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Purification, characterization and potential detergent industry application of a thermostable α-amylase from *Bacillus licheniformis* RA31

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α-Amylase is an endoamylase catalysing the degradation of starch into maltose, maltotriose and glucose. The enzyme isolated from microbial sources possess unique properties of thermostability thus making it a useful tool in the detergent industry. Here, we explored a strain of thermophilic bacteria *Bacillus licheniformis* for its potential application in detergent industry. The *B. licheniformis* RA31 was isolated from soil samples of hot spring in Rampur district of Himachal Pradesh, India and grown on optimized media to produce thermostable α-amylase. The enzyme was ethanol precipitated, purified (12.93 fold, 55.52% yield and 621.93 U/mg specific activity) to homogeneity with a single band on SDS-PAGE (66 kDa) and native-PAGE (68 kDa). Purified enzyme displayed best activity in pH 8 buffer and ~80% activity was retained in pH 7 and 10. It showed temperature optima at 70°C. Its activity was decreased at 70°C (70% after 4 h), 80°C (65% after 4 h) and 90°C (50% after 1 h). The enzyme was stimulated (126%; 5 mM) by barium chloride. It was relatively stable in the presence of commercial detergents (109-125%), SDS (84%), Tween 20 (88%), EDTA (72%) and β-ME (70% at 10 mM). K_m and V_{max} for the enzymatic hydrolysis of starch were 0.339 mg/mL and 1.450 mg/min, respectively. The enzyme revealed the highest specificity towards wheat starch granule (140% after 1 h) and SEM analysis displayed its biodegradation (2-10 h). Improved cleaning efficiency of potato curry stained fine cotton clothes were observed with enzyme assisted detergent advance treatment (0.02% w/v). The enzyme showed potential applications in detergent industry.

Keywords: Laundry, Stain removal

Starch is an abundant, inexpensive, versatile and renewable resource on earth. It is present in the form of major storage carbohydrate in plants. It is commercially obtained from various sources including corn-82%, wheat-8% (cereals), potato-5% (tubers) and cassava-5% (root)[1]. Normal native starches on average contain 20-30% amylose and 70-80% amylopectin[2]. Amylose is of moderate size (10^6 Da) and generally have linear backbone of α-(1-4)-linked glucan, while amylopectin is the largest biopolymer (10^8 Da) and about 5% are branched off to another chain of α-(1-6) glycosidic bonds[3]. According to projections, the global modified starch market will grow at a compounded annual growth rate (CAGR) of 4.62% from 2022 to 2027.

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Abbreviations: β-ME, β-Mercaptoethanol; DMSO, Dimethyl sulfoxide; EDTA: Ethylenediamine; tetraacetic acid, PMSF, Phenylmethylsulfonyl fluoride

α-Amylase (1,4-α-glucan glucanohydrolase; EC 3.2.1.1) is an endo-acting glycosyl hydrolases which cleave α-1,4-glucosidic linkages in starch, glycogen, and related carbohydrates[4], yielding glucose, oligosaccharides, and/or dextrin. It is primarily present in plants, animals, bacteria and fungi. It is the main representative of GH13 family, showing evolutionarily diversty with major industrial applications[5].

Thermophile microorganisms have been isolated from habitats like hot spring of Yellowstone National Park, USA[6], Tibetan Plateau China[7], Zavarzin thermal spring, Russia[8], Kagoshima hot spring, Japan[9] , Pharaoh's Bath hot spring in Egypt[10]. Geothermal areas of Turkey[11], Taptapani spring, Odisha India[12], Manikaran and Yumthang hot springs, Indian Himalayas (Himachal Pradesh and North Sikkim district of Sikkim, respectively)[13], and hot spring in Rampur district of Himachal Pradesh India[14]. The thermophiles mostly produce thermostable enzyme[15]. Applications of thermozymes are continuously increasing because of their stable, faster and

economical processing[16]. The commercial microbial strains producing α-amylase are *Bacillus subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *Aspergillus oryzae*[17]. Novozymes produced commercial α-amylases *like Liquozyme X, Duramyl®, Stainzyme®, Stainzyme®* Plus, Termamyl® and Termamyl® Ultra for efficient removal of starch stains from rice, grain, potato, etc.[18]. More than 50% of enzymes used in industrial processes are from fungi, >33% from bacteria, 8% from animals and 4% from plants[19].

α-amylase is used in house hold detergent formulations to remove gelatinized starchy stains (e.g. rice, pasta, gravy, potato, corn, baby food, etc.) from the fabric. It must be active and stable in the presence of detergent ingredients, such as other enzymes, surfactants, builders, various agents (antiredeposition, bleaching, oxidizing, sequestering, alkaline, suds control), etc. at the cleaning pH and temperatures during the washing process/storage. Complete and efficient stains removal requires the joint affords of all the detergent ingredients and mechanical functions of washing machines. The enzyme is widely used in various industries like food (baking, starch liquefaction, brewing, *etc.*), detergent, pharmaceutical, textile (desizing, warp sizing), pulp and paper, cosmetics, *etc.*[20].

In the present study, we have made an attempt to purify and characterize the thermostable α-amylase from thermophilic *Bacillus licheniformis* RA3 and explore its potential for degradation of raw starches, compatibility and cleaning efficiency with the commercial detergents.

Materials and Methods

Materials

DEAE-cellulose, gluteraldehyde and Sephadex G-100 were procured from Sigma (UK). Acrylamide, ammonium persulphate, bis-acrylamide, bromophenol blue, β-ME, coomassie brilliant blue R-250, phenol/chloroform/ isoamyl alcohol, saturated phenol and TEMED were from Amresco (USA). Tween 20, Triton X-100 (1% v/v), Folin ciocalteau's phenol were supplied by Merck (USA). Commercial detergents (Ariel, Ariel fresh clean, Ariel oxy blue, Henko, Mr. white, Rin advance, Surf excel quick wash, Surf excel blue, Surf excel, Tide, Tide plus, Vanish, Ezee and Safe wash) were purchased from local market. Molecular weight markers from Sigma (USA), all other chemicals and biochemicals were purchased from Himedia (India).

