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Optimization of the xylanase production with the newly isolated *Bacillus aerophilus* KGJ2

[Yeni izole edilen *Bacillus aerophilus* KGJ2 den xylanase üretiminin optimizasyonu]*

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ABSTRACT

Objective: The objective of the present study was to isolate a potential and novel *Bacillus* strain from paper mill effluent for the production of an industrially important xylanase.

Material and Methods: A potent xylanase producing microorganism was isolated from paper mill effluent based on zone of clearance on xylan agar medium. The strain was identified based on 16S rRNA analysis and biochemical characterization**.** Xylanase produced by the isolated strain was partially purified and characterized for its activity and stability.

Results: The xylanase produced by this *Bacillus aerophilus* KGJ2 was thermophilic, shows higher activity and stability at 70°C. Xylanase had activity peak at pH 4.0 and was very acid stable. Birchwood xylan and beef extract were identified as best suited carbon source and nitrogen source, respectively.

Conclusion: The results confirm that *Bacillus aerophilus* KGJ2 produced a unique acido-thermotolerant xylanase.

Key Words: Paper mill effluent, *Bacillus aerophilus* KGJ2, xylanase, thermophilic.

Conflict of Interest: Authors have no conflict of interest.

ÖZET

Amaç: Bu çalışmanı amacı endüstüriyel önem taşıyan xylanase üretiminde kullanmak için kağıt fabrikalarından potansiyel ve özgün bir *Bacillus* suşu izole etmektir.

Yöntemler: Xylanase üretebilecek bir mikroorganizma, kağıt fabrikası atıklarından xylan agar ortamında temiz bir bölge oluşturma yeteneği karşılaştırılarak seçilmiştir. Elde edilen suş 16S rRNA analizi ve biyokimyasal karakterizasyon yöntemleri ile belirlenmiştir. Bu sış tarafından üretilen xylanase enzimi kısmen saflaştırılmış ve aktivite ve dayanıklılık özelliklerine göre karakterize edilmiştir.

Bulgular: *Bacillus aerophilus* KGJ2 'den izole edilen xylanase 70 °C'de aktivite göstererek termofilik bir protein olarak belirlenmiştir. Saflaştırılan xylanase pH 4.0'da en yüksek aktivitesini göstermiştir. En verimli xylanase üretimi için Birchwood xylan ve beef extract karbon ve nitrojen kaynağı olarak kullanılmıştır. The results confirm that *Bacillus aerophilus* KGJ2 produced a unique acido-thermotolerant xylanase.

Sonuç: bu çalışmanın sonuçunda *Bacillus aerophilus* KGJ2 suşu tarafından üretilen xylanase'in asido-thermotolerant bir enzim olduğu bulunmuştur.

Anahtar Kelimeler: Kağıt fabrikası atığı, *Bacillus aerophilus* KGJ2, xylanase, thermofilik.

Introduction

Xylan is the second abundant structural polysaccharides other than cellulose present in the lignocellulosic materials, especially in plants [1]. Xylan can be used to produce a numerous value added products including natural food sweetner xylitol. It is also used in various medical treatments like diabetics [2, 3]. Xylan is the linear homopolymer back bone made up of β-1,4 glycosyl bonds [4, 5]. The complex structure of the xylan is completely hydrolyzed to hexose and pentose monomers by different enzymes.

Xylanase (E.C.3.2.1.8) is one among the enzymes used in the biodegradation of xylan in a complex process by which it hydrolyses the β-1,4 glycosyl bond. Microorganisms like bacteria, actinomycetes and fungi are rich sources of xylanases [5]. Bacteria, especially *Bacillus sp.*, are capable of producing alkaline thermostable xylanases. Earlier reports state that *Bacillus SSP-34*, *Bacillus stearothermophilus* strain T6, *Streptomyces cuspidoporus, Bacillus sp. strain NCL 87-6, Bacillus circulans AB 16,Bacillus pumilus* SV-85S, were used successfully in the production of xylanases [6-9].

Xylanases are industrially attractive enzymes with wide range of applications including pulp treatment, textile processing, ethanol production, brewing industry, and waste treatment [10]. Bacterial xylanases show higher efficiency than fungal xylanases upon hydrolysis of xylan [11]. Relative to fungal xylanases, bacterial xylanases are preferred for the following reasons. Most of the industrial applications of xylanase require low pH. This pH is not suitable for fungal xylanase. Next major problem is low yield of fungal xylanase on fermenter studies. Often, industrial applications require very high agitation speed. This will disrupt the fungal biomass and results in reduction in xylanase titers [12]. Recently many efforts had been devoted for the production of xylanases from microorganisms isolated from industrial effluents, because these organisms may produce thermostable, acidophilic and or alkaliphilic enzymes [13].

