectinase and cellulase

Each isolate was screened for the production of different enzymes. For the screening of alpha-amylase activity, all the five isolates of *B. licheniformis* were grown on 0.5% starch agar plates and incubated at 37ºC for 24 hours. After incubation the plates were flooded with iodine-potassium iodide solution for alpha amylase activity (Teodoro and Martins, 2000; Qader *et al*., 2006).

Screening for protease activity was performed by growing the isolates on 0.1% casein agar plates at 37ºC for 24 hours (Bjorklind and Arvidson, 1977). For visualization of the clear zones of activity around the growing culture the plates were flooded with 0.1 % Coomassie brilliant blue R250 followed by destaining using 40% methanol and 10% acetic acid.

Pectinase activity of the culture was evaluated by growing the bacterial strains in a medium containing 0.25% pectin (Soares *et al*., 1999). All the isolates were grown at 37ºC for 24 hours and activity zones were detected using iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide in 330 ml deionized water) (Fernandes-Salomao *et al*., 1996).

The selected isolates were grown for 24 hours in a medium containing 1.0% carboxymethyl cellulose (CMC). Hydrolysis of CMC by cellulase enzyme was verified by staining the plates with Congo-Red solution (0.1%) followed by washing with 1M solution of NaCl to intensify the zones (Ramesh *et al*., 2008).

Production medium and assay procedure of alpha-amylase, glucoamylase, protease and pectinase activities of the isolates

All the cultures were grown at 37°C for 48 hours for the production of alpha-amylase and glucoamylase in the medium containing (g L^{-1}): starch, 15.0; yeast extract, 10.0; peptone, 10.0; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 0.1; $CaCl_2$, 0.01 (pH 7.5). enzyme activity of alpha-amylase was estimated using 1.0 % soluble starch as a substrate and the reducing sugar released as maltose equivalent was detected by 3,5-dinitrosalicyclic acid (DNS) method (Miller, 1959). The unit of alpha-amylase is defined as the amount of enzyme which liberates 1.0 µmol of maltose per minute at 50°C in phosphate buffer of pH 7.5 For the estimation of glucoamylase activity, 1.5% soluble starch was used as substrate and was determined by the increase in glucose that was released after the digestion of starch under standard assay conditions. Glucose was measured by glucose oxidase and peroxidase method (Bergmeyer and Bernt, 1974). One unit of glucoamylase activity is defined as the amount of enzyme that liberates 1.0 µmole of glucose at 45°C in potassium phosphate buffer of pH 7.0.

For the production of protease the cultures were grown at 37°C for 24 hours in the medium containing (g L^{-1}): yeast extract, 5.0; peptone, 5.0; dextrose, 30.0; K_2HPO_4, 0.1; $CaCl_2$, 0.01 (pH 7.0). For protease estimation, the method described by Anson (1938) was followed with slight modifications. Crude enzyme (0.25 ml) was incubated with 1.0 ml of substrate (0.5 % casein in 50 mM Tris buffer, pH 8.5). The reaction was stopped using 110 mM TCA (Trichloroacetic acid). The reaction mixture was centrifuged at 1000 rpm for 10 minutes and was detected for the presence of tyrosine residues as described by Folin and Ciocalteu (1927). Unit activity of protease is defined as the amount of enzyme that hydrolyzes casein to produce color that is equivalent to 1.0 µmol of tyrosine per minute at pH 7.0 at 40°C.

Pectinase was produced at 37°C for 24 hours in a medium containing (g L^{-1}): pectin, 5.0; yeast extract, 5.0; ammonium sulphate, 2.5; K_2HPO_4 2.5; KH_2PO_4, 2.0 (pH 7.5). Pectinase activity was assayed in terms of polygalacturonase activity using DNS (Miller, 1959). The reducing sugar released as galacturonic acid was detected using 0.25 % citrus pectin as substrate in the assay. One unit of pectinase activity is defined as the amount of enzyme required to release one µmole of galacturonic acid per minute at 50°C in phosphate buffer (pH 7.0). Cellulase activity was not quantified but only screened for enzyme production. The values presented in all the tables are mean of replicate results with p-value of <0.005 and standard deviation of ± 2%.