Bacterial isolate

Soil and water samples were collected from hot water spring in Rampur district of Himachal Pradesh, India and processed in the lab. Serially diluted water/soil samples were spread on starch (1% w/v) and LB agar plates followed by incubation at 50°C for 24 h. Isolates obtained were further sub cultured to obtain pure culture. The pure isolates were flooded with Lugol's reagent (mixture of iodine-potassium iodide). Formation of clear zones around the colonies indicated positive results. Four most amylolytic bacterial isolates were grown in the liquid medium at different temperature and pH. Efficient α-amylase producing bacterial isolate RA31 was selected for further studies. The 16S rRNA gene of bacterial isolate RA31 was amplified and sequenced. Gene sequence was submitted in NCBI database with accession number KT989882. It was identified as *Bacillus licheniformis* and is carrying *amyN* gene[14].

Enzyme production

Submerged fermentation was optimized for the production of α-amylase from *B. licheniformis* RA31 in 250 mL flasks containing soluble starch (1.5% w/v), tryptone and gelatin (0.5% w/v each), $MgCl_2$ (0.2% w/v), K_2HPO_4 (0.016 % w/v) at pH 8, inoculum age/size (24 h/0.4% v/v) and incubated at 50°C for 72 h. *B. licheniformis* RA31 was maintained on solid media in Petri plates and stored at 4°C for routine purpose while glycerol stocks were prepared for long term storage.

Protein and enzyme assay

The protein concentration was estimated by Lowry method using bovine serum albumin as a standard[21]. Amylase activity was estimated and quantified using glucose standard curve[22]. One unit (U) of enzyme activity was defined as the amount of the enzyme which released 1 μM of glucose per mL per minute at standard assay conditions. The specific activity was defined as U/mg protein.

Enzyme purification

Four days old culture was centrifuged at 10,000 rpm for 10 min at 4°C and supernatant was concentrated by ultrafiltration through a 10 kDa molecular weight cut-off membrane (Amicon USA). The concentrated crude enzyme was precipitated with pre cooled absolute ethanol (1:7 volume). The precipitates were recovered by centrifugation at 12,000 rpm for 60 min at 4°C. Precipitates were dissolved in minimum volume of buffer (0.1M

Tris-HCl buffer, pH 8). DEAE-cellulose column (15×1.5 cm) was equilibrated using 0.1M Tris-HCl, pH 8 buffer. Protein sample (2.5 mL) was loaded in the column and was eluted with two bed volumes of same buffer followed by a linear gradient of 0-1M sodium chloride in buffer. Fractions were collected at a flow rate of 0.5 ml/min. Fractions corresponding to the highest enzyme activity were pooled (2-9) and concentrated. Absorbance at 280 nm was measured for each fraction to determine the concentration of eluted protein.

Sephadex G-100 (1% w/v) suspended in 0.1M Tris-HCl buffer, pH 8 was swollen for 5 h in boiling water bath and packed in the column. Protein sample (2.5 mL) was loaded on the sephadex G-100 column. The column was eluted with same buffer in 2 mL of 60 fractions with a flow rate of 0.5 mL/min. The protein concentration and enzyme activity was estimated according to the standard protocols. The fractions corresponding to the highest enzyme activity were pooled (7-13), concentrated and selected for characterization of enzyme.

Characterization of α-amylase

Gel electrophoresis

SDS–PAGE was performed on 7.5% polyacrylamide gels as per standard method[23]. The molecular weight of purified amylase was determined with pre-stained protein ladder of 10 recombinant proteins ranging between 10-170 kDa (catalog no #SM0671 Fermentas). Native PAGE (4.5-10%) was performed as described in Manufacturer's Instructions manual (MKR 137, Sigma Chemicals). Urease (272, 545 kDa), BSA (66, 132 kDa), chicken egg albumin (45 kDa), carbonic anhydrase (29 kDa) and β–lactalbumin (14.2 kDa) were used as standard molecular mass markers.

Zymogram analysis

Native PAGE 7.5% was prepared and sample was loaded in duplicate in the wells. After the electrophoresis, the native gel was cut into two parts. First half of the gel was stained with coomassie brilliant blue R-250 dye staining solution to confirm subunit compositions. The second part was overlaid on agarose gel composed of 1% w/v soluble starch and 2% w/v agarose followed by incubation at 37°C for 24 h. After the incubation, the agarose gel was washed and stained with Lugol's reagent (mixture of iodine-potassium iodide). A sharp and clear band as a zone of clearance, against the black background indicated the enzymatic hydrolysis of the starch.

Effect of pH and temperature

The appropriate volume of α-amylase was incubated with soluble starch 1% w/v in different pH buffers (0.1M) *i.e* sodium citrate buffer (pH 4-5), sodium phosphate buffer (pH 6-8), sodium glycine buffer (pH 9-10). The optimal pH was determined by measuring the enzyme activity. Enzyme was also incubated with buffers to check the stability at regular intervals of time. The appropriate volume of α-amylase was incubated with soluble starch 1% w/v at different temperature range from 40-90°C. The optimal temperature was determined by measuring the enzyme activity. Enzyme was also incubated to check the stability at regular intervals of time using standard assay.

Effect of metal ions, detergent, surfactant, metal chelator, reducing agent, protein inhibitor and organic solvents on the enzyme activity

The appropriate volume of α-amylase was incubated with soluble starch (1% w/v) individually with sulphates and chlorides (5 mM) of metal ions; SDS, Tween 20, Triton X-100, EDTA, PMSF, β-ME (5 and 10 mM); organic solvents (10 and 20% v/v) such as acetic acid, acetone, acetonotrile, ethanol, methanol, propanol, butanol, chloroform, DMSO, toluene, glycerine, diethyl ether, hydrogen peroxide and H_2O_2) for 10 min at 50°C and enzyme activity was checked by standard assay conditions using appropriate controls.