In this paper, a novel xylanase from *Bacillus aerophilus* KGJ2, from paper mill effluent was isolated. It had shown maximum growth at pH at 4.0 and at a temperature of 70 ^{0}C and this enzyme was active under acidic and high temperature conditions. These characteristics of xylanase suggest it may have greater potential in various biotechnological applications. To the best of our knowledge, this is the first report on xylanase producing *Bacillus sp. (B. aerophilus* KGJ2) isolated from a paper mill effluent.

Materials and Methods

Isolation and screening of xylanase producing bacteria

The paper mill effluent samples used in this study were collected from Tamil Nadu Paper Limited, Karur District, Tamil Nadu, India. Xylanase producing organism was isolated by using birch wood xylan agar medium (pH 7.0) containing (w v^{-1}) 0.5% peptone, 0.1% NaCl, 0.2% K_2HPO_4, 0.01% $CaCl_2$, 0.01% $MgSO_4$, 0.1% yeast extract, 1.5% bacteriological agar and 0.5% birch-wood xylan. Plates were incubated at 37°C for 24 h. Six xylanase positive bacterial cultures were screened based on the zone of clearance on the petri-plate around the bacterial colony. Based on the greater clear zone one microorganism was selected for further study.

Bacterial identification and phylogenetic analysis

The cultural, morphological and biochemical properties of the selected organisms were studied according to the methods in Bergeys Manual of Systematic Bacteriology [14]. The molecular characterization was done by using 16S rRNA sequencing technique [15]. The genomic DNA was extracted from the given organism using standard procedures [16]. The 16S rRNA gene amplification was performed using the universal primers. Forward primer: 5'AGAGTTTGATCCTGGCTCAG-3' and Reverse Primer: 5'-ACGGCTACCTTGTTACGACTT-3'. PCR product was directly sequenced using the big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA,USA) and the data was analyzed for its similarity by using BLAST [17] and by using MEGA 4.1 software [18] the phylogenetic analysis was done and the tree obtained by means of neighbor-joining method was analyzed for related organisms [19].

Submerged fermentation and xylanase production

Bacterial culture (cell count 1.52x10^7 cfu/ml) was transferred into the medium (basal liquid) containing (w v^{-1}) 0.5 % birch-wood xylan, 0.5 % peptone, 0.1% NaCl, 0.2% K_2HPO_4, 0.01% $CaCl_2$, 0.01% $MgSO_4$ and 0.1% yeast extract, at pH 4.0 and it was incubated in an orbital shaker maintained at 150 RPM at 37°C for 48 h.

Effect of various parameters on xylanase production

The carbon source such as sucrose, fructose, lactose, starch, xylan, sesame oil cake, ground nut oil cake, neem oil cake and various lignocellulosic materials such as paper waste, coir, rice straw, coffee waste and sugarcane bagasse (5 g/L) were chosen for the production of enzymes. Carbon source gained maximum production was taken and used for further studies. Influence of nitrogen sources like yeast extract, beef extract, glycine, urea, tryptone, peptone, ammonium nitrate, ammonium sulphate, ammonium chloride and potassium nitrate (5g/L), were used. Effects of initial pH (2.0-10.0) and temperature (30 ^{0}C -90 ^{0}C) on enzyme production and activity were also studied. A control devoid of carbon and nitrogen source was also kept.

Xylanase enzyme extraction from submerged fermentation.

Samples obtained from submerged fermentation was centrifuged at 10,000 × g for 10 min at 4 ºC, the supernatant was collected and used as crude enzyme solution. Then the enzyme solution (0.1 ml) was added to 0.5 mL of 0.5% birch-wood xylan solution in 20 mM sodium citrate buffer (pH 4.0). The enzyme reaction was performed at 70 ºC for 5 min and its xylanase activity was determined by DNS method [20].

Partial Purification of Xylanase

About 50 ml of the crude enzyme (supernatant) was partially purified by precipitating with 60% saturation of ammonium sulphate. The pellet was obtained by centrifuging the precipitate at 10,000 × g for 30 min at 4 ºC and it was dissolved in 20 mM Sodium citrate buffer (pH 4.0). The precipitated ammonium sulphate solution was further dialyzed and kept over night and then examined for enzyme activity.