RESULTS

In the current study thirty five bacterial strains belonging to genus *Bacillus* were isolated and purified from the mixed culture obtained from the vegetative soil field and environment samples. Among them only 05 isolates were identified as *Bacillus licheniformis* on the basis of

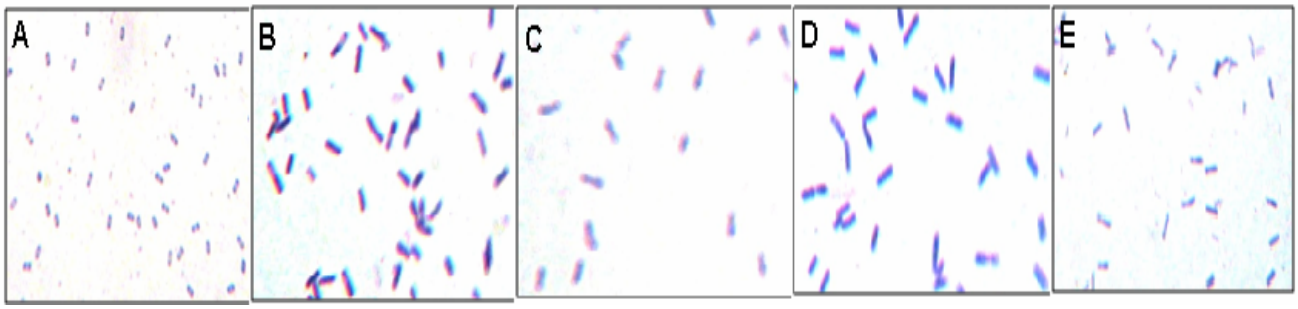

Fig. 1: Microscopic characteristics of *B. licheniformis*.
Key: **A**, KIBGE-IB1; **B**, KIBGE-IB2; **C**, KIBGE-IB3; **D**, KIBGE-IB4; **E**, KIBGE-IB5.

Table 1: Morphological, physiological and biochemical characteristics of *Bacillus licheniformis*.

Parameters	**Characteristics of *Bacillus licheniformis***				
	KIBGE-IB1	**KIBGE-IB2**	**KIBGE-IB3**	**KIBGE-IB4**	**KIBGE-IB5**
GenBank Accession	GU216258	GU216259	GU216260	GU216261	GU216262
Colony Morphology	Large opaque adherent irregular edges	Large opaque adherent irregular edges	Large opaque adherent irregular edges	Large opaque adherent irregular edges	Large opaque adherent irregular edges
Gram Reaction	Positive	Positive	Positive	Positive	Positive
Cell Morphology	Short rods in chains	Rods in chains	Rods in chains	Large rods	Large rods
Spore Formation	+	+	+	+	+
Catalase	+	+	+	+	+
Urease	+	+	+	+	+
Nitrate Reduction	+	+	+	+	+
Citrate Utilization	+	+	+	+	+
NaCl (%)	7-10	7-12	7-12	7-12	7-12
Growth Temperature	30°-55°C	30°-55°C	30°-55°C	30°-55°C	30°-55°C
Growth pH	3-10	3-10	4-11	4-11	4-11
Growth on					
SDA	+	+	+	+	+
Nutrient broth	+	+	+	+	+
Starch agar	+	+	+	+	+
Skimmed milk	+	+	+	+	+
Casein agar	+	+	+	+	+
Pectin agar	+	+	+	+	+
Saccharide utilization					
Glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannose	+	+	+	+	+
Lactose	–	–	–	–	–
Xylose	–	–	–	–	–

phenotypic features. Chemotaxonomic characteristics were further confirmed by 16S rDNA analysis. Microscopic characteristics of all the five predominant isolates revealed that all of them are rod-shaped Gram-positive and spore forming organism with varying cell size (fig. 1). All the isolates were aerobes or facultative anaerobes in nature, with a wide diversity in physiological properties, particularly with respect to temperature for growth, pH and salt concentration. Main physicochemical properties of these 05 isolates and their cultivation features on various media are summarized in table 1. Assimilation of different carbohydrates is also presented.

