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Production of amylase from *Bacillus subtilis* sp. strain KR1 under solid state fermentation on different agrowastes

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1. Introduction

Industrialization has resulted in rapid progress in the field of technology which has opened new avenues in the field of research. Microbial enzymes are one of the most sought out industrial products with amylases being one of the prominent enzymes. Amylases are used in starch industry for saccharification of starch and in laundry detergents (Van der Marrel et al., 2002). Amylases are generally produced by submerged fermentation (SmF). SmF is a costly affair especially for amylase as the soluble starch, most commonly used for its production is expensive. A bright emerging alternative is to use solid-state fermentation involving low cost agro industrial wastes (Pandey, 2003). Solid- state fermentation (SSF) is a fermentation process carried out on a solid matrix in presence of little or no water. SSF brings the cultured microorganism in close proximity of substrate. Important factors governing the success of SSF are the selection of microorganism and substrate, optimization of process parameters and purification of end products. SSF models are developed according to the relationship between microbial physiology and various physio-chemical factors affecting the fermentation process. Among these factors, moisture content and nature of solid substrate are most vital determinants affecting SSF parameters. Selection of moisture level is dependent on the microorganism and also on nature of substrate. Initially, fungi and yeast were considered ideal microorganisms for SSF in accordance with the theoretical concept of water activity. However, now it's well known that bacteria can be cultured under SSF to produce various products (Gupta et al., 2008; Mukherjee et al., 2008; Chinn et al., 2007). Amylase production by SSF using substrates like date waste by *Bacillus licheniformis* AT70 (Afrisham et al., 2016), wheat straw by *Bacillus* sp. BBXS-2 (Qureshi et al., 2016), apple pomace by *Macrophomina phaseolina* (Kaur et al., 2012), wheat bran by *Aspergillus oryzae* (Chen et al., 2014) and soy bread waste by *Aspergillus oryzae* S2 (Cerda et al., 2016) has been reported.

Lignocellulose is a complex carbohydrate made up of lignin and carbohydrate polymers like cellulose and hemicelluloses, pectins and traces of salts, minerals and ash (Singh et al., 2014). It is a great source of cheap carbohydrates and has been used over past few decades as raw material for the production of high value products like bioethanol, enzymes, organic acids and biodegradable plastics (Ravindran and Jaiswal, 2016). Lignocellulosic materials are highly complex structures which make them recalcitrant in nature and so are not directly used in microbial processes. To overcome recalcitrant nature, these materials are subjected to pretreatments and enzymatic hydrolysis to release fermentable sugars which can serve as a source of nutrients and energy for the microbes. Pretreatment can be physical, chemical, biological or a combination of all these processes (Kumar and Wyman, 2013; Karimi and Chisti, 2015).

Last few decades has seen a rapid surge in characterization of enzymes capable of converting abundant renewable biomass to fermentable sugars and ultimately to bioethanol (Bansal et al., 2012; Diaz et al., 2015). It has been previously reported that pretreatment induces certain changes in the structure of substrates like wheat bran which increases the yield of enzymes like amylases, proteases, cellulases (Salim et al., 2017).

The present world scenario emphasizes on a cost effective sustainable approach for production of commercial products so an effort was made to ascertain the efficacy of *Bacillus* sp. Strain KR1 for its ability to utilize various agro- wastes in its natural as well as pretreated wheat straw for copious production of amylase. The ability of the organism to act more readily on pretreated substrates in comparison of control for production of amylase makes it a possible candidate for bioethanol production using simultaneous saccharification and fermentation (SSF) technique, a cost effective method for production of biofuel often involving co-culture systems comprising of lignocellulosic enzymes and amylolytic enzymes. It is a strategy which combines enzymatic hydrolysis and fermentation of sugars for increased cellulosic conversion to biofuel (Brethauer and Studer, 2014).

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2. Material and methods

2.1. Isolation and screening of amylase producing microorganisms

Soil samples were aseptically collected from different regions of Patna district. Sample sites harboring vegetable wastes, industrial wastes and other starch containing wastes were selected. Serial dilution method was used for the isolation of microorganisms. Soil samples was properly mixed with 10 ml of normal saline. Serial dilutions were plated on Nutrient Agar (NA) (0.5% peptone, 0.3% beef extract and 1.5% Agar) plates and incubated for 24 h at 37 °C. After incubation, colonies were purified by transferring on NA plates. Colonies were stored on NA slants. For screening of amylase producing microorganisms, purified colonies were streaked on Starch Agar (SA)(1% starch, 0.5% peptone, 0.3% beef extract and 1.5% Agar) plates and incubated for 48 h at 37 °C. After 48 h plates were flooded with Lugol's iodine solution. Starch forms a blue colored complex on interacting with iodine. However, if the starch present in the medium has been hydrolyzed by the microorganism, a halo, transparent zone is observed surrounding the microorganism. Halo zone formation around the colony is indicative of amylase production (Abd-Elhalem et al., 2015).

