



Full Length Article

Studies on Chitinase isolation and thermostability between *Bacillus Circulance* Strain L2 and *Bacillus Licheniformis* Strain 2J-1

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ABSTRACT

Bacillus circulance strain L2 and *Bacillus licheniformis* strain 2J-1 were isolated from soil samples of different places of Akola district and were identified by 16s rDNA sequencing. *Bacillus circulance* strain L2 was grown on media containing 1% colloidal chitin having temperature 40°C and pH 7, maximum enzyme production was observed for the period of 5 days. *Bacillus licheniformis* strain 2J-1 was also grown on media containing 1% colloidal chitin having temperature 50°C and pH 7, maximum enzyme production was observed for the period of 6 days. Chitinase was purified through ammonium sulfate precipitation, dialysis and gel filtration chromatography, chitinase optimum temperature was found at 40°C and pH 7.5 for *Bacillus circulance* strain L2 and optimum temperature 45°C and pH 7.3 for *Bacillus licheniformis* strain 2J-1. Thermostability studies were carried out and finding of this studies reveal that *Bacillus licheniformis* strain 2J is more thermostable and higher chitinase producer as compare to *Bacillus circulance* strain L2 which is useful for industries and biotechnological applications.

Key words: : *Bacillus* *circulance* strain L2, *Bacillus* *licheniformis* strain 2J-1, Chitinase, Chitinase isolation, , Thermo stable chitinase

INTRODUCTION

Chitinases (EC 3.2.1.14), a group of enzymes capable of degrading chitin to low-molecular-weight products produce by many microorganisms such as *Bacillus amyloliquefaciens* SM3 (Merina *et al.*, 2012), *Aspergillus terreus* (Ghanem *et al.*, 2010), *Serratia marcescens* SMG (Das, 2011), bacteria including *Aeromonas* (Sitrit, 1995), *Alteromonas* (Tsujiibo *et al.*, 1993), *Bacillus* (Watanabe *et al.*, 1990), *Serratia* (Jones *et al.*, 1986), *Streptomyces* (Blaak and Schrempf, 1995), *Enterobacter* (Chernin *et al.*, 1995), *Vibrio* (Bassler *et al.*, 1991) and *Escherichia* (West and Colwell, 1984). Chitinase producer were isolated from different environments including soil (Kuddus and Ahmad, 2013; Khan and Khan, 2011; Wang, 1997),

shellfish waste (Wang and Hwang, 2001), marine sediment (Annamalai *et al.*, 2010), soil samples of hot spring (Dai *et al.*, 2011), Chilli rhizosphere (Narasimhan and Shivakumar, 2012) etc. The biodegradation of chitin requires the synergistic action of several hydrolytic enzymes for efficient and complete breakdown. The combined action of endochitinases (EC 3.2.1.14) and exochitinases [(chitobiosidases) and β -N-acetyl hexosaminidase (EC 3.2.1.82)] results in the degradation of chitin polymer into the soluble N-acetyl D-glucosamine (Gkargkas *et al.*, 2004). Chitinase enzymes have a wide range of biotechnological applications, especially in the production of chito-oligosaccharides and N-acetyl D-glucosamine (Pichyangkura *et al.*, 2002),

biocontrol of pathogenic fungi (Chernin *et al.*, 1997; Mathivanan *et al.*, 1998; Farag *et al.*, 2014), preparation of sphaeroplasts and protoplasts from yeast and fungal species (Mizuno *et al.*, 1997; Balasubramaniam *et al.*, 2003) and bioconversion of chitin waste to single cell protein (Vyas and Deshpande, 1991). Because of its thermo stability chitinase enzymes are widespread in gram-positive bacteria, e.g., spore-forming genera *Bacillus* and *Clostridium*.

This paper describes studies on chitinase isolation of *Bacillus circulans* strain L2 and *Bacillus licheniformis* strain 2J-1 from soil samples of Akola district. Main objective and Scope of this study is estimation of more thermo stable and higher chitinase producer between two studied *Bacillus* spp.

MATERIALS AND METHODS

Collection of soil sample

Different places like agricultural land, bank of river and play ground were selected for collection of soil samples. Upper part of soil approximately 10-15 cm was removed and about 500 gm of soil was collected from four different corners and middle region of above mentioned places of Akola district in sterile polythene bags with the help of sterile sickle. The samples were preserved at 4°C till further use for microbial analysis.