Phylogenies of the bacterial isolates

Fig. 2 illustrates the purified PCR product of all the 05 isolates for 16S rDNA. The bands visualized on 1.0 % agarose gel showed the sequence size of about 1500 bp. 16S rDNA sequences for all the five isolates of *B.*

licheniformis were used to generate the phylogenetic tree (fig. 3). The isolates received the following accession numbers: GU216258, GU216259, GU216260, GU216261 and GU216262 for *B. licheniformis* KIBGE-IB1, *B. licheniformis* KIBGE-IB2, *B. licheniformis* KIBGE-IB3, *B. licheniformis* KIBGE-IB4 and *B. licheniformis* KIBGE-IB5, respectively.

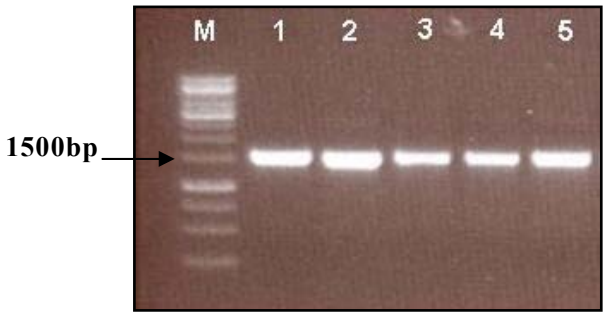

Fig. 2: Agarose gel electrophoresis of purified PCR product for 16S rDNA analysis.
Key: M, Marker (1Kb)**; 1**, KIBGE-IB1; **2**, KIBGE-IB2; **3**, KIBGE-IB3; **4**, KIBGE-IB4; **5**, KIBGE-IB5

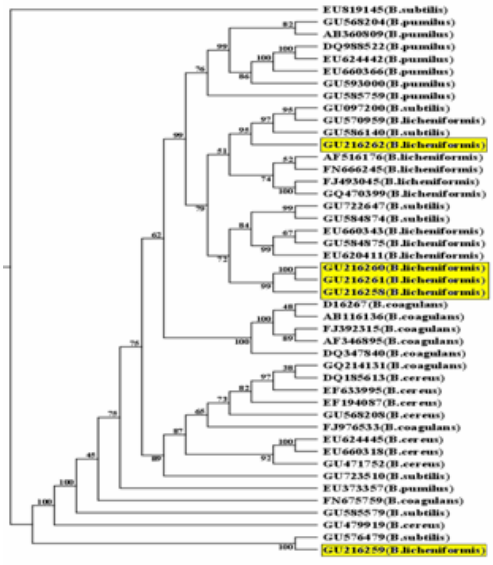

Fig. 3: Phylogenetic tree constructed based on 16S rDNA gene sequence analysis from the isolates obtained from Karachi, Pakistan with reference sequences available at NCBI through BLAST analysis. Sequences highlighted are from the current study.

Screening for production of industrial enzymes

All the isolates were screened for the production of different enzymes. Figs. 4, 5, 6 and 7 present the data for screening of alpha-amylase, protease, pectinase and cellulase, respectively. It was observed that the clearing zone for each isolate in the presence of specific substrate was different for all the enzymes representing that all of them are competent of producing these enzymes but with varying titers.