TLC analysis

Nine hundred microlitre of starch (1% w/v) was incubated with 3U of enzyme at 50°C. The enzymatic reaction was stopped at various interval of time and the reaction mixture was precipitated with ethanol. The sugars present in the supernatant were spotted on TLC (Kieselgurh 60 P254 plates from Merck, India). A mixture of soluble starch, glucose, maltose and dextrin were used as a standard. TLC plate was put in the developing chamber saturated with ethyl acetate: isopropanol: water: pyridine (26:14:7:2 v/v). The enzymatic hydrolysis of soluble starch was detected by spraying the TLC plates with diphenylamine reagent followed by drying at 100°C for 15 min and Rf values were calculated.

Kinetic constants

The enzyme was incubated with various concentrations of starch (0.1-10%; w/v) for 10 min at 50°C. Concentration of reducing sugar was measured by DNS method. K_m and V_{max} values were determined from Lineweaver-Burk plot.

Substrate specificity

The enzyme was incubated with different grain starch individually (1% w/v; soluble starch (control), gram flour, wheat flour, millet flour, rice flour, water chest flour, oat flour, corn flour as substrates) in sodium phosphate buffer (0.1M; pH 8) at 50°C and 150 rpm. Samples were taken at regular intervals of time and enzyme activity was measured at standard assay conditions using soluble starch as control.

Enzymatic degradation of wheat starch granules-SEM analysis

One millilitre enzyme was incubated with wheat flour (1% w/v) in 9 mL sodium phosphate buffer (0.1M; pH 8) at 50°C, 150 rpm. Samples were taken at regular intervals of time, centrifuged (8000 rpm; 5 min) and enzyme activity of supernatant was measured at standard assay conditions. Wheat starch pellet was washed thrice and suspended in sodium phosphate buffer. Pellet suspension was stubbed on the adhesive tape for SEM analysis and photographed using a scanning electron microscopy (SEM Jeol JSM 6100).

Enzyme stability in commercial detergents

The enzyme was incubated with various detergents individually (Ariel, Ariel fresh clean, Ariel oxy blue, Ezee, Henko, Mr. white, Rin advance, Safe wash, Surf excel, Surf excel blue, Surf excel quick wash, Tide, Tide plus and Vanish) prepared in the concentration of 2.4 g/L as standard used commercially and 0.2, 0.5 and 1% w/v in combination of enzyme with detergent to check the stability of enzyme in these detergents. The enzyme activity was estimated at standard assay conditions.

The enzyme assisted detergent treatment to potato curry stained cotton clothes

The clean cotton cloth (5×5 cm) was stained with starch solution (potato curry) followed by drying at 50°C for 30 min. The dried stained pieces of cloth were placed in three beakers that contained (i) only water (ii) 0.2% w/v detergent solution (iii) 0.2% w/v detergent and 0.5 mL of purified enzyme respectively. The beaker was then incubated at 45°C under shaking conditions. After incubation, the cloth pieces were removed and washing efficiency was visually analyzed.

Results and Discussion

Purification of α-amylase from *Bacillus licheniformis* RA31 α-amylase was purified to 12.93 fold with 55.52% yield and 621.93 U/mg specific activity (Table 1). The protein was purified to homogeneity on SDS-PAGE with estimated molecular weight to be 65 kDa when compared to the pre-stained protein ladder (Fig. 1C; lanes 2 & 3). Native-PAGE with single band (70 kDa) confirms that the protein has been purified and the clear zone on zymogram corresponding to the purified band (Fig. 1A; lane 4 and 1B**)**.

Table 1 — Summary of purification of α-amylase from *Bacillus licheniformis* RA31

Purification steps	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	10753.6	223.8	48.1	1	100
Ethanol precipitation	7991.71	116.06	68.9	1.43	74.32
DEAE cellulose	6800.39	65.28	104.17	2.17	63.24
Sephadex G-100	5970.65	9.61	621.3	12.93	55.52

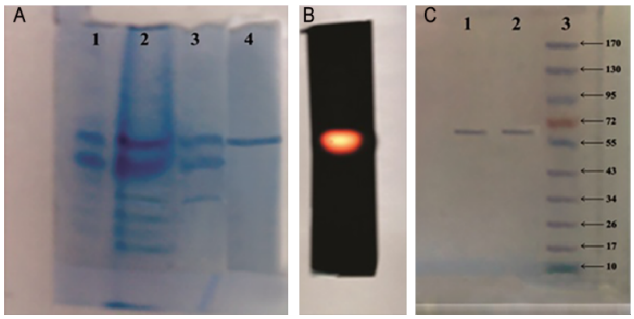

Fig. 1 — Native-PAGE and SDS-PAGE of α-amylase from *Bacillus licheniformis* RA3. (A) Native-PAGE with crude enzyme in lane 1, Precipitated protein in lane 2, Protein after DEAE chromatography in lane 3, and Protein after Sephadex G-100 in lane 4; (B) Native-PAGE; Zymogram corresponding to the purified protein in lane 4; and (C) SDS-PAGE; lanes 6 and 7 purified protein and lane 8 protein ladder.

Wide variation in molecular weight of α-amylases was determined using SDS-PAGE from *Geobacillus* sp. IIPTN (97 kDa)[24], *Anoxybacillus* sp. AH1 (85 kDa)[25], *Bacillus licheniformis* So-B3 (74 kDa)[26], *B. mojavensis* SO-10 (73 kDa)[27], *B. subtilis* XL8 (70.4 kDa)[28], *B. megaterium* (67 kDa)[29], *Geobacillus* sp. (62 kDa)[30], *Bacillus* sp. FW2 (55 kDa)[31], *Pseudomonas balearica* VITPS19 (47 kDa)[32], *Streptomyces* sp. Al-Dhabi-46 (44 kDa)[33] and *Bacillus* sp. Cos (38.02 kDa)[34].

Native molecular mass ranging from 38-68 kDa were reported for the enzymes from *Bacillus* sp. Cos[34], *B. subtilis* US586[35], *B. subtilis* Y25, *B. amyloliquefaciens* BH072[36], *B. licheniformis* B4-423[37], *Geobacillus* sp.K1C[38], *B. subtilis* PF1[39], *B. persicus*, *Alkalibacillus* sp.[40] and *B. subtilis*[41].