Result and Discussion

Screening and identification of bacteria with high xylanase activity

Bacteria are generally identified based on their morphological and biochemical characteristics studies. The initial screening showed that the isolate was aerobic, gram-positive, motile bacilli with short rods. Biochemical tests showed positive reactions for catalase, triple sugar iron test, simmon's citrate and methyl red test and negative for Voges Proskeur, indole and Macconkey agar test (Table 1). The bacterial isolate was confirmed as *Bacillus aerophilus* KGJ2 with 16S rRNA sequencing analysis. The sequence was deposited in Gene Bank (Accession No. JX027507). The phylogenetic relationship of this bacterium could be inferred using neighbor joining method (Fig 1).

Table 1. Morphological and biochemical characteristics of *Bacillus aerophilus* KGJ2

TEST	OBSERVATION
Shape	Rod
Gram reaction	Gram positive
Motility	Motile
Catalase	+
Oxidase	+
Indole	-
MR	+
VP	-
Simmons citrate	+
Triple iron sugar	+
Amylase	+
Pectinase	+
Lipase	+
Arginine	+
Lysine	-
Dextrose	+
Fructose	+
Lactose	+
Sorbitol	-
Sucrose	+
Xylose	-
Gelatinase	+
Cellulose	+
Starch	+

Effect of carbon source on xylanase production

Carbon source is an essential variable in microbial fermentation medium, which is vital for the cellular growth and metabolism. Fig. 2 illustrates the effect of carbon source on xylanase production. Among the carbon sources tested, birchwood xylan yielded highest xylanase (113.83 U/mL) production. Although lignocellulosic substrates such as rice straw, sugarcane bagasse, groundnut oil cake, sesame oil cake, neem oil cake, coir and others have been found to support production of xylanase, the maximum enzyme activity was found in birch wood xylan. Purified xylan is an excellent substrate for efficient production of xylanase, as the low molecular weight intermediate compounds derived from xylan are efficient xylanase inducers. The use of such substrates has proven to be efficient in xylanase production. Moreover, xylan selectively induces xylanases, with low cellulase activity in a number of microorganisms. These results are in agreement with those reported in literature [21 – 23]. Garg *et al*., 2009 [21] and Roy Narayanan *et al*., 2004 [22] showed that increased xylanase activity on birch wood xylan and oat spelt xylan by using *Bacillus halodurans* MTCC 9512 and *Bacillus* sp., isolated from alkali soda lake respectively. Similarly, birch wood xylan was proven to be the best carbon sources for xylanase production by marine *Bacillus pumillus* strain, GESF-1 [23].

Effect of nitrogen sources on xylanase production

The impact of inorganic and complex nitrogen sources was evaluated on xylanase production by *Bacillus aerophilus* KGJ2 by replacing peptone and yeast in basal medium with other nitrogen sources. It was found that the control, were basal medium was used, yielded the maximum xylanase production. Among all nitrogen sources studied, beef extract supported the maximum enzyme production other than the basal medium [Fig.3]. Similar observations were also reported by Suvarnalaxmi *et al*., 2008 [24]. In general, both