Preparation of colloidal chitin:

Colloidal chitin was prepared by the method of Rod-riguez-Kabana *et al.*, 1983. 50 gm chitin was finely crushed with mortar and pestle followed by grinding in mixer. This chitin was partially hydrolyzed with 400 ml 10 N HCl for 2-3 hrs with continuous shaking at room temperature. After shaking to specified time chitin appears in colloidal form. The colloidal chitin was washed several times with large volumes of distilled water to adjust pH to 7.0.

Preparation of Chitin agar plates having calcofluor white M2R dye:

Chitin agar plates were prepared using nutrient media with colloidal chitin and calcofluor white M2R dye (0.001%) as per method of Vadiya *et al.*, 2003, having following composition
yeast extract:1.5g, peptone:5g, colloidal chitin:10g, NaCl:2.5g, calcofluor white M2R dye:0.005g, agar:10g, Distilled water:500ml, pH:7.

Microorganism Isolation

Initial screening has been performed by serial dilutions technique. Soil diluted samples were

inoculated on chitin agar plates containing calcofluor white M2R dye, after incubation at 37°C for 3- 5 days well grown colonies having zone of clearance were selected for Gram staining, and morphological characters. After confirmation of bacillus spp. these colonies were used for preparation of pure cultures and further identification test.

Identification of the isolates

Isolates were identified through 16S rDNA sequencing. Physiological and biochemical properties of isolates were also determined according to Bergey's Manual of systematic Bacteriology

Culture condition for chitinase production

production of Chitinase was done in culture media in rotating shake flask at 100 rpm in 500ml conical flask containing 100 ml production media having following concentration and cultural condition.

For *Bacillus circulans* strain L2 :

1% colloidal chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% K_2HPO_4 , 0.01% FeSO_4 , 0.01% ZnSO_4 , 0.01% MnCl_2 , 0.02% $(\text{NH}_4)_2\text{SO}_4$, pH 7, Temperature 40°C, 100rpm for 05 days.

For *Bacillus licheniformis* strain 2J-1:

1% colloidal chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4 , 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% K_2HPO_4 , 0.01% FeSO_4 , 0.01% ZnSO_4 , 0.01% MnCl_2 , 0.02% $(\text{NH}_4)_2\text{SO}_4$, pH 7, Temperature 50°C, 100rpm for 06 days.

Chitinase Assay

Chitinase activity was determined colorimetrically at 540nm by detecting the amount of *N*-acetylglucosamine (GlcNAc) released from chitin substrate (Sigma protocol: SCCHIT01.001).

Unit definition: One unit will liberate 1.0 mg of *N*-acetyl-D-glucosamine from chitin per hour in two step reaction with β -*N*-Acetylglucosaminidase under the specified condition.

Protein concentration was measured according to Bradford's method (1976) using bovine serum albumin as the standard.

Purification of chitinase

The cultures from shake flask level were harvested after specified time, as mentioned above; cells were removed by centrifugation at 6000 x g for 30 mins at 4 °C. The cell free supernatant was used as crude enzyme. The crude chitinase was precipitated with ammonium sulphate at 80% saturation levels and allowed to stand overnight at 4 °C.

Next day precipitates were collected , centrifuged at 10000 x g at 4 °C for 30 min. , dialyzed against 50mM citrate phosphate buffer pH 7 for 24 hrs. and loaded on gel filtration column (1.5 x 15cm) , sephadex G-100 , 2ml active fractions were pooled.

Optimization of temperature and pH:

Effect of temperature: Optimum temperature was measured by incubating the reaction mixture at different temperature to assay the enzyme activity. Thermal stability was examine by exposure of the enzyme to different temperature for 120 min. and enzyme activity was measure under the assay condition.