2.2. Assay of amylase activity

Amylase assay was performed by DNSA method as described previously (Miller, 1953) with certain modifications. The assay mixture contained 500 µl of soluble starch (1% w/v), 500 µl of 0.1M phosphate buffer and appropriately diluted enzyme solutions with pH adjusted to 7. The reaction was performed at 50 °C for 15 min. The reaction was stopped by the addition of 1 ml of 3, 5-dinitrosalicylic acid reagent. 2 ml of distilled water was also added. The mixture was boiled for 5 min and solution was rapidly cooled in ice water. Absorbance was read at 540 nm in systronics UV-VIS spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required that catalyzed the liberation of reducing sugar equivalent to one µmol of D-glucose per min under assay conditions.

2.3. Morphological and biochemical characterization of the bacterial strain

The bacterial isolate was subjected to morphological and biochemical characterization such as cell shape, gram staining, spore formation, growth in thyoglycollate broth, catalase test, citrate test, Methyl-Red (MR) and Voges Proskauer (VP) test, carbohydrate fermentation test and indole test. Biochemical tests were performed according to Bergey's Manual of systemic Bacteriology (2005).

2.4. Molecular characterization of the bacterial strain

Identification of the organisms was done on the basis of 16S rDNA based molecular technique. DNA was isolated from the bacterial cultures and quality was evaluated on 0.8% agarose gel. Isolated DNA was amplified with 16S rRNA specific primers (8F & 1492R) using Veriti® 99 well Thermal cycler (model No.9902). The PCR product was enzymatically purified and further subjected to Sanger sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

2.5. Phylogenetic analysis

The evolutionary tree was inferred using the Neighbor-Joining Method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsentein, 1985) was taken to represent the evolutionary history of the taxa analysed (Felsentein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base difference per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were total of 1492 positions in the final dataset. Evolutionary analysis was conducted in MEGA7 (Kumar et al., 2016).

2.6. Amylase production on different Agrowastes under solid-state fermentation

2.6.1. Enzyme extraction

The enzyme from the fermented bacterial was extracted twice with 50 ml of 1 mM phosphate buffer (pH 7.0). Extraction was done by soaking the fermented solids with phosphate buffer for 30 min. The contents were mixed by shaking for 30 min at 30 °C on a rotary shaker at 150 rpm. The slurry was squeezed through a cheese cloth. The extracts were pooled and centrifuged at 4 °C for 30 min at 6000 rpm to separate small wheat bran particles, cells and spores. The brown clear supernatant was used as the source of amylase (Ellaiah et al., 2002).

2.6.2. Different agro-residues as solid substrate for amylase production

In solid-state fermentation (SSF), the choice of a suitable substrate for production of an enzyme is an important factor. The selection involves screening of a number of agro-waste residues for optimum enzyme production. In this study, five substrates namely wheat bran, rice bran, corn bran, maize bran and wheat straw were selected as substrate for amylase production. The fermentation was carried out by taking 5 g each of the selected substrate separately in a 250 ml Erlenmeyer flask, to which 10 ml of distilled water was added. The flasks were inoculated using 1% (v/w) of the selected bacterial inoculum and incubated at 37 °C for 72 h. Amylase activity was determined in the cell free supernatant.

2.6.3. Effect of inoculum level on amylase production

Culture flasks (250 ml) containing optimized substrate (5 g) moistened with 10 ml distilled water were autoclaved and inoculated with different amounts (2, 4, 6, 8, 10 and 12% v/w) of bacterial culture.

2.6.4. Effect of incubation period on amylase production

Incubation time for amylase production was optimized. Erlenmeyer flasks (250 ml) containing optimized substrate were inoculated with optimum inoculum concentration (10%) of the selected bacterial strain. The inoculated flasks were incubated for 120 h and enzyme was extracted after every 24 h and amylase activity performed. The optimum incubation period found was followed for further experiments.

2.6.5. Effect of moistening agents on amylase production

The effect of salt solutions on the production of amylase in SSF was studied using five mineral salt solutions prepared in distilled water which were employed as Moistening Agents (MA). In addition to moistening agents, distilled water was also used as a moistening agent. The substrate was moistened with selected mineral salt solution separately and was autoclaved. Fermentation studies was carried under optimized conditions.

2.6.6. Effect of moistening levels on amylase production

Production of amylase at different moisture levels was studied by varying the ratio of amount of substrate to the volume of salt solutions. Selected substrates to different volumes of optimum moistening agent for KR1 (1:1.0, 1:2, 1:3, 1:4 and 1:5 w/v) were taken separately in different flasks. The fermentation was carried out under optimized conditions. The optimized moisture level ratio was used for further studies.

2.6.7. Effect of pH on amylase production

In order to determine the effect of initial pH on production of

amylase by KR1, the pH of the selected moistening agent was varied from 5 to 10 with 0.5 unit interval using 0.1 N HCl and 0.1 N NaOH. The optimum initial pH of the substrate was used to carry out subsequent experiments.

2.6.8. Effect of incubation temperature on amylase production

The optimum temperature for the production of amylase was determined for KR1 by incubating the organism under optimum conditions at different temperatures from 30 to 45 °C with an interval of 1 °C. The optimum temperature was used for further experiments.

2.6.9. Effect of supplementary carbon sources on amylase production

The effect of various carbon supplements on amylase production KR1 was evaluated by adding carbon sources(1% w/w) such as soluble starch, corn starch, potato starch, arabinose, maltose, galactose, mannose lactose, sucrose, glucose, fructose, ribose and xylose. The best carbon supplement was used for further studies.