Characterization of purified α-amylase javensis

Effect of pH

Purified α-amylase from *B. licheniformis* RA31 showed no activity in acidic pH range (4-5) but after

that the activity started increasing and reached to maximum at pH 8. Similar pH optima was observed for the enzymes from *Streptomyces* sp. Al-Dhabi-46[33], *Geobacillus* sp. nov.[42], *Bacillus* MRS6, *B. licheniformis* AT70[43], *B. subtilis* Y25 and *Anoxybacillus* sp[44]. Higher pH maxima (9) was observed for the enzymes from *B. mojavensis* SA[45], *Bacillus* sp. BCC 01-50[46] and *Anoxybacillus* sp. YIM 342[47]. Lower pH optima in the range of 5-7 was observed for the enzymes from *B. subtilis* XL8[38], *Bacillus* sp. Cos[34], *B. subtilis* strain[41], *Geobacillus* bacterium (K1C)[42], *B. amyloliquefaciens* BH072, *B. licheniformis* B4-423[43], *B. licheniformis* So-B3[26], *B. subtilis*, *B. amyloliquefaciens* M37[48] and recombinant AmyZ1 gene cloned from and expressed in *E. coli*[49].

Purified α-amylase from *B. licheniformis* RA31 was optimally stable (100%) at pH 8 for more than 24 h and was reduced to ~80% at pH 7 and 9. Its stability was further decreased to 62, 49 and 39% at pH 10, 5 and 4, respectively after 8 h (Fig. 2A). pH stability of the enzymes in the range of 4-9 was reported from *Bacillus subtilis* XL8[28], *Bacillus* sp. Cos[34], *B. subtilis* strain[35], *Bacilli* sp., *Parageobacillus thermoglucosidasius* Pharon1 (MG965879)[10] and *Pontibacillus* sp. ZY recombinant AmyZ1 gene cloned and expressed in *E. coli*[49].

Effect of temperature

Purified α-amylase from *B. licheniformis* RA31 showed optimum activity (100%) at 80°C. It decreased to 85, 80 and 50% at 85, 90 and 100°C, respectively. Activity ranging 60-85% was observed between 40 and 75°C. Similar temperature optimum (80°C) was observed for the enzyme from *Geobacillus* bacterium (K1C)[37], *Anoxybacillus* sp. YIM 342[47], *A. flavithermus* SO-13 and *B. licheniformis* AS08E[50]. Wide range of temperature optima (100-70°C) was reported for the enzyme from different sources like *B. licheniformis* B4-423[38] at 100°C; *B. licheniformis* ATCC 9945a at 90°C[51]; *Bacillus subtilis* XL8[28]; *Bacillus* sp. Cos[34], *Geobacillus* sp. nov.[42] at 75°C; *B. mojavensis* A21 and *B. subtilis* QM3 at 70°C[52].

Purified α-amylase from *B. licheniformis* RA31 showed thermostability in range of 50-90°C. Enzyme activity was decreased to 70 and 65% at 70 and 80°C, respectively after 4 h. But at 90°C, it was sharply reduced to 50% after 1 h (Fig. 2B). Similarly, the enzyme from *Bacillus* sp. Cos and *B. licheniformis* So-B3 showed thermostability at 70°C, decreased continuously towards higher temperatures and the enzyme able to retain 40-46% activity at 100 and 90°C[34]. The enzyme from *Geobacillus* bacterium (K1C) retained 50% activity at 70 and 80°C for 6 and 2 h, respectively[38]. The enzyme from *Anoxybacillus* sp. YIM 342 was stable at 70-80°C[47]. α-Amylase from extremophoilic *Bacillus* sp. FW2 exhibited activity of 89 and 58% at 45 and 75°C, respectively in 2 h[31]. The enzyme of *B. amyloliquifaciens* TSWK1-1 was relatively stable for 24 h at 60°C, 12 h at 70°C and up to 3 h at 90°C[53]. α-Amylase from *B. subtilis* Y25 showed 70.39% enzyme activity in 30 min at 60°C.

Effect of metal ions, detergent, surfactant, metal chelator, reducing agent, protein inhibitor and organic solvents on the enzyme activity

Metal ions

Effect of metal ions on activity of purified α-amylase from *B. licheniformis* RA31 was studied at 5 mM. The enzyme was induced in the presence of

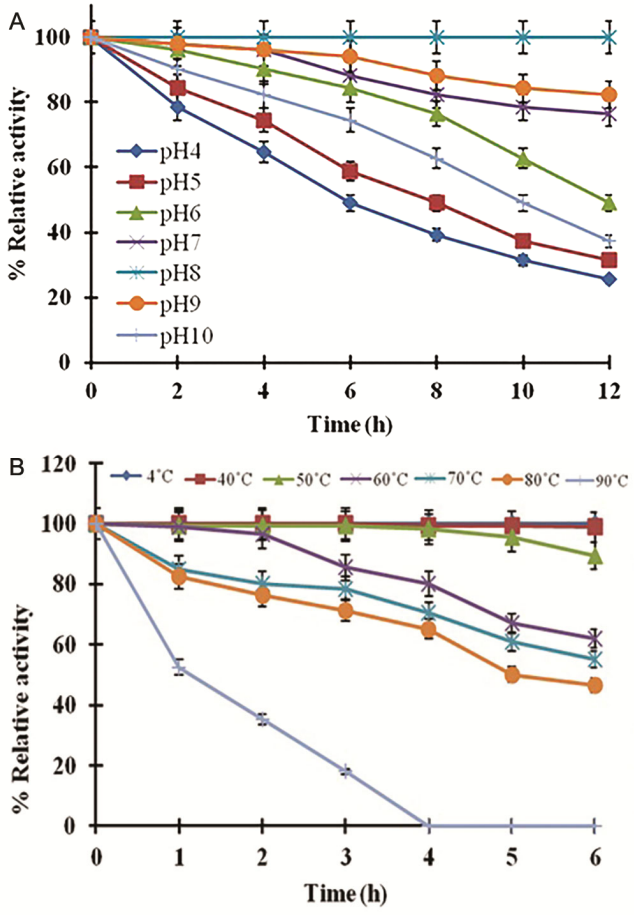

Fig. 2 — Effect of (A) pH; and (B) temperature on enzyme stability. [Each data point represents mean of three independent assays (standard errors were < 5% of the means)]

sulphate and chloride of barium to 116 and 126%, respectively. Decreased activity to 41% in the presence of calcium chloride and 100% with other metals (Al^{3+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{+}) was reported (Table 2).