Effect of pH: Optimum pH for the chitinase activity was measured at different pH values. The buffers used were: citric acid –Na2HPO4 buffer (pH 3-7), Phosphate buffer (pH 7-8), Boric acid-NaOH buffer (pH 8-9.5) (Alkaline Borate Buffer), phosphate-NaOH buffer (pH 9.5-12)

RESULTS AND DISCUSSION

Microorganism Isolation

after incubation of chitin agar plate having calcofluor white M2R dye at 37°C for 3- 6 days well grown colonies having zone of clearance were observed (Fig.1)

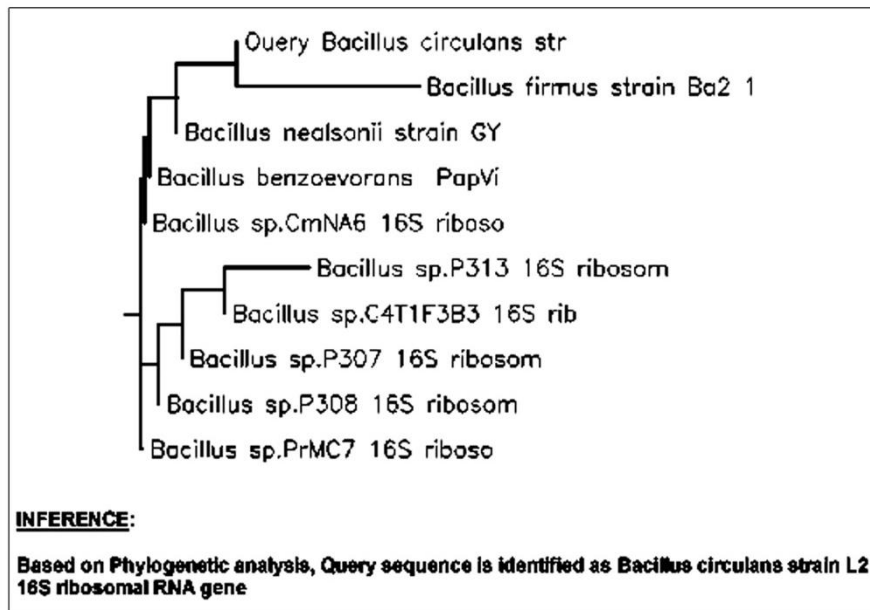


Fig. 2 Phylogenetic tree of Bacillus Circulance strain L2 (Query Bacillus) based on 16s rDNA analysis

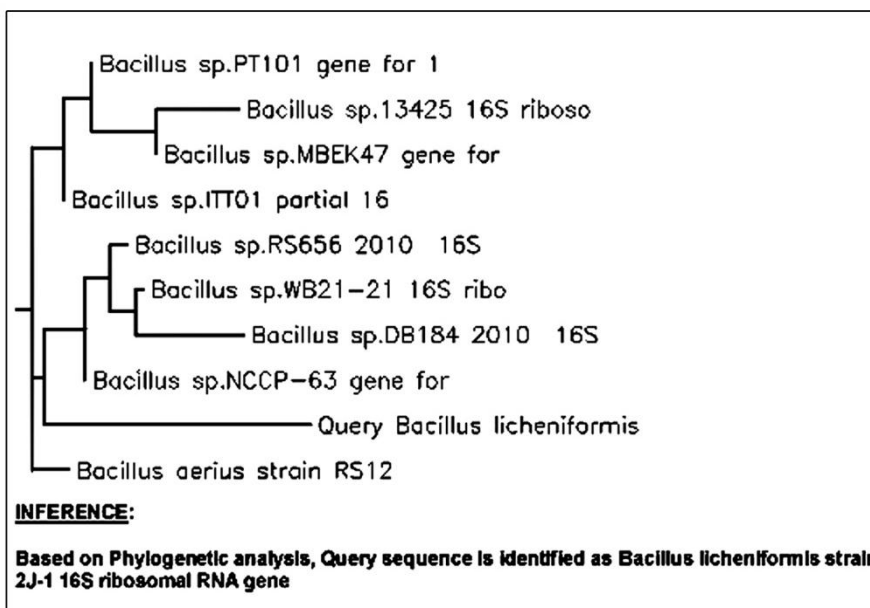
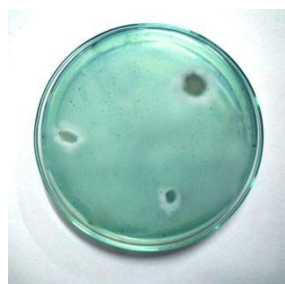
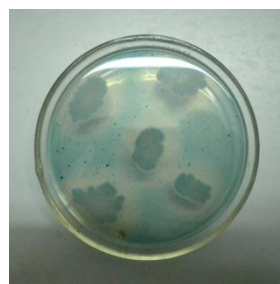