2.6.10. Effect of supplementary nitrogen sources on amylase production

Various nitrogen sources such as beef extract, yeast extract, peptone, tryptone, urea, sodium nitrate, ammonium acetate, ammonium chloride, ammonium sulphate and ammonium mono hydrogen phosphate were added at 1% w/w separately into the optimized fermentation medium to select the best nitrogen supplement for amylase production. The most suitable supplement was used for further experiments.

2.6.11. Effect of metal ion on amylase production

Effect of different metal ions on the production of amylase production was studied by incubating the culture medium with mineral salts such as potassium chloride, cupric chloride, cuprous chloride, magnesium chloride, manganese chloride, cobalt chloride, barium chloride, calcium chloride, nickel chloride, ferric chloride, ferrous chloride, zinc chloride, chromium chloride and mercury chloride each at concentration level of 1 mM under optimum conditions. Amylase activity was assayed as per standard protocol.

2.6.12. Pretreated wheat straw

Pretreated wheat straw was procured from Microbial and Molecular Genetics Laboratory, Department of Botany, Patna University. Wheat straw was subjected to alkali, steam and irradiation pretreatment. Irradiation of wheat straw was originally done at Bhabha Atomic Research Centre, Mumbai using a 60Co-γ irradiation device.

2.6.13. Amylase production from pretreated wheat straw

Wheat straw subjected to various pretreatment processes such as alkali, steam and irradiation as well as their various combinations was used as a substrate for amylase production under solid-state fermentation (SSF). Untreated wheat straw was used as a control with 100% enzyme activity and relative amylase activity (%) was calculated under standard conditions.

2.7. Statistical analysis

The graphs were prepared in Prism3 Software. One way ANOVA (Non-parametric) was done with the help of Tukey's multiple comparison test with $p \leq 0.0001$ considered highly significant.

3. Results

3.1. Identification of tested bacterial isolate

Primary screening resulted in 72 amylolytic bacterial isolates and based on halo zone formation and starch ratio formation (SHF), KR1 showed highest amylase activity and was selected for biochemical and molecular identification.

3.1.1. 16S rDNA gene sequence identification

Molecular identification of the isolated bacterial strain was carried out on the basis of 16S rDNA sequence analysis. The results showed that 16S rDNA gene partial sequence is about 1519 bp long consisting of both variable and conserved regions. The resulted sequence was compared with bacterial species recorded in the GenBank data base using DNAMAN program and identified as *Bacillus subtilis* with the highest similarity of 99%. It has been assigned with NCBI accession number KX345353.1.

Based on multiple sequence alignment done with the help of CLUSTALW program (Thompson et al., 1994) and phylogenetic tree mapped on the basis of neighbor joining method, the organism was found to be showing 99% sequence homology with *Bacillus subtilis* IHB B1026 shown in Fig. 1.

3.2. Solid-sate fermentation studies

In solid-state fermentation (SSF), the choice of a suitable substrate for production of an enzyme is an important factor. Among the substrates, wheat bran was the most efficient substrate for amylase production by KR1 (Fig. 2.). The organism KR1 showed highest production with wheat bran (82.60 ± 0.636 U/gds) followed by rice bran (80.51 ± 0.257 U/gds). Lowest amylase production took place with wheat straw as the substrate (69.37 ± 0.932 U/gds).

The effect of different inoculum concentration on amylase production by KR1 was monitored (Fig. 3.). The production of amylase increased with increase in inoculum size. Optimum amylase production took place with 10% v/w inoculum for KR1 (86.014 ± 0.464 U/gds).

Amylase production by KR1 was studied at different incubation period upto 120 h (Fig. 4.). KR1 showed optimum amylase production after 48 h (82.02 ± 0.351 U/gds). Decline in amylase production was observed with further increase in incubation time. KR1 showed lowest amylase production after 24 h (32.39 ± 0.595 U/gds).

In solid-state fermentation (SSF), the nature of the moistening agents is an important factor governing the success of a process. Different mineral salt solutions and distilled water were used as moistening agents for amylase production (Fig. 5.). Among different mineral salt solutions, MAII resulted in highest amylase production for KR1 after 48 h of incubation (81.68 ± 1.07 U/gds). For KR1, MAVI was the least effective moistening agent among different salt solutions (36.73 ± 0.834 U/gds) while lowest amylase production took place in distilled water (35.12 ± 0.715 U/gds).

For the organism, effect of moisture level on amylase production was studied and the amount of enzyme produced was quantified under optimized conditions (Fig. 6.). The organism KR1 showed highest amylase production when wheat bran was used with MAII in the ratio of 1:2 (82.02 ± 0.748 U/gds). Amylase production decreased at both high (1:3, 1:4, 1:5) and low moisture levels (1:1).

The effect of various initial pH of the medium on amylase production was studied for the organism KR1 (Table 1.). KR1 showed optimum amylase production at pH 7.5 (83.874 ± 0.716 U/gds). Amylase production declined at pH 8.0 (81.24 ± 0.621 U/gds) and above. KR1 showed lowest amylase titer at pH 10.0.