Similar results were reported for the enzyme from *Geobacillus* sp. nov.[42], *B. methylotrophicus*[54], *B. amyloliquefaciens*[55] and *G. thermoleovorans*[56], where activity increased in the presence of Ba^{2+} at 10 mM to 158%, 5 mM to 135%, 1 mM to 224% and 5 mM to 104%, respectively. The enzyme from *B. licheniformis* So-B3 was activated by Ca^{2+} and Mn^{2+} showing a relative activity of 113 and 110%, respectively at 1.5 mM[26]. In another study, Ca^{2+}, Na^{+}, K^{+} and Mg^{2+} increased relative activity in a range of 130-110% in the enzyme from *B. subtilis* QM3. Metals that inhibited the enzyme from different bacterial sources were Fe^{3+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+} and Na^{+} at 10 mM (*Streptomyces* sp. Al-Dhabi-46)[33]; Ca^{2+}, Mn^{2+} and Cu^{2+} at 1 and 5 mM (*Geobacillus* (K1C)[30]; Ca^{2+} at 10 mM (*G. thermoleovorans*)[57]; Fe^{2+} (10 mM), Mn^{2+} (10-100 mM), Mg^{2+} and Sr^{2+} (100 mM) (*B. licheniformis*)[58]; Fe^{2+}, Zn^{2+} and Cu^{2+} at 1.5 mM (*B. mojavensis* SO-10[27] and *A. flavithermus*)[59] and Hg^{2+} at 1.5 mM (*B. licheniformis* So-B3)[26].

Detergent

Purified α-amylase from *B. licheniformis* RA31 in the presence of 5 and 10 mM SDS had decreased the relative activity to 94 and 84%, respectively (Table 3). Similarly, the enzymes from other bacterial sources were relatively stable and retained activity in the presence of SDS like 98.5% activity in 10 mM from *B. methylotrophicus*[54], 75% in 0.1% (w/v) from *Marinobacter* sp. EMB8[59], 73% in 1.5 mM from *A. ayderensis* FMB1[11] and 60% in 0.1, 0.2% from *Geobacillus* (K1C)[37]. However, increased activity of 126% was reported from *Alkalibacillus* sp[40].

Surfactants

Purified α-amylase from *B. licheniformis* RA31 in the presence of 5 and 10 mM Tween 20 revealed 94 and 88% activity, respectively (Table 3). Similarly, the enzymes from other bacterial sources were relatively stable and retained activity in the presence of Tween 20 such as ~80% in 0.1 and 0.5% from *Geobacillus* (K1C)[37] and 64.8% in 10 mM from *B. methylotrophicus*[53] was reported in the presence of 5 and 10 mM Triton X-100. In contrast, enzymes from various sources retained more activity in its presence like ~80% activity in 0.1 and 0.5% from *Geobacillus* (K1C)[38], 75% in 0.1% (w/v) from *Marinobacter* sp. EMB8[60]. However, the enzyme from *B. subtilis* PF1 was induced by 141%[39]. α-Amylase from a thermophilic actinobacteria strain *Actinomadura keratinilytica* Cpt29 showed 90% reduction in activity in Triton X-100 (2%) but was not affected by the addition of Tween 80 (10%)[61].

Metal chelator

Purified α-amylase from *B. licheniformis* RA31 presented relative activity to 89 and 72% at 5 and 10 mM EDTA, respectively (Table 3). Variable

Table 2 — Effect of metal ions on α-amylase activity.

Metal ions ($)	*Relative enzyme activity (%)	Metal ions (†)	*Relative enzyme activity (%)
Al^{3+}	Nil	Al^{3+}	Nil
Ba^{2+}	116	Ba^{2+}	126
Ca^{2+}	47	Ca^{2+}	41
Fe^{3+}	Nil	Fe^{3+}	Nil
Fe^{2+}	Nil	Fe^{2+}	Nil
K^{+}	71	K^{+}	86
Mn^{2+}	Nil	Mn^{2+}	Nil
Mg^{2+}	100	Mg^{2+}	98
Na^{+}	99	Na^{+}	98
Zn^{2+}	Nil	Zn^{2+}	Nil
Cu^{2+}	Nil	Cu^{2+}	Nil
Ni^{+}	Nil	Ni^{+}	Nil

[*Each data point represents mean of three independent assays. ($) Metal sulphates (†) metal chlorides]

Table 3 — Effect of additives (detergents/surfactants/chelating agent/protein inhibitor) and organic solvents on α-amylase [*Relative enzyme activity (%)] from *Bacillus licheniformis* RA31

Additives	5 mM	10 mM
Control	100	100
SDS	94	84
Tween 20	94	88
Triton X-100	28	10
EDTA	89	72
BME	89	70
PMSF	0	0
Organic solvents	10%	20%
Control	100	100
Acetic acid	0	0
Acetone	61	43
Acetonotrile	59	41
Ethanol	60	49
Methanol	58	41
Propanol	60	42
Butanol	63	44
Chloroform	30	11
DMSO	12	7
Toluene	51	25
Glycerine	44	8
Diethyl ether	0	0
H_2O_2	0	0

[*Each data point represents mean of three independent assays]

decreased activity was reported by different researchers like 95% activity from *Bacillus* sp. A3-15 in 5 mM EDTA[62], 70% from *B. licheniformis* in 1 mM[39], 64-78% from *B. mojavensis* SO-10 in 1-10 mM[27], 56% from *Geobacillus* sp. nov. in 10 mM[42], 50% from *B. subtilis* QM3, 35% from *B. licheniformis* So-B3 in 5 mM[26], 22% from *B. methylotrophicus* in 10 mM[54], 48% from *B. licheniformis* AS08E in 2 mM[51], 40% from *Alkalibacillus* sp. in 2 mM[40], 13-20% from *Anoxybacillus flavithermus* in 1-10 mM[63]. Significantly reduced activity of the enzyme in 1 and 5 mM EDTA was reported from *Geobacillus* (K1C)[37]. Complete loss of activity with 5 mM EDTA was observed from *Halophilic marinobacter*[60].