Fig.3: Phylogenetic tree of Bacillus licheniformis strain 2J-1 (Query Bacillus) based on 16s rDNA analysis



A: *Bacillus circulance* strain L2



B: *Bacillus licheniformis* strain 2J-1

Fig 1 Chitin agar plates showing zone of clearance

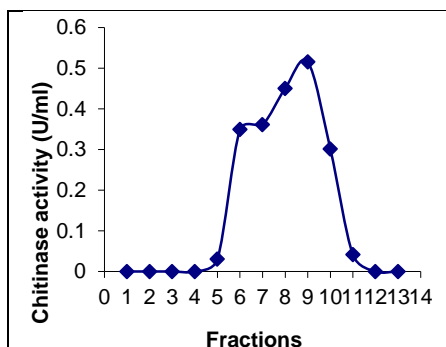


Fig.6: Gel filtration chromatography for *Bacillus circulance* strain L2

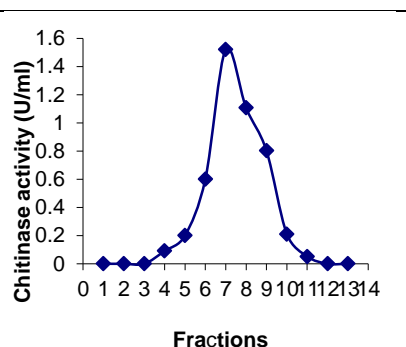


Fig.7: Gel filtration chromatography for *Bacillus licheniformis* strain 2J-1

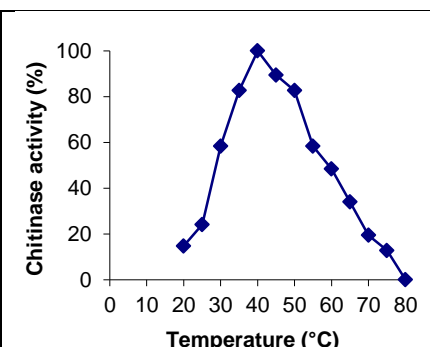


Fig.8: Effect of temperature on chitinase activity for *Bacillus circulance* strain L2

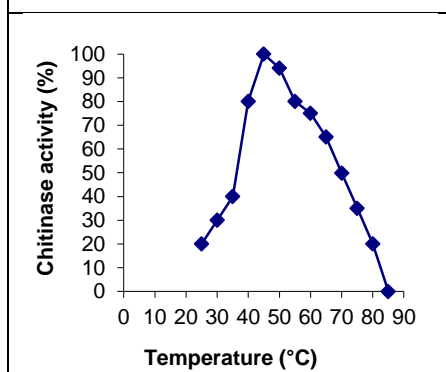


Fig.9: Effect of temperature on chitinase activity for *Bacillus licheniformis* strain 2J-1

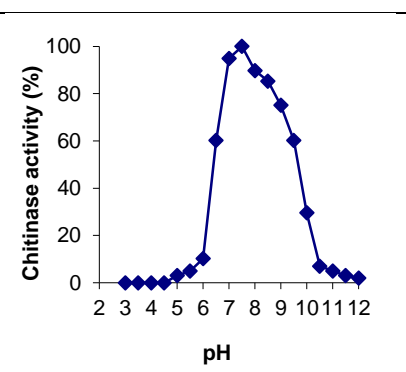


Fig.10: Effect of pH on chitinase activity for *Bacillus circulance* strain L2

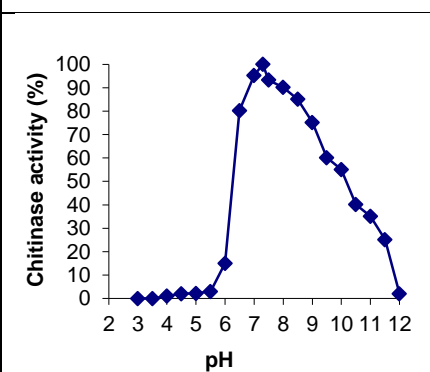


Fig.11: Effect of pH on chitinase activity for *Bacillus licheniformis* strain 2J-1