Effect of temperature on amylase production was studied by incubating the organism under optimized conditions at various temperatures (30–45 °C) conditions (Table 1.). The organism KR1 showed highest amylase production at 40 °C (91.22 ± 0.971 U/gds). The production declined at higher temperatures. Lowest amylase production took place at 30 °C (23.74 ± 0.654 U/gds).

The effect of different carbon supplements on amylase production was examined (Table 2.). The organism KR1 showed highest amylase production with soluble starch (126.15 ± 0.844 U/gds) followed by corn starch (109.51 ± 1.31 U/gds). Among simple sugars, maltose was the best supplement (89.37 ± 0.864 U/gds). Glucose and fructose repressed amylase production.

The effect of different nitrogen supplements on amylase production

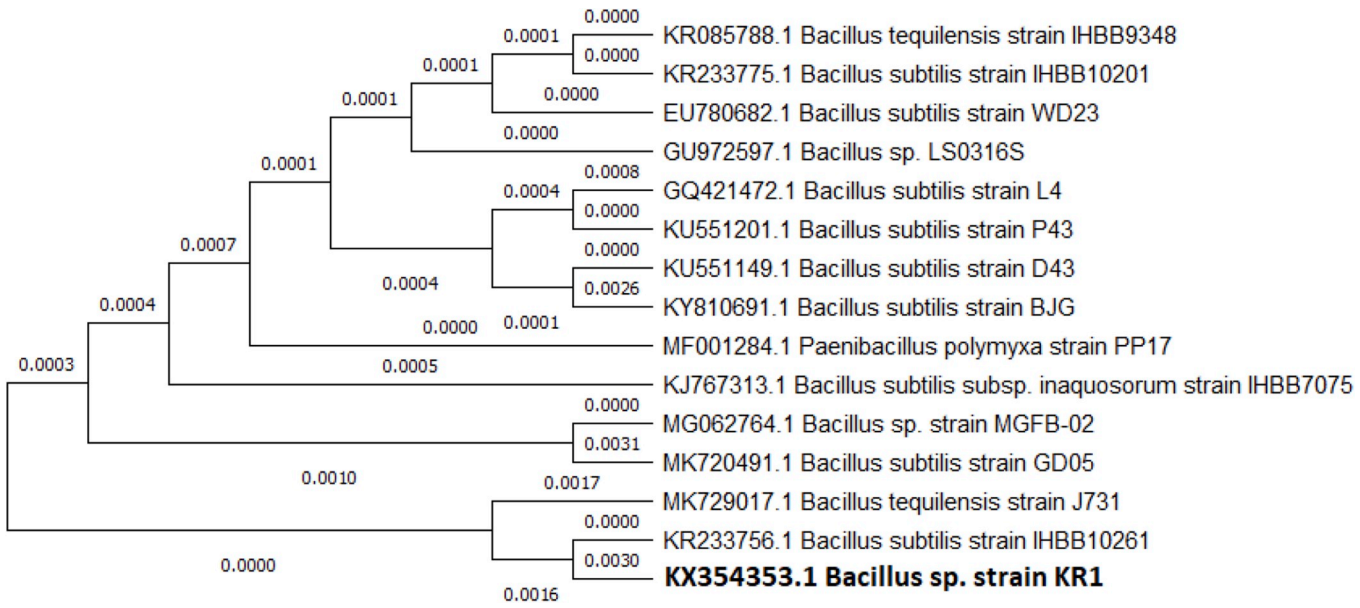

Fig. 1. Phylogenetic tree of *Bacillus* sp. strain KRI based on 16S rRNA gene sequence based on neighbor-joining method.

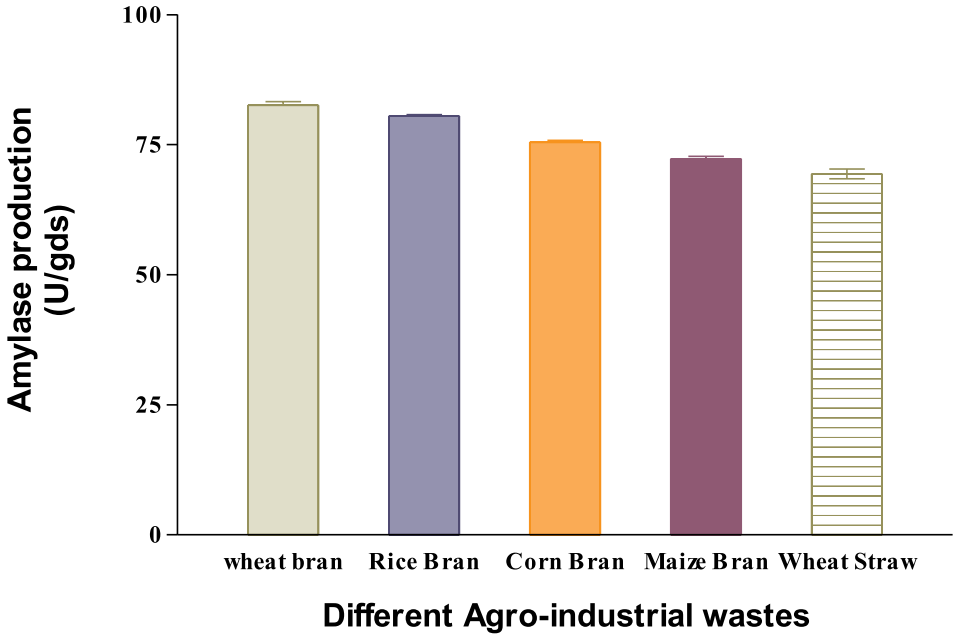

Fig. 2. Effect of different agro wastes on production of amylase by *Bacillus* sp. KR1. The graph depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.001$ statistically significant.