DISCUSSION

Among several organisms, bacteria have now become the choice of selection for various industrial processes. One of the main reasons is that they can be genetically modified to produce the desired activity and minimize the undesired products. Screening of a large number of microorganisms is an important step in selecting a highly potent microbial culture for multipurpose utilization. Many studies have been conducted previously in order to search and select new bacterial isolates that can substitute fungi by bacterial isolates for the production of various enzymes at industrial scale. Current study is an attempt to isolate potent bacterial culture especially *B. licheniformis* that has the capability of producing multiple enzymes. Along with the constitutive enzymes produced by these bacterial isolates, they could also be able to produce some of the inducible enzymes when specific substrates are used. In this study the main focus was to isolate Bacillus species because they are considered as attractive hosts for producing heterologous enzymes and they can be easily genetically modified and transformed. Secondly, they have an enormous potential to secrete their own proteins into an extracellular medium.

Bacterial isolates obtained and selected in the present study showed a prokaryotic diversityamong the microbial community and belongs to the phylum *Firmicutes*. Among thirty five (35) bacterial strains which belonged to genus *Bacillus*, only five (05) isolates were identified as *B. licheniformis* on the basis of taxonomic characteristics. They were further confirmed by 16S rDNA sequence analysis. The sequences for the closest neighbors were used for constructing phylogenetic tree. Total forty sequences available in the NCBI Gen Bank database were aligned and it was found that *Bacillus licheniformis* KIBGE-IB1, IB3 and IB4 are clustered in one group and are closely related to *B. licheniformis* group. However, IB2 and IB5 are closely related to previously isolated *Bacillus subtilis* (GU576479) and (GU586140), respectively (fig. 3).

All the isolates were screened for the production of different enzymes and all of them were capable to produce various enzymes in the presence of specific substrate. Initially plate-screening method was used for this purpose. When these isolates were grown under specific condition with specific substrates they showed noticeable clearing zones around their colonies on the agar plates. They showed the capability of producing alpha-amylase, protease, pectinase and cellulase. All of these enzymes have a great utility in various industries. The screening method using starch is a general qualitative method for the entire amylolytic enzymes, hence it was not possible to screen for glucoamylase. After confirmation of the enzymes by different screening methods, they were quantified using specific assay

Table 2: Production of different enzymes by various isolates of *Bacillus licheniformis*

B. licheniformis	Alpha-Amylase (U/mg)	Glucoamylase (U/mg)	Protease (U/mg)	Pectinase (U/mg)
KIBGE-IB1	2621±52	174±3.50	740±14.8	240±4.80
KIBGE-IB2	1328±26	86±1.70	1950±39.0	358±7.16
KIBGE-IB3	2783±55	797±15.9	1025±20.5	620±12.4
KIBGE-IB4	3250±65	35±0.70	650±13.0	591±11.8
KIBGE-IB5	2567±51	72±1.44	598±11.9	270±5.40

Standard deviation, ±2%; p-value, <0.005; n=3.

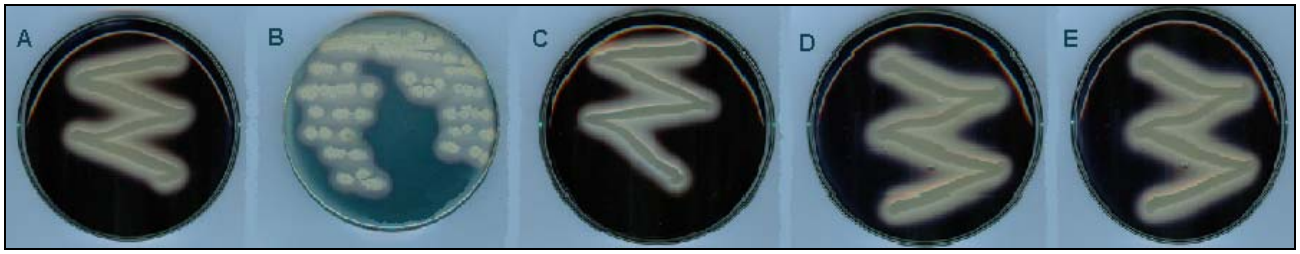

Fig. 4: Screening for alpha-amylase production by different strains of *B. licheniformis*.
Key: **A**, KIBGE-IB1; **B**, KIBGE-IB2; **C**, KIBGE-IB3; **D**, KIBGE-IB4; **E**, KIBGE-IB5.