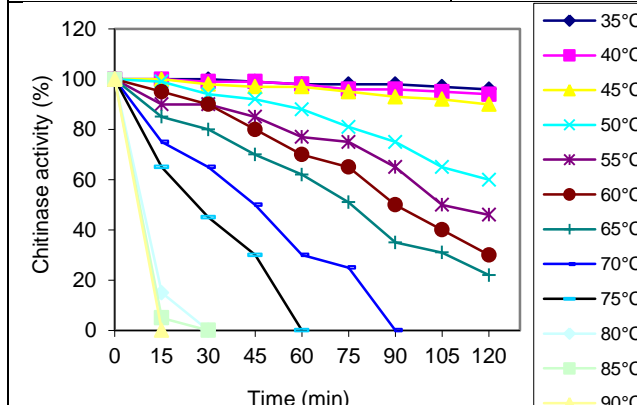


Fig.12 Thermo stability of chitinase from *Bacillus circulance* strain L2

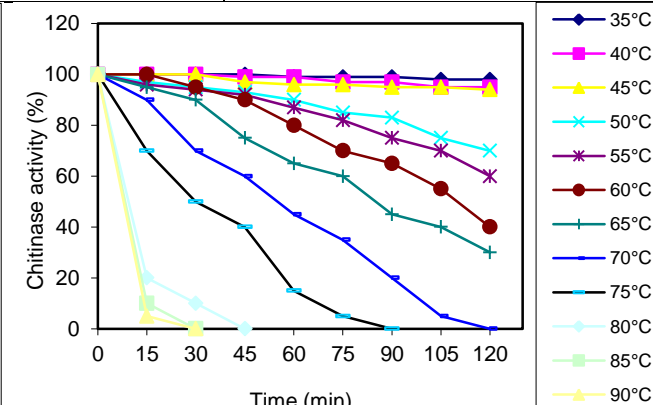


Fig.13. Thermo stability of chitinase from *Bacillus licheniformis* strain 2J-1

Table 1: Biochemical characteristics of isolates from different soil.

Tests		*B1	⁰ B2
Catalase		+	+
Anaerobic growth		-	+
Voges-preskauer test		-	+
Acid from :	D-Glucose	+	+
	L-Arabinose	+	+
	D-Xylose	+	+
	D-Mannitol	+	+
Gas from glucose		-	-
Hydrolysis of	Casein	+	+
	Gelatin	+	+
	Starch	+	+
Plate assay of chitin		+	+
Degradation of Tyrosine		-	-
Deamination of phenyl alanine		-	-
Egg-yolk Lecithinase		-	-
Nitrate reduced to nitrite		-	+
Formation of Indol		-	-
Growth at pH	6.8 Nutrient broth	+	+
	5.7 Nutrient broth	+	+
Growth in presence of lysozymes		+	+
Growth in NaCl conc.	2%	+	+
	5%	+	+
	7%	-	+
	10%	-	-
Growth at diff.Temp.	5°	-	-
	10°	-	-
	30°	+	+
	40°	+	+
	50°	-	+
	55°	-	+
65°	-	-	

*B1= *Bacillus circulance* strain L2, ⁰B2 = *Bacillus licheniformis* strain 2J-1

Identification of the isolates

Through 16s rDNA sequencing isolates have been identified as *Bacillus circulance* strain L2 and *Bacillus licheniformis* strain 2J-1 . Fig 2 and fig 3 showing phylogenetic tree of *Bacillus circulance* strain L2 and *Bacillus licheniformis* strain 2J-1

respectively . Table 1 showing biochemical characters of isolates.

Purification of chitinase

After centrifugation ,cell free supernatant was precipitated with ammonium sulphate at 80% saturation level followed by dialysis and column chromatographic separation.

Fig 6 and Fig.7 showing protein elution pattern of gel filtration chromatography for *Bacillus circulance* strain L2 and *Bacillus licheniformis* strain 2J-1 respectively. Active fractions were pooled and preserved for further use.