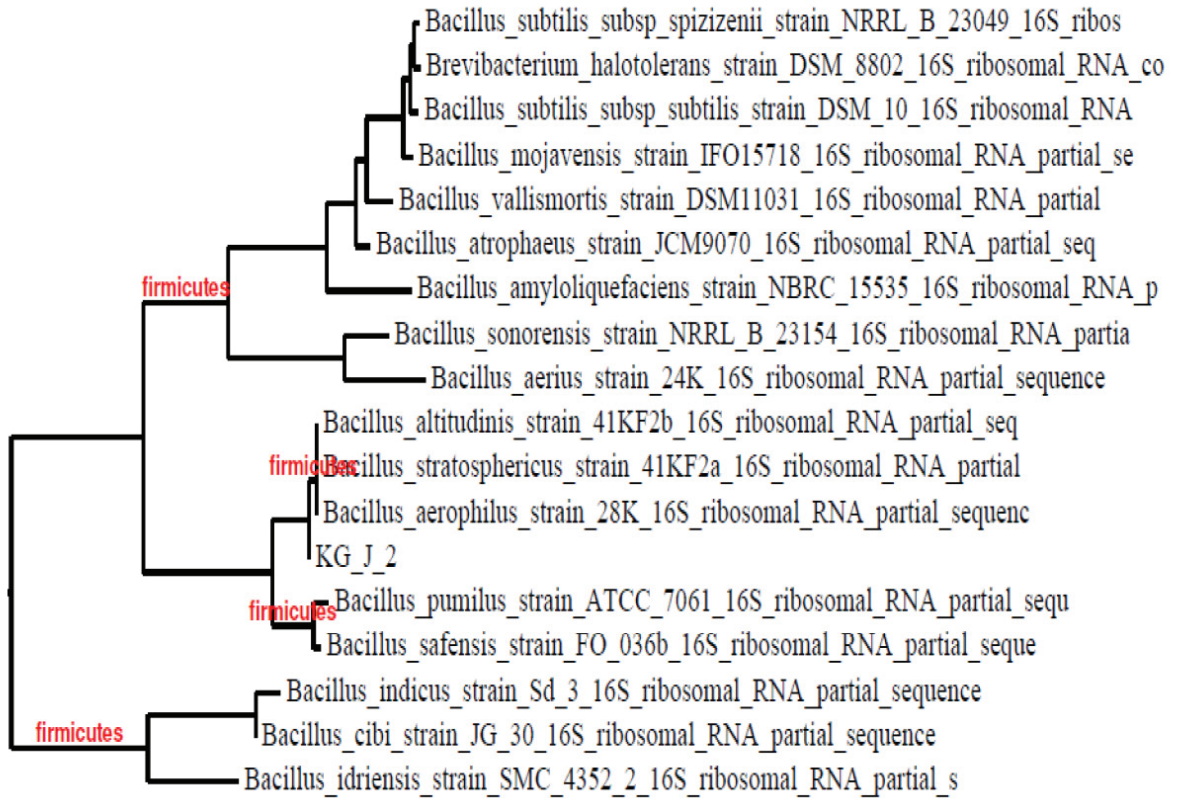

Figure 1. Phylogenetic tree generated based on the sequesnce of 16S rRNA indicating the relatedness of *Bacillus aerophilus* KGJ2