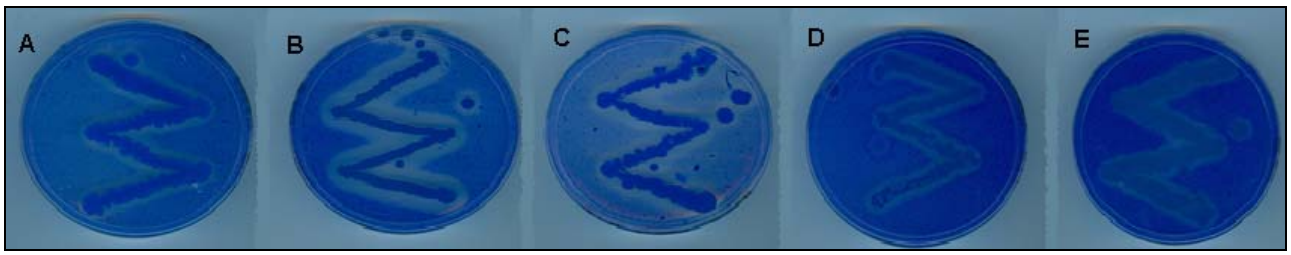

Fig. 5: Screening for protease production by different strains of *B. licheniformis*.
Key: **A**, KIBGE-IB1; **B**, KIBGE IB2; **C**, KIBGE-IB3; **D**, KIBGE-IB4; **E**, KIBGE-IB5.

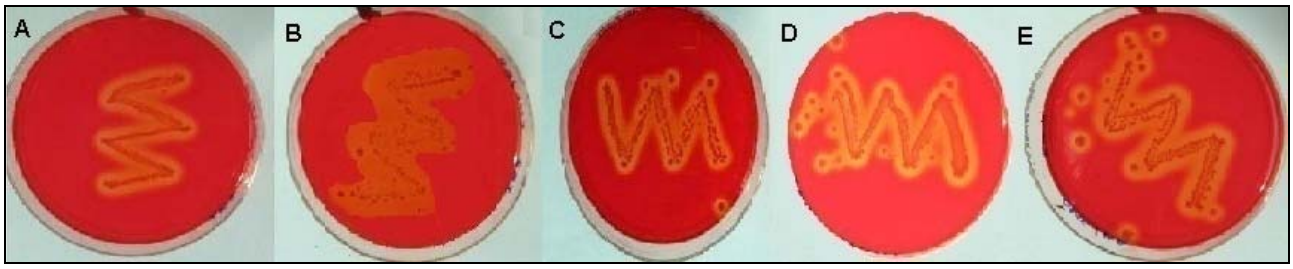

Fig. 6: Screening for pectinase production by different strains of *B. licheniformis*.
Key: **A**, KIBGE-IB1; **B**, KIBGE-IB2; **C**, KIBGE-IB3; **D**, KIBGE-IB4; **E**, KIBGE-IB5.