Protein inhibitor

Purified α-amylase from *B. licheniformis* RA31 was completely inhibited at 5 and 10 mM of PMSF (Table 3). Similar results were reported for the enzyme from *Anoxybacillus* sp. AH1[25], *Nesterenkonia* sp.[64] and *B. thermooleovorans* NP54[55]. However, enzyme activity in PMSF decreased to 65-77% in 1-10 mM from *B. mojavensis* SO-10[27], 66-71% in 1-10 mM from *B. licheniformis* So-B3[26], 89% in 5 mM from *B. subtilis* PF1[31], 87.9% in 10 mM from *B. methylotrophicus*[54], 77% in 10 mM from *Geo-bacillus* sp. nov.[42], 36% in 2 mM from *B. licheni-formis* AS08E[59] and 45-91% in 1-10 mM from *A. flavithermus*[63]. On the contrary, increased activity (131%) in 2 mM was reported from *Alkalibacillus*[40].

Reducing agent

Purified α-amylase from *B. licheniformis* RA31 showed relative activity to 89 and 70% in 5 and 10 mM of β-ME respectively (Table 3). Broad range of activity was retained (80-98%) in 1-10 mM from *B. mojavensis* SO-10[27], 1-10 mM from *A. flavithermus*[63] and *Geobacillus* (K1C) in 1 and 5 mM β-ME[38]. While enzyme from *B. licheniformis* So-B3 depicted 65-79% relative activity was observed in 1-10 mM of β-ME[26]. However, complete inhibition was reported for the enzyme (1% v/v) from *Alkalibacillus* sp.[40] and increased activity to 136% in 10 mM β-ME was observed from *Nesterenkonia* sp[64].

Organic solvents

Purified α-amylase from *B. licheniformis* RA31 was mixed with 10 and 20% v/v water miscible and immiscible organic solvents for 1 h. Maximum weakened activity was observed in 20% of all organic solvents. The relative enzyme activity was decreased to 41-49% in water-miscible organic solvents (acetone, acetonotrile, ethanol, methanol propanol and butanol). Strongly inhibition was observed in water-immiscible organic solvents (DMSO, 7%; glycerine, 8%; chloroform, 11%; and toluene, 25%) and complete inhibition in glacial acetic acid, diethyl ether and hydrogen peroxide (Table 3). Nearly similar results were reported with the enzyme from *Geobacillus thermoleovorans* NP54 in 20% v/v of acetone (100%), methanol (55%), ethanol (60%), propanol (14%), complete inhibition of activity in butanol and chloroform[55]. The relative activity of the enzyme from *G. thermoleovorans* in 15 and 25% v/v of ethanol was (73, 43%) and butanol (56, 60%), respectively[65]. The relative activity of the enzyme from *B. subtilis* PF1 in H_2O_2, glycerol (0.50% v/v) and DMSO (1% v/v) was reduced to 81, 51 and 30%, respectively[39]. The enzyme from *Geobacillus* (K1C) presented high tolerance to acetone and benzene; 50% activity was retained in the presence of propanol, ethanol, methanol and only 30% in case of isoamylalcohol[38]. Amylase from *Actinomadura keratinilytica* sp.Cpt29 showed considerable tolerance towards various organic solvents as it retains 70-94% of its activity in the presence of ethanol, acetone, and DMSO (10%)[61].

Kinetic constants

Purified α-amylase from *B. licheniformis* RA31 was incubated with various concentrations of starch (0.1-10%; w/v) for 10 min at 50°C. Concentration of reducing sugar was measured by DNS method at standard assay conditions. A Lineweaver-Burk plot depicted the K_m and V_{max} of 0.339 mg/mL and 1.450 mg/min, respectively (Fig. 3). However,

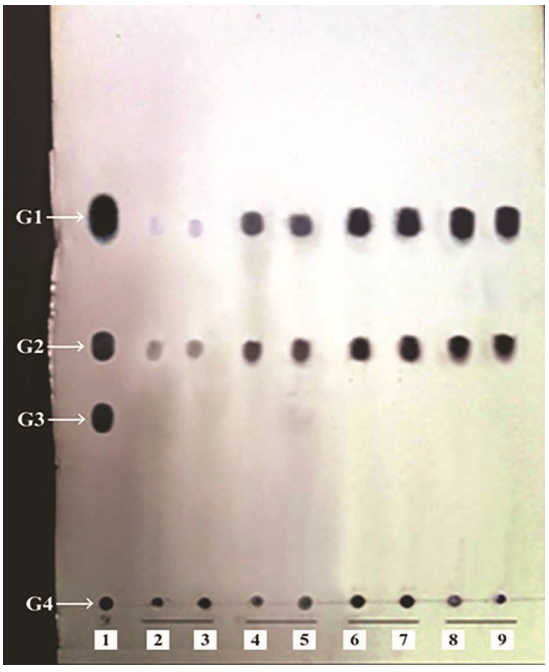

Fig. 3 — Lineweaver–Burk plot for the determination of K_m/V_{max}.

variable values of K_m & V_{max} were reported for the enzymes from *B. subtilis* XL8 (4.9 mg/mL and 1188 µmol/mL/mg)[28], *Bacillus persicus* (1.053 mg/mL and 356 µM/min), *Bacillus* sp. MTCC 1434 (2.85 g/L and 1.12 g/L)[66], *Exiguobacterium* (5.88 mg/mL and 250 µmol/min/mL)[12], *A. flavithermus* (0.005 mM and 3.5 µmol/min)[63], *B. megaterium* KAN1 (0.65 mg/mL and 1.568 mg/min), *B. licheniformis* So-B3 (0.004 mM and 3.07 µmol/min)[26], *B. subtilis* Y25 (53.98 mg/ml and 314.10 U) and *B. amyloliquifaciens* (0.6 mg/mL and 2632 µmol/mL/min)[53].