was examined (Table 2.). Among the various organic and inorganic nitrogen sources, yeast extract acted as the best supplement for amylase production (93.06 ± 0.958 U/gds). For KR1, urea repressed amylase production (75.16 ± 0.469 U/gds).

Metal ions are known to affect microbial enzyme production. The effect of various metal ions on amylase production was studied (Table 3.). For KR1, Ca^{2+} was the best metal supplement (103.43 ± 0.64 U/gds) followed by Mg^{+2} (97.98 ± 1.27 U/gds). Mn^{2+}, Fe^{2+}, Fe^{3+}, K^{+} and Ba^{2+} ions also stimulated amylase production. Lowest amylase production took place with Hg^{2+} (18.92 ± U/gds) and Cr^{3+} (53.32 ± 1.00 U/gds) ions. Cu^{+}, Ni^{2+}, Cu^{2+}, Zn^{2+} also repressed amylase production.

Pretreated wheat straw by chemical and physical methods was used as a substrate for amylase production under solid-state fermentation (SSF) conditions. Relative amylase activity (%) was calculated considering enzyme activity of untreated wheat straw as 100% (Table 4.). It was found that amylase production was enhanced after pretreatment processes. Combination of steam and irradiation (50 kGy) was found to be the most effective inducer of amylase for KR1 (201%). For KR1, alkali and irradiation (50 KGy) was least effective inducer of amylase production (103%).

4. Discussion

Among different agro-residues used for amylase production, wheat bran was the most efficient substrate for amylase production by *Bacillus* sp. KR1. High amylase production using wheat bran as a substrate has been previously reported for *Bacillus megaterium* (El-shishtawy et al.,

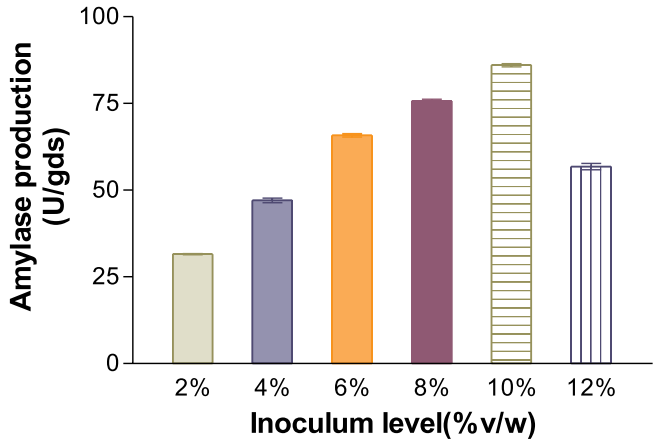

Fig. 3. Effect of different inoculum size on production of amylase. The graph depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.001$ statistically significant.

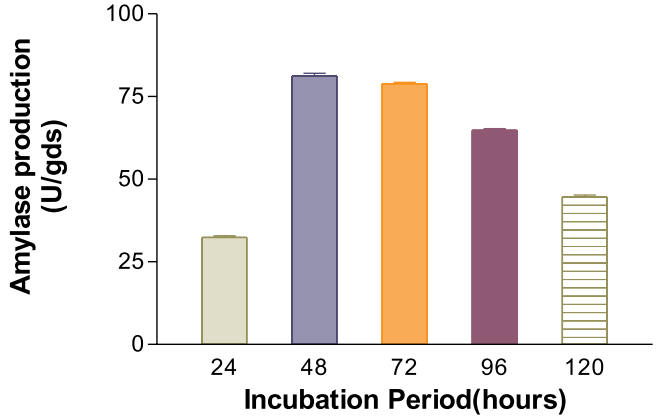

Fig. 4. Effect of different incubation periods on production of amylase. The graph depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.001$ statistically significant.

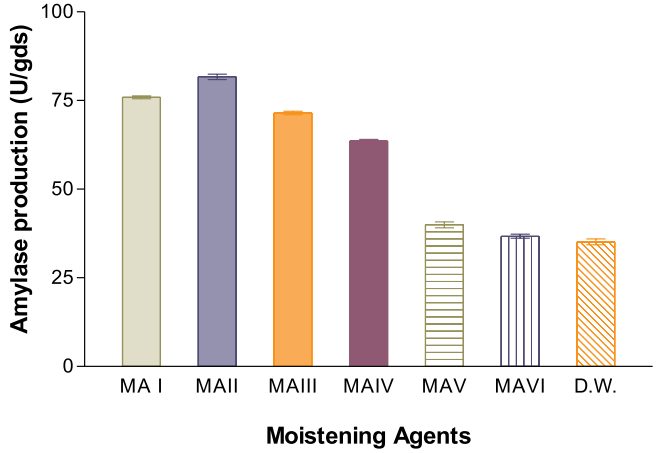

Fig. 5. Effect of different moistening agents (MAI-VI & distilled water) on production of amylase. The graph depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.001$ statistically significant.