Optimization of Temperature and pH:

Fig.8 and fig.9 showing the effect of temperature on enzyme activity for *Bacillus circulance* strain L2 and *Bacillus licheniformis* strain 2J-1 respectively. Optimum temperature for *Bacillus circulance* strain L2 is 40°C and for *Bacillus licheniformis* strain 2J-1 is 45°C.

Fig.10 and fig.11 showing the effect of pH on chitinase activity for for *Bacillus circulance* strain L2 and *Bacillus licheniformis* strain 2J-1 respectively. Optimum pH for *Bacillus circulance* strain L2 is 7.5 and for *Bacillus licheniformis* strain 2J-1 is 7.3

Thermo stability studies:

Fig. 12 and Fig.13 showing the thermo stability studies of chitinase isolated from *Bacillus circulance* strain L2 and *Bacillus licheniformis* strain 2J-1 respectively. Chitinase from *Bacillus circulance* strain L2 is remarkably stable upto temp.55°C, moderately stable for temp.60°C and 65°C and poorly stable above temp.70°C . chitinase from *Bacillus licheniformis* strain 2J-1 remarkable stable up to temp.60°C ,moderately stable for temp. 65 and 70°C, poorly stable above temp.75°C. On the other hand Dai *et al.*(2011) studied thermo stability of chitinase enzyme from *Bacillus* sp. Hu1 and reported that chitinase activity was retained at 60° for 100 min., Bansode and Bajekal(2006) studied thermo stability of chitinase enzyme isolated from different microorganism of Lonar lake and reported that all chitinases having stability range from 25°C to 60°C.

Chitinolytic *Bacillus* have been isolated from soil samples of Akola distt. by observing zone of clearance for 3-6 days using colloidal chitin, similar zone of clearance have been observed by Kuddus and Ahmad (2013) using colloidal Chitin (without dye) for isolation of chitinolytic bacterial from soil samples.

In this study isolates have been identified as *Bacillus circulance* strain L2 and *Bacillus licheniformis* strain 2J-1 through 16S rDNA sequencing similarly Singh(2010) identified *Paenibacillus* sp. D1 by 16S rDNA sequencing the NCBI GeneBank accession no. for which was given as DQ908925, Merina *et al.*(2012) identified *Bacillus amyloliquefaciens* SM3 by performing polymerase chain reaction (PCR) to amplify the 16S rRNA gene. In this study crude enzyme was precipitated at 80% ammonium sulphate saturation level which is comparable with Dai *et al.*(2011) use 80% ammonium sulphate for chitinase purification from thermophilic *Bacillus* sp. Hu1, Annamalai *et al.*(2010) use 60% ammonium sulphate for chitinase precipitation from *Micrococcus* sp.AG84. In purification steps for gel filtration chromatography active fractions (for chitinase activity) were found between 06 to 10 for *Bacillus circulance* strain L2 and 06 to 09 for *Bacillus licheniformis* strain 2J-1. Chitinase optimum temperature for *Bacillus circulance* strain L2 is 40°C and for *Bacillus licheniformis* strain 2J-1 is 45°C, chitinase optimum pH for *Bacillus circulance* strain L2 is 7.5 and for *Bacillus licheniformis* strain 2J-1 is 7.3 which is comparable as reported by Annamalai *et al.* (2010) that chitinase activity from *Micrococcus* sp.AG84 was found to be maximum at 45°C and pH of 8.0. Ghanem *et al.*(2010) reported higher chitinase activity from *Aspergillus terreus* at temperature of 50°C and pH 6, Merina *et al.*(2012) reported that maximum chitinase activity from *Bacillus amyloliquefaciens* SM3 was observed at 35° and pH of 7.5, Kuddus and Ahmad(2013) reported maximum chitinase production for *Aeromonas hydrophila* HS4 at 37 °C and pH 8.0 after 24–48 h of incubation and for *Aeromonas punctata* HS6 at 37 °C and pH 7 after 48 h incubation, Sing(2010) reported optimum temperature and pH for *Paenibacillus* sp. D1 as 50°C and 5.0 respectively. Thermostability studies have been carried out along with zone of clearance study and concluded that *Bacillus licheniformis* strain 2J-1 is more thermostable and higher chitinase producer than *Bacillus circulance* strain L2. More thermo stability of chitinase from *Bacillus licheniformis* strain 2J-1 makes it more useful for industrial significance and biotechnological applications.

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