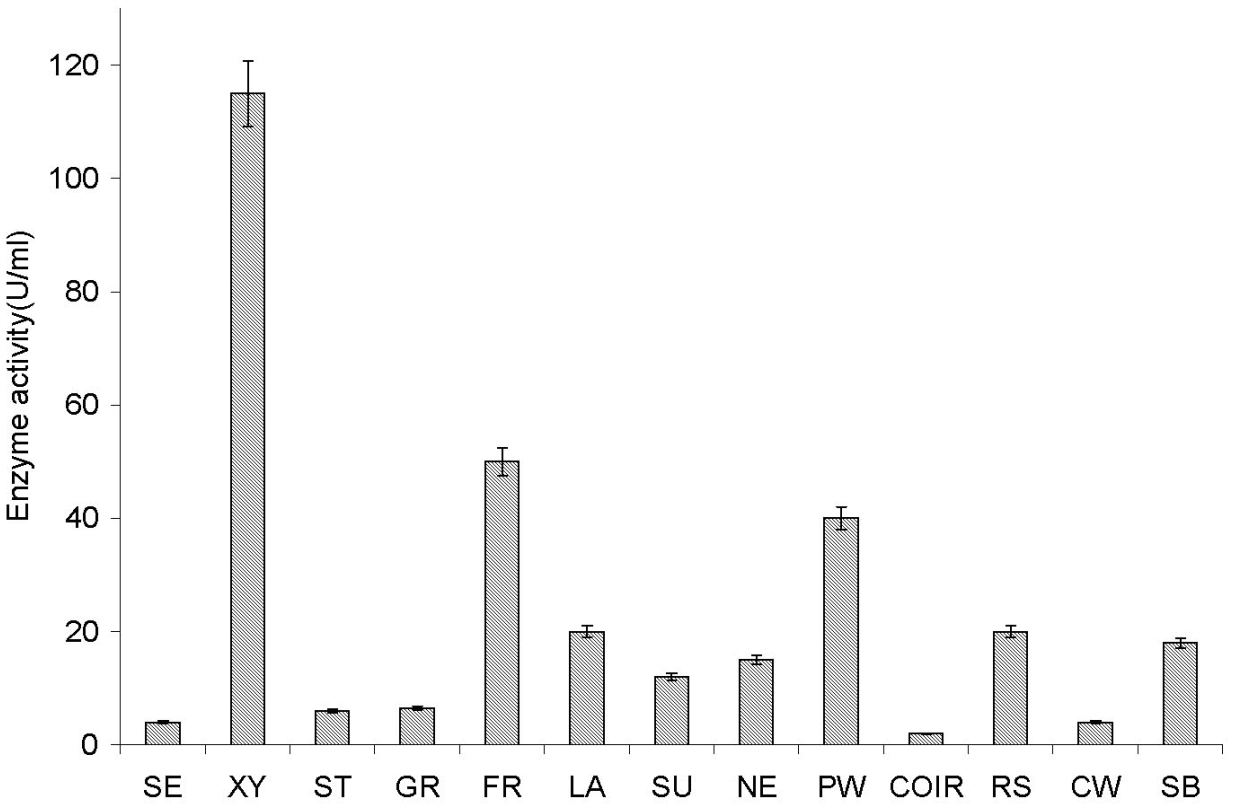

Figure 2. Xylanase production using different carbon sources. [SE: sesame; XY: Xylan; ST: starch; GR: ground nut; FR: Fructose; LA: lactose; SU: sucrose; NE: neem; PW: paper waste; COIR: coir; RS: rice straw; CW: coffee waste; SB: sugarcane bagasse]

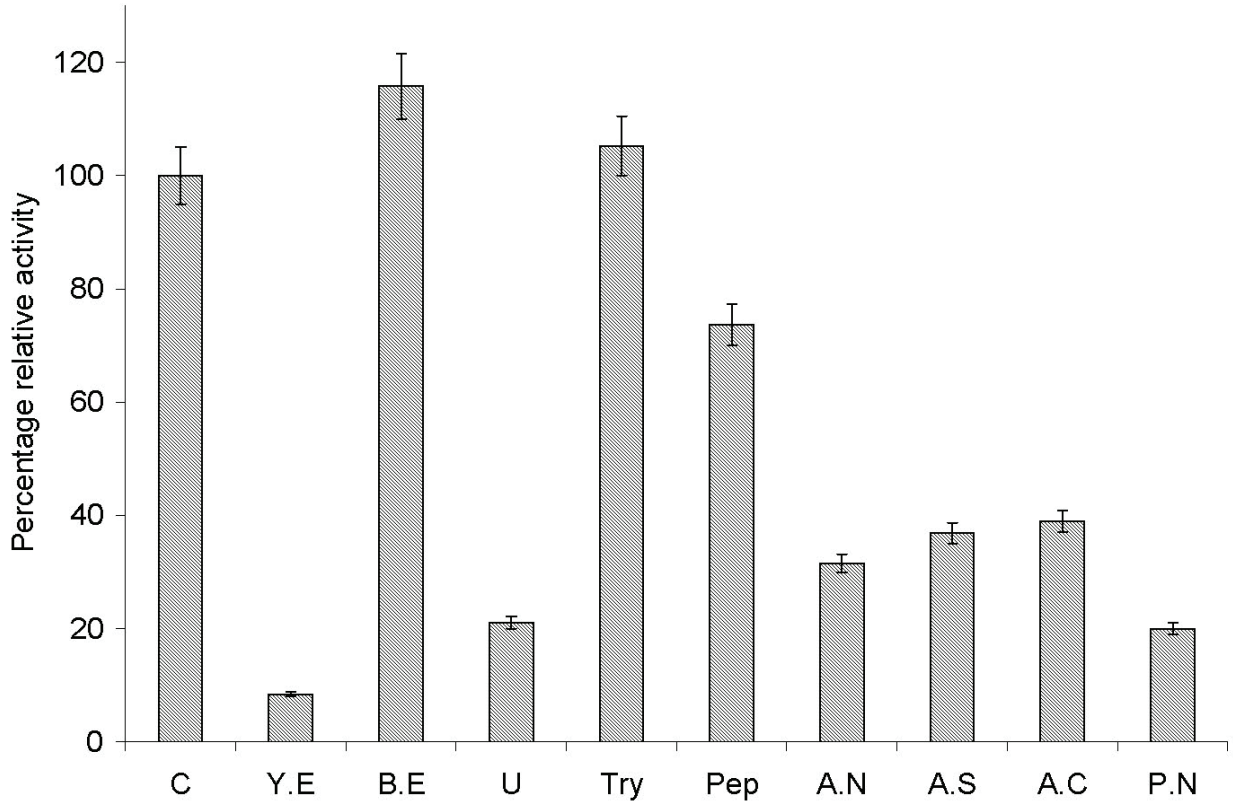

Figure 3. Effect of different nitrogen source (5g/ L) on xylanase production by *Bacillus aerophilus* KGJ2. [C – Control (basal medium); Y.E – Yeast extract; B.E – Beef extract; U – Urea; TRY – Tryptone; PEP – Peptone; A.N – Ammonium nitrate; A.S – Ammonium sulphate; A.C – Ammonium chloride; P.N – Potassium nitrate]