2014), *Bacillus subtilis* (Nimkar et al., 2010), *Bacillus subtilis* MTCC 121 (Raul et al., 2014), Bacillus *licheniformis* RT7PE1 (Tabassum et al., 2014), *Bacillus* sp. (Bozic et al., 2014), *Bacillus amyloliquefaciens* MTCC (Saha et al., 2014), *Bacillus amyloliquefaciens* KCP2 (Prajapati et al.,

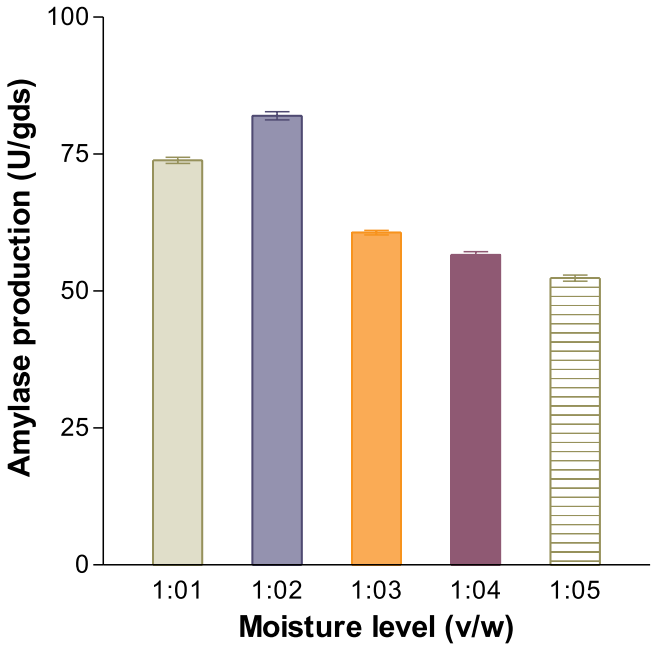

Fig. 6. Effect of different moistening levels on production of amylase. The graph depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.001$ statistically significant.

Table 1
Effect of incubation pH and temperature on amylase production (U/gds) by KR1. The table depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.0001$ statistically significant.

Factors		Amylase Production (U/gds)
pH	5.0	22.57 ± 0.844
	5.5	32.49 ± 0.654
	6.0	49.62 ± 0.552
	6.5	62.80 ± 0.350
	7.0	74.53 ± 0.848
	7.5	83.87 ± 0.717
	8.0	81.24 ± 0.621
	8.5	67.77 ± 0.591
	9.0	49.28 ± 1.066
	9.5	33.27 ± 1.258
	10.0	14.30 ± 0.891
Temperature(°C)	30	23.74 ± 0.654
	31	28.70 ± 0.319
	32	41.25 ± 0.597
	33	46.60 ± 0.632
	34	52.44 ± 0.514
	35	59.93 ± 0.341
	36	66.65 ± 0.508
	37	69.76 ± 0.589
	38	82.65 ± 0.542
	39	84.99 ± 1.144
	40	91.22 ± 0.972
	41	85.47 ± 0.644
	42	78.96 ± 0.843
	43	74.82 ± 0.877
	44	63.87 ± 1.180
	45	58.38 ± 1.076

2015) and *Bacillus amyloliquefaciens* (Gangadharan et al., 2006). On the contrary, good amylase production using corn gluten meal and mustard oil seed cake as substrates have been reported from *B. amyloliquefaciens* and *Bacillus* sp. (Tanyildizi et al., 2007; Saxena and Singh, 2011). Rice bran under submerged fermentation conditions was found to be best source amylase production from *Streptomyces* MSC702 (Singh et al., 2012).

Inoculum size greatly affects amylase production in solid-state

Table 2
Effect of supplementary carbon and nitrogen sources on amylase production (U/gds) by KR1. The table depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.0001$ statistically significant.

Factors		Amylase Production (U/gds)
Carbon Sources	Control	82.31 ± 0.636
	Glucose	52.25 ± 0.803
	Mannose	83.33 ± 0.843
	Galactose	82.90 ± 0.669
	Fructose	53.41 ± 0.636
	Arabinose	83.43 ± 0.592
	sucrose	85.43 ± 0.764
	lactose	82.65 ± 1.195
	maltose	89.37 ± 0.864
	xylose	84.16 ± 0.432
	ribose	76.62 ± 0.506
	soluble starch	126.15 ± 0.844
	corn starch	109.51 ± 1.311
	potato starch	100.17 ± 0.901
Nitrogen Sources	Control	82.31 ± 0.636
	$(NH_4)_2HPO_4$	90.68 ± 0.548
	NH_4Cl	84.50 ± 0.421
	$(NH_4)_2SO_4$	84.74 ± 0.319
	$NH_4CH_3CO_2$	87.37 ± 0.844
	$NaNO_2$	83.38 ± 0.947
	Peptone	82.36 ± 0.638
	Tryptone	82.94 ± 0.257
	Yeast extract	93.06 ± 0.958
	Beef extract	88.59 ± 1.025
	Urea	75.16 ± 0.469

Table 3
Effect of metal ions on amylase production (U/gds) by KR1. The table depicts the enzyme activity of triplicate experiments (Mean ± SE) with $p \leq 0.001$ statistically significant.