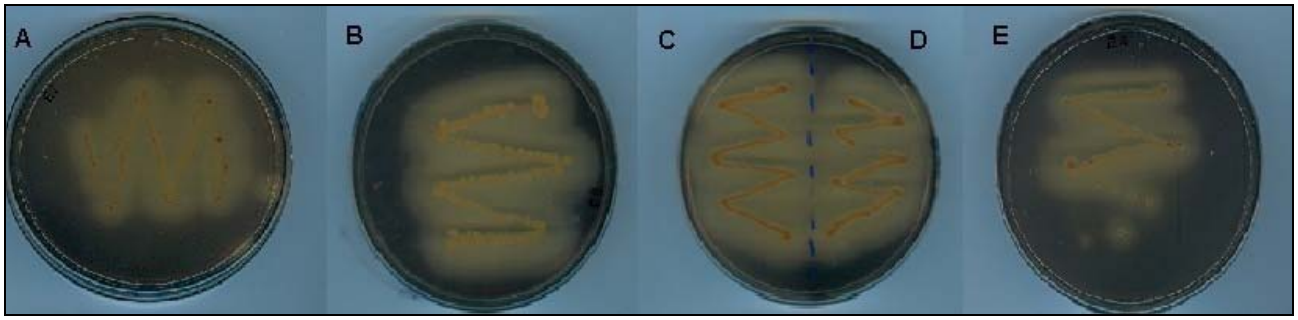

Fig. 7: Screening for cellulase production by different strains of *B. licheniformis*.
Key: **A**, KIBGE-IB1; **B**, KIBGE-IB2; **C**, KIBGE-IB3; **D**, KIBGE-IB4; **E**, KIBGE-IB5.

methods under standard conditions. Among all the isolates, KIBGE-IB4 and KIBGE-IB3 showed maximum alpha-amylase and glucoamylase activities, respectively (table 2). However, it is a possibility that their enzyme production capability will increase after complete optimization of various physical and chemical parameters.

Majority of the currently used industrial enzymes are hydrolytic in action and are used for the degradation of various natural substances (El-Enshasy *et al*., 2008). Amylolytic enzymes from bacterial strains particularly alpha-amylase and glucoamylase are currently being used for various industrial purposes (Soccol *et al.,* 1994; Soccol *et al.,* 1992). Previously, production of bacterial alpha-amylases have been genetically improved by applying a range of strategies based on molecular cloning tools (Niu *et al*., 2009) but it is much easier and feasible to explore new industrial strains and optimize fermentation conditions as compared to genetic modifications. Proteases also remains the dominant enzyme type, because of their extensive uses in different sectors including food, pharmaceutical and exclusively in the detergent industries (Kalisz, 1988). *B. licheniformis* KIBGE IB1, IB2, IB3, IB4 and IB5 have an immense ability to grow at wide pH range therefore, proteases produced by them can be used in the preparation of biological washing powders because all these strains are well adapted to alkaline conditions and produce alkaline proteases. The protease produced by strain KIBGE IB-2 was most active near pH 8.5.

Strains belonging to genus *Bacillus* have played an important role in biodegradation and bioconversion of various macro-molecules particularly pectin. Pectinases is a group of enzymes that catalyzes the degradation of pectic polymers present in the plant's cell wall and can also be produced by many yeast and fungi. On commercial scale, fungal pectinases are preferable due to high enzyme yield. However, the fungal pectinases are not encouraged to be used for the production of vegetable purees and other food preparations because the pH values of these food products are close to physiological pH (Chesson and Codner, 1978) whereas, fungal pectinases are mostly active in between 3.0 to 5.5 pH (Ueda *et al*., 1982). Cultivation of bacteria for the production of pectin degrading enzymes having neutral pH values are of great importance. Neutral pectinases is used in processing of fruits as well as vegetable nectars and for the improvement of color during wine production. It is also used to improve extraction yields of oil from plant tissues and also for the liquefaction of plant tissues (Stressler, 1971; Bailey and Pessa, 1990). In this study we have successfully screened various pectinase producing *B. licheniformis* strains that produces neutral pectinases. It was found that the maximum pectinase production was by *B. licheniformis* KIBGE IB-3 but as KIBGE IB-4 also produced nearly the same amount of enzyme therefore, it has been presumed that these strains can be potential candidates for the production of pectinase after further optimization of fermentation conditions.

CONCLUSION

B. licheniformis have the ability to survive under harsh industrial conditions and the enzymes produced by them have now replaced several organic and inorganic chemical processes. Bacterial isolates are currently being used for the production of different products on large scale as compared to fungi. Isolation and characterization of predominant *B. licheniformis* strains capable of producing various enzymes was performed. The results demonstrated that all these newly discovered isolates of *B. licheniformis* have great potential for producing high titers of different extracellular enzymes of commercial importance. Optimization of growth parameters is of prime importance in industrial production of enzymes therefore, it is now underway.

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