TLC analysis

Purified α-amylase from *B. licheniformis* RA31 reported hydrolysis of starch by the formation of maltose and glucose in the initial stage which increased in intensity with the time and reached maximum after 3 h of hydrolysis (Fig. 4; Lane 6-7). Similar products (maltose and glucose) were also detected after hydrolysis of potato and wheat starch by the enzyme from *B. licheniformis* AS08E[50]. However, contrasting products by numerous scientists like reported maltose, maltotriose and maltopentaose from *B. licheniformis* NH1 and maltose, maltotriose and maltotetraose from *B. mojavensis* A21 strain[52]; glucose, maltose and maltotriose from *Anoxybacillus* sp. YIM 342[47]; maltose and maltotriose from *Amphibacillus* sp. NM-RA2; maltohexaose from *B. halodurans* LBK 34[64]. TLC results from soluble wheat starch degradation by α-amylase from *B. licheniformis* So-B3 resulted in formation of maltose, maltotriose, maltotetraose and maltooligosaccharides after 15 and 30 min of hydrolysis followed by complete hydrolysis into glucose (240 min)[26].

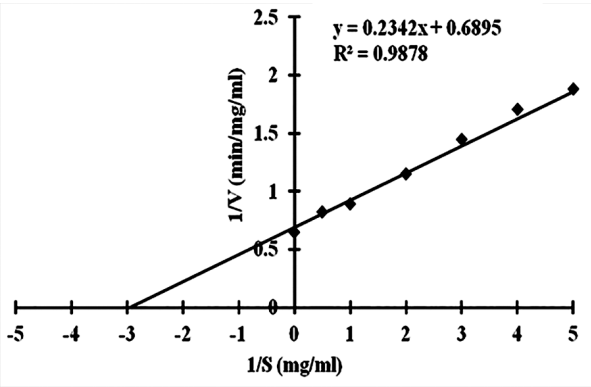

Fig. 4 — Enzymatic hydrolysis of soluble starch at 50°C using thin layer chromatography. [Lane 1. Marker (G1) as glucose, (G2) as maltose, (G3) as dextrin, (G4) as soluble starch Reaction mixture spotted in duplicate: Lane 2-3 after 1 h; Lane 4-5 after 2 h; Lane 6-7 after 3 h; Lane 8-9 after 4 h]

Potential application in detergent industry

α-Amylases in laundry detergent formulations hydrolyze the starch food stains, such as rice, pasta, potato, corn, baby food and improve cleaning efficiency of the detergents. The enzyme was purified from *B. licheniformis* RA31; experiments were performed on its substrate specificity, SEM analysis, stability in commercial detergents, treatment to potato curry stained cotton clothes and the results are discussed below.

Substrate specificity

Purified α-amylase from *B. licheniformis* RA31 was incubated with different raw grain flours individually. The rate of starch hydrolysis was optimum for wheat (140%) followed by rice (120%), gram flour (100%), corn (90%), oat (50%), water chest (45%), millet (40%) and sorghum flours (30%) after 1 h treatment. The enzyme from *Geobacillus* (K1C) showed a broad substrate specificity toward raw starches (10%; w/v) derived from rice (35.7%), wheat (58.6%), corn (43%) and insoluble potato (16.4%)[37]. Recombinant AmyZ1 gene cloned from *Pontibacillus* sp. ZY and expressed in *E. coli* showed a broad substrate specificity toward raw starches (30%; w/v) derived from rice (52%), corn (47%), and wheat (38%) after 4 h incubation[49]. The enzyme from *Anoxybacillus* displayed 100, 100, 95 and 90% hydrolysis of wheat, potato, corn, rice starch (0.5% w/v; 30 min), respectively[59]. The enzyme from *B. mojavensis* SO-10 hydrolyzed starch (1.0% w/v; at 50°C for 4 h) from corn (39.2%) and wheat (36.7%)[27]. However, the enzyme from *Bacillus* sp. was unable to hydrolyse wheat.

Enzymatic degradation of wheat starch granules-SEM analysis

The raw wheat flour was treated with purified α-amylase from *B. licheniformis* RA31 for different intervals of time. SEM analysis demonstrated the gradual degradation of granules by forming depressions and finally holes *w.r.t.* control (Fig. 5).

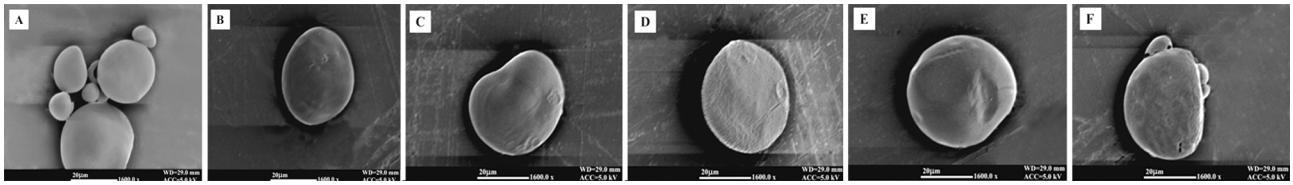

Fig. 5 — Scanning electron microscopy of wheat granules degradation by enzyme. (A) Raw wheat without enzyme treatment; and (B-F) Raw wheat with enzyme treatment(s) after 2, 4, 6, 8, 10 h, respectively.

SEM analysis demonstrated the action mechanism of enzyme on wheat starch granule *w.r.t.* control (Fig. 5). First the enzyme acted on outer surface of the granule and formed cracks and pits (Fig. 5; 2-8 h). Then, it created tunnels towards the centre which leads to breakdown of the granule (Fig. 5; 10 h).