Factors		Amylase Production (U/gds)
Metal Ions	Control	93.26 ± 0.607
	KCl	94.72 ± 1.822
	CuCl	64.75 ± 0.621
	$MgCl_2$	97.98 ± 1.279
	$MnCl_2$	95.01 ± 0.674
	$CoCl_2$	81.97 ± 0.796
	$CaCl_2$	103.43 ± 0.643
	$NiCl_2$	66.21 ± 0.783
	$BaCl_2$	96.03 ± 0.804
	$CuCl_2$	64.36 ± 0.843
	$FeCl_2$	96.86 ± 0.984
	$ZnCl_2$	82.46 ± 0.735
	$FeCl_3$	99.92 ± 0.844
	$CrCl_3$	53.32 ± 1.005
	$HgCl_2$	18.92 ± 0.759

Table 4
Amylase production from different pretreated wheat straw, A-control, B-1%NaOH, C-100 kGy, D-1%NaOH + steam+50 kGy, E-1%NaOH + steam+100 kGy, F-1%NaOH+50 kGy, G-1% NaOH+100 kGy, H-Steam+50 kGy and I-Steam+100 kGy. The table depicts the relative enzyme activity (%) of triplicate experiments (Mean ± SE) with $p \leq 0.0001$ considered highly significant.

Pretreatment Method	KR1
A	100
B	105.43 ± 0.42
C	178.64 ± 1.01
D	108.22 ± 0.58
E	113.73 ± 0.87
F	103.47 ± 0.607
G	107.39 ± 1.00
H	201.05 ± 0.58
I	179.32 ± 0.85

fermentation (SSF). In present investigation, it was found that KR1 produced maximum amylase at 10% v/w inoculum level and similar finding has been reported for *Bacillus subtilis* ATCC6633 (Maity et al., 2015). On the contrary, optimum amylase production at 20% v/w has been observed for *B.subtilis* (Baysal et al., 2003). Cells growth rate at lower inoculums level is slow so it takes longer in order to accomplish efficient utilization of substrate which results in lower enzyme production (Mukherjee et al., 2008; Ramachandran et al., 2004).

The incubation period is influenced by culture conditions as well as growth rate and enzyme production. For KR1, maximum amylase production was observed after 48 h of incubation. Optimum amylase production after 72 h has been previously reported for *B.amyloliquefaciens* (Gangadharan et al., 2006), *B. subtilis* DM-03 (Mukherjee et al., 2009), *Bacillus* sp. (Saxena and Singh, 2011). Maximum amylase production after 48 h of incubation has been reported for *B. amyloliquefaciens* MTCC1270 (Saha et al., 2014), *B. subtilis* ATCC 6633 (Maity et al., 2015) and *Bacillus* sp. MRS6 (Sahoo et al., 2016). On the contrary optimum amylase production under SSF after 24 hours has been reported for *B. amyloliquefaciens* (Tanyildizi et al., 2007).

Different moistening agents (MA I-V) used for amylase production, for KR1, MAII resulted in significant increase in amylase yield. Mineral salt solutions have been used to enhance amylase production for *Bacillus coagulans* (Babu and Satyanarayana, 1995). MAII contains salts like $MgSO_4$, NaH_2PO_4 and K_2HPO_4 and amylase production in presence of these salts could lower the overall cost of industrial production. On the contrary, tap water (pH 8.5-9.0) was found to be most effective moistening agent for amylase production by *B.subtilis* DM-03 (Mukherjee et al., 2009), *Bacillus* PS-07 (Sodhi et al., 2005) and *B. subtilis* DM-03 (Das et al., 2004).

Among different moisture levels used for amylase production, 1:2 w/v was found to be optimum for KR1. Similar findings have been reported for *Bacillus* sp. KR-8104 (Hashemi et al., 2011) and *Bacillus megatherium* (El-Shishtawy et al., 2014). Optimum amylase production at less than 100 moisture level has been reported (Ramachandran et al., 2004). Therefore, it may be inferred that different strains of *bacillus* have different optimum moisture level. It has been argued that lower moisture level causes reduction in the solubility of the nutrients in SSF resulting in lower degree of swelling and water moisture (Sodhi et al., 2005). On the other hand, higher moisture level beyond the optimum level decreases the porosity as well as modifies the structure of the solid fermentable substrate which leads to development of stickiness, reduction in gaseous exchange and lower oxygen transfer ultimately interfering with microbial metabolism (Baysal et al., 2003; Das et al., 2004; Sodhi et al., 2005; Prakasham et al., 2006; Mukherjee et al., 2008).