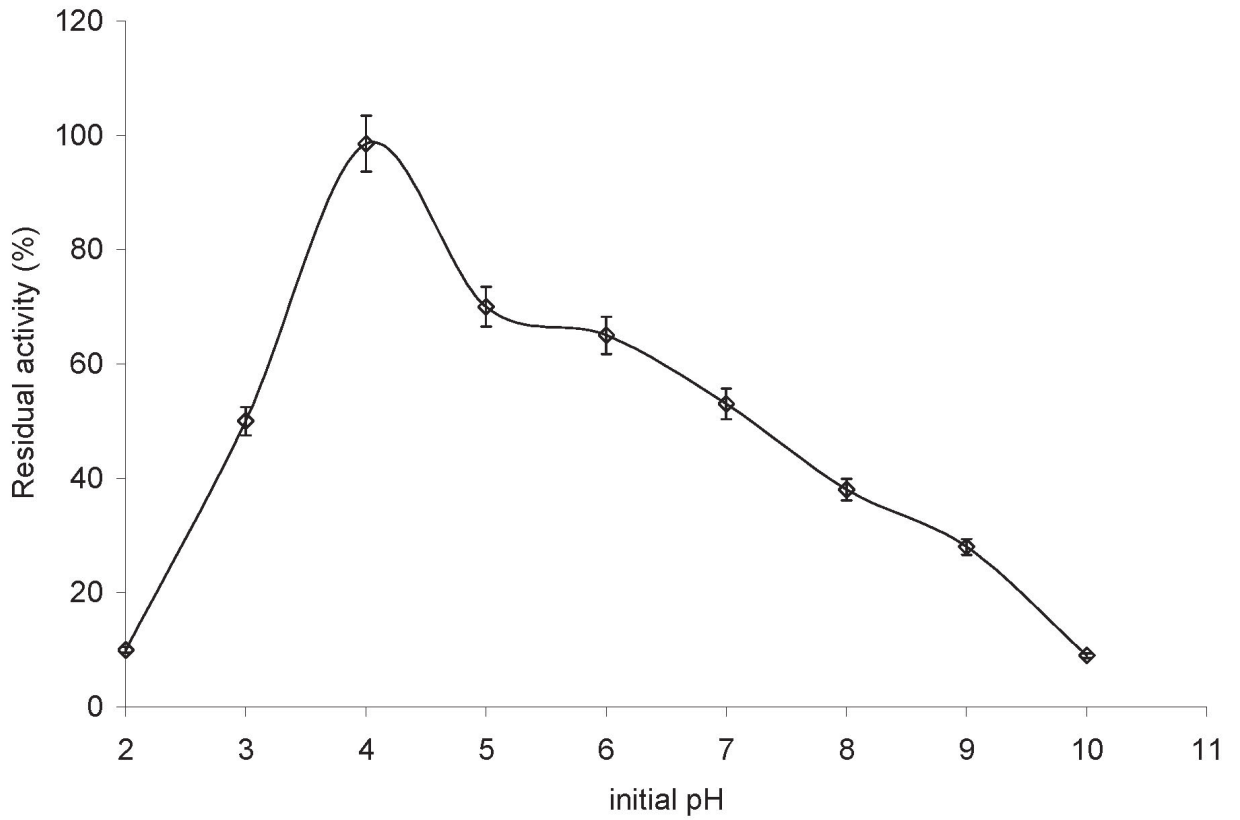

Figure 4. Effect of initial pH on activity of *Bacillus aerophilus* KGJ2 xylanase. The activity assay was conducted using following buffers: sodium citrate (pH 2 – 6), sodium phosphate (pH 7), tris-HCl (pH 8) and glycine -NaOH (pH 9–10)