Similar degradations were reported with the enzymes from *B. licheniformis* ATCC to corn granules[67]; *Bacillus* sp. to kuttu, rice, water chest, oat, corn and soluble starch; *B. licheniformis* AS08E to potato[50]; *B. subtilis* AS-S01a to wheat[68]; *Geobacillus thermoleovorans* to wheat and corn[65] and *B. licheniformis* ATCC 9945a to dry-milled corn, wheat and triticale flour[57]. Lin *et al.*[69] suggested that the enzymatic degradation of native starch granules occur in two ways (exo and endo-corrosion). In e*xo*-corrosion, the enzyme degrades the outer surface of granule and creates characteristic fissures and pits. In endo-corrosion, the enzyme creates a tunnel leading to the centre of the granule, which loosens the structural entity of granule consequently leading to breakdown[69].

Enzyme stability in commercial detergents

Purified α-amylase from *B. licheniformis* RA31 was incubated with different concentrations of detergents (0.2-1% w/v). It was observed that enzyme was highly stable in the presence of three detergents (0.2% w/v) like Ariel (125%), Surf excel (109%) and Rin advance (125%). The enzyme lost its relative activity in Mr. White and Ariel fresh clean (90%), Ariel oxy blue and Henko (78%), Surf excel quick wash, Surf excel blue, Safe wash, Tide plus and Tide (71%), Ezee (56%) and Vanish (40%) (Table 4).

Similar results with decreased enzyme activity were reported for the enzyme from *B. licheniformis* AS08E in commercial detergents like Safed-85%; Tide and Surf excel-70%; Fena Ultra-68%; Sunlight, Henko and Wheel-60%; and Nirma-50%[50]. The enzyme from *Streptomyces* sp. lost 15% activity with 0.2% v/v Surf[70]. *Bacillus* sp. FW2'S enzyme showed activity 50.4%-Omo, 54.7%-Tursil and 64.4%-Alo used in 0.5%[31]. The enzyme from *B. amyloliquefaciens*[71] and *B. subtilis* PF1[39] retained ~90% activity in 0.7% w/v Surf excel blue > Surf excel > Fena Detergent bar > Tide> Active wheel gold > Rin supreme bar.

Enzyme assisted detergent treatment to potato curry stained cotton clothes

Washing efficiency was visualized on three different cloth materials stained with potato curry. Improved cleaning efficiency of potato curry stained fine cotton clothes were observed (Uniform removal of starch stains and softness of the fabrics) after the enzyme assisted Rin detergent treatment (0.02% w/v). Washing efficiency was better in all the three fabrics when washed with the 0.2% Rin advance detergent supplemented with enzyme, rather than just detergent or its increased concentration to 0.5% w/v. (Fig. 6).

Similar results of the enzyme assisted detergent treatments were reported for alkaliphilic α-amylase from *B. licheniformis* AS08E, *B. subtilis* PF1[39] and *Exiguobacterium*[12]. The addition of enzymes to detergent formulations have various advantages like savings of energy by lowering wash temperature, reducing loads of harmful chemicals to the

Table 4 — Effect of commercial detergents on α-amylase [*Relative enzyme activity (%)] from *Bacillus licheniformis* RA31

Control	100
Ariel	125
Ariel fresh clean	90
Ariel oxy blue	78
Ezee	56
Henko	78
Mr.white	90
Rin advance	125
Safe wash	71
Surf excel	109
Surf excel blue	71
Surf excel quick wash	75
Tide	71
Tide plus	71
Vanish	40

[*Each data point represents mean of three independent assays]

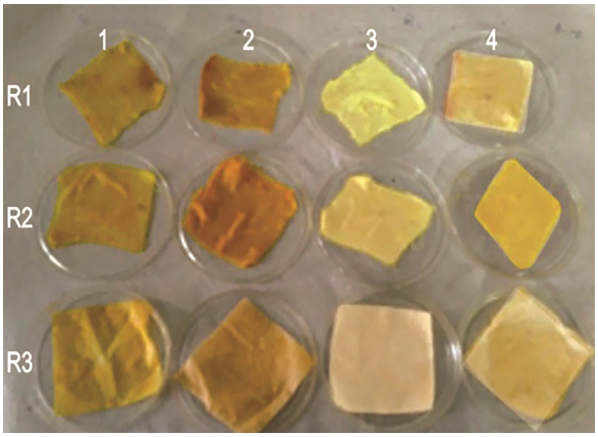

Fig. 6 — Washing efficiency of Rin detergent along with α-amylase from *Bacillus licheniformis* RA31. [Starch stained cotton cloth pieces (Mix quality R1; Pure quality R2; Fine quality R3) washed with: Lane 1: Distilled water only; Lane 2: Rin detergent (0.2% w/v) only; Lane 3: Rin detergent (0.2% w/v) + enzyme; Lane 4: Rin detergent (0.5% w/v) + enzyme]

environment, slashing the environmental impact of the cleaning process[72] and improving efficiency of stains removal at milder conditions[50].

Conclusion

Thermophilic *Bacillus licheniformis* RA31 was grown on optimized media to produce thermostable extracellular α-amylase. The enzyme was ethanol precipitated, purified using DEAE-cellulose and Sephdex G-100 column chromatography's to homogeneity. The purified enzyme displayed broad range of pH and thermal stability. It was also stable in the presence of SDS, Tween 20, EDTA and β-ME. It showed high tolerance (41-49%) in water-miscible organic solvents but stronger inhibition (7-25%) in water-immiscible organic solvents at 20% (v/v). The enzyme revealed the highest specificity to wheat starch granules (140%) after 1 h. The enzyme is highly stable in commercial detergents (*i.e.* Ariel, Surf excel and Rin advance). The enzyme assisted detergent treatment to potato curry stained cotton clothes showed better cleaning efficiency. Hence, it can be concluded that α-amylase from thermophilic and alkaliphilic *B. licheniformis* RA31 has future applications in detergent industry.

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Conflict of Interest

Authors declare no competing interests.

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