Optimum initial pH for amylase production was 7.5 for KR1. Significant increase in amylase titer was observed for KR1 in the pH range of 6.5-8.0. Optimum initial pH 7.0 has been previously reported for *B. amyloliquefaciens* (Tanyildizi et al., 2007), *Bacillus subtilis* (Kokab et al., 2003), *Bacillus subtilis* (Konsula and Liakopoulou-Kyriakides, 2004), *Bacillus subtilis* (Asgher et al., 2007), *Bacillus* sp. (De Souza and Martins, 2000), *Bacillus licheniformis* (Haq et al., 2005). Similarly optimum initial pH 8.0 has been reported for *Bacillus* sp. MRS6 (Sahoo et al., 2016). On the contrary optimum initial pH of 8.5 has been reported for B.*licheniformis* SPT 27 (Aiyer and Modi, 2005).

Optimum incubation temperature for amylase production for KR1 was 40 °C. Optimum amylase production at 40 °C under optimized conditions has been previously reported for *B. subtilis* MTCC 121 (Raul et al., 2014). Maximum amylase yield at 37 °C has been previously reported for *B. amyloliquefaciens* (Gangadharan et al., 2006), *B. amyloliquefaciens* (Saha et al., 2014). On the contrary optimum incubation temperature of 60 °C, 45 °C, 35 °C and 33 °C has been reported for amylase production under SSF (Tanyildizi et al., 2007; Unakal et al., 2012; Bozic et al., 2014; El-shishtawy et al., 2014).

Among different supplementary carbon sources used for amylase production, soluble starch was the most efficient inducer of amylase synthesis for KR1. This finding is in coherence with many reports

suggesting starch as the best inducer of amylase production. Starch as the best inducer has been reported for *Bacillus subtilis* 65 (Hayashida and Teramoto, 1988), *Bacillus* sp. IMD 434 (Hamilton et al., 1999), *Bacillus thermooleovorans* (Narang and Satyanarayana, 2001), *Bacillus subtilis* (Konsula and Kyriakides, 2004), *Bacillus* sp 1–3 (Goyal et al., 2005), *Bacillus* DM-03 (Mukherjee et al., 2009). On the contrary, glucose as the best inducer of amylase production for *Bacillus megatherium* (El-shishtawy et al., 2014) has been reported. There are reports in which supplementary carbon sources did not increase amylase yield (Babu and Satyanarayana, 1995).

Among different inorganic and organic nitrogen sources used as a supplement for amylase production, yeast extract showed significant increase in amylase titer for KR1. Similar findings have been observed for *Bacillus* sp. IM435 (Hamilton et al., 1999), *Bacillus* sp. (De Souza and Martins; 2000; Oliveira Santos and Martins, 2003; Thipperswamy et al., 2006) and *B. subtilis* JS 2004 (Asgher et al., 2007). Ammonium sulphate as optimum supplementary nitrogen source for amylase production has been reported for *B.amyloliquefaciens* KCP2 (Prajapati et al., 2015). Ammonium nitrate as the best nitrogen source for induction of amylase production has been reported (Hashemi et al., 2010). On the other hand no significant increase in amylase production was noticed in presence of various supplementary nitrogen sources for *B. amyloliquefaciens* (Gangadharan et al., 2006) and *B.subtilis* DM-03 (Mukherjee et al., 2009).

Lignocellulosic wastes are a complex biomass consisting of molecules like lignin, cellulose, hemicelluloses and others (Ravindran and Jaiswal, 2016). It is being widely used to produce bioethanol, organic acids and others. Wheat straw represents a by-product left after harvesting of grains. It has immense potential to generate bioethanol. In present investigation, wheat straw, a lignocellulosic waste was pretreated by various chemical as well as physical methods and its efficacy as a substrate for amylase production under solid-state fermentation (SSF) was ascertained. Experimental studies revealed that pretreatment significantly enhanced amylase production with combinatorial methods being more effective. Pretreatment of lignocellulosic wastes has been previously used to ascertain microbial enzyme production by *Bacillus* sp. TMF-1 (Salim et al., 2017). The organism can be potentially used in a co-culture system for biofuel production. Previously co-culture of *Bacillus subtilis* and *Saccahromyces cerevisiae* has been used for bioethanol production (Tantipaibulvut et al., 2015). Similarly simultaneous saccharification and fermentation of mango peel have increased bioethanol yield (Somda et al., 2011). Researchers have also carried out dark fermentation of starch for biohydrogen production using mixed bacterial culture of *Bacillus* sp. and *Brevumdimonas* sp. (Bao et al., 2012). So it was concluded that strain under examination readily utilize agro-wastes to produce amylase under solid-state fermentation which could prove beneficial at industrial level.

5. Conclusion

The present endeavor was aimed to ascertain the potency of *Bacillus* sp. strain KR1 for its ability to degrade various agro-industrial wastes as well as optimization of various production conditions. The isolate showed significant potency in production of amylase by degrading various ecofriendly natural substrates. In addition it also showed promise in degrading pretreated wheat straw which makes the organism a promising candidate in the lignocellulosic industry. However a pretreatment study is only of preliminary nature and exhaustive investigation is needed. The organism was also thermotolerant, halotolerant and alkaliphilic organism (data not shown) which further enhances its potential industrial applications.

Conflicts of interest

The authors have declared no conflict of interests.

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