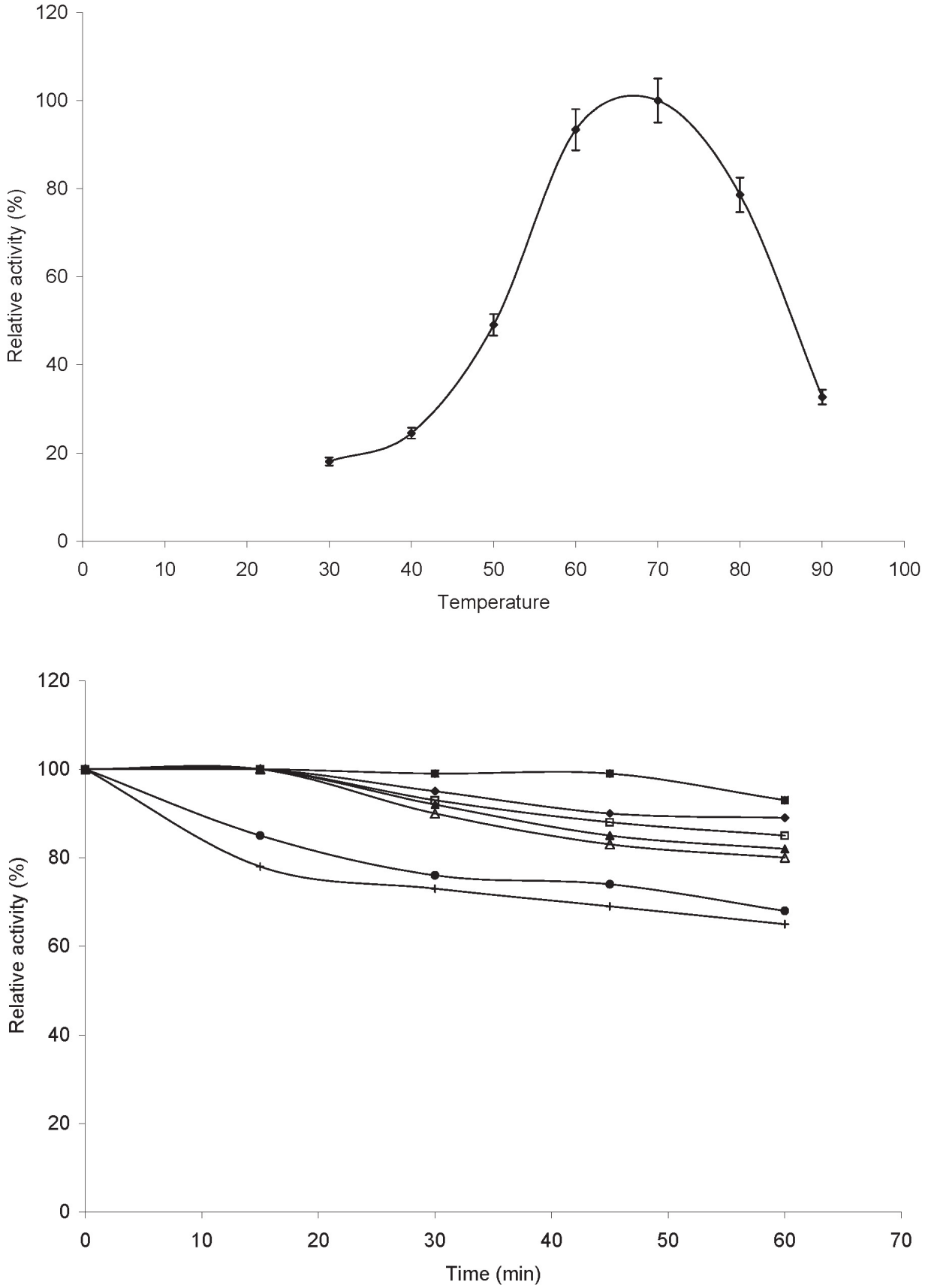

Figure 5. (a) Effect of temperature on xylanase activity. **(b)** Thermostability of *Bacillus aerophilus* KGJ2 xylanase. The enzyme was pre-incubated at different temperatures (30-90 °C) and residual activity was assayed after 1 h.

organic and inorganic sources were used efficiently for xylanase production. Giridhar *et al*., 2010 [25] have also reported that *Gracillabacillus* sp. showed similar effects in organic and inorganic nitrogen sources on cultivation and production. The optimum xylanalytic activity produced by *B.mojavensis* A21 was achieved with inorganic nitrogen source like ammonium chloride followed by yeast extract and soy peptone [26]. Highest xylanase production was achieved due to the presence of nitrogen sources, carbohydrates and other compounds in the fermentation medium, which is easily utilized by the growing microorganism and enhance the xylanase producing ability [27]. Along with beef extract, calcium chlorides in the fermentation medium act as a growth supplements and promote extra cellular xylanase production [28]. Thus, the beef extract along with growth supplements in the fermentation medium shows a significant effect on *Bacillus aerophilus* KGJ2 xylanase production.

Effect of initial pH

Growth of any microorganism is highly influenced by the medium pH as pH influences the transport of nutrients as well as the enzymatic systems in microorganism [8]. If the pH of the medium is unfavorable, the growth and hence xylanase production may be limited due to substrate inaccessibility. So, initial pH of the medium is an important parameter for enzyme production. To study the influence of initial pH on xylanase enzyme production, the birch wood xylan containing fermentation medium was adjusted using various buffers, such as sodium citrate (pH 2.0–6.0), sodium phosphate (pH 7.0), Tris-hydrochloride (pH 8.0) and glycine – sodium hydroxide (pH 9.0–10.0) each at 20 mM in the xylanase enzyme at the optimum temperature at 70 ºC. The result of specific activity of xylanase enzyme in submerged fermentation is shown in Fig. 4. It was noted that xylanase production was minimum at pH 2.0 because of low growth rate of cells at this initial pH. Maximum xylanase production was appeared at an initial pH of 4.0. Similar pH optimum for xylanase production was reported by various researchers. *Bacillus* sp., 11-1S, a strain of an acidophilic bacteria produced an extra cellular xylanase on the growth of xylan. The enzyme had a pH optimum for activity at 4.0 and its stability range was pH 2.0-6.0 [29]. Similar acidophilic bacteria was also found in *Bacillus* sp.such as *Bacillus coagulans* [30], *Bacillus acidocaldarius*; a new species of the genus *Bacillus*. [31], and *Bacillus acidoterrestris* [32].

Effect of temperature activity and stability

Influence of temperature on xylanase activity and stability on submerged fermentation is one of the important parameter. Fig. 5a & Fig. 5b shows the activity of partially purified xylanase which was minimum (11.2 U/ml) at 30 ^{0}C and the maximal (62 U/ml) at about 70 ^{0}C. More than 80% of the maximum activity was retained between 30 ^{0}C to 60 ^{0}C and about 100% activity was retained at 70 ^{0}C. Our observations showed that the xylanase from *Bacillus aerophilus* KGJ2 could be useful for industrial applications at the temperature range of 40 ^{0}C –70 °C. Xylanase with similar temperature optima had been reported from *Bacillus licheniformis* in the broad range of 40 ^{0}C to 100 ^{0}C [28]. In *Bacillus* sp., 11-1S the temperature optimum was 80 ^{0}C. However, the enzyme retained full activity after incubation at 70 ^{0}C for 15 min. [29]. Similarly *Bacillus steareothermophilus* shows highest reaction rate at 75 ^{0}C and its relative activity was 100% [11]. Thermal stabile xylanase finds potential applications in many industries [33 - 36].

Xylanase enzyme produced by *Bacillus aerophilus* KGJ2, shows an interesting characteristics and properties and it appears to be a prospective candidate for application in feed and food industries. Particularly it is interesting to see that it shows an activity and stability at pH 4.0 and temperature 70 ^{0}C. Xylanase with low pH optimum and good stability under acidic conditions are suitable for feed industry due to extremely low pH prevailing in the digestive tract. With the application of this acid-stable enzyme, the nutrient availability in ruminant feed can be improved [37, 38]. Specific applications of acidophilic xylanase also include its use in maceration and clarification of fruit juices [39]. The acidophilic xylanase obtained from *Bacillus aerophilus* KGJ2 has advantage of utilizing efficiently under harsh conditions in feed and food industry.

Conclusion

The xylanase producing bacterial culture was successfully isolated from paper mill effluent and it could be identified as *Bacillus aerophilus* KGJ2. The xylanase enzyme was purified and characterized for its activity and stability. It showed optimal activity at pH 4.0 and at a temperature of 70 °C, which revealed that it was acidophilic and thermotolerant nature. From these results, the enzyme produced by the isolated strain, *Bacillus aerophilus* KGJ2, appear to have potential application in animal feed industry.

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Conflict of Interest: We declare that there is no conflict of interest.

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