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ORIGINAL PAPER

Optimization of novel hyperthermostable β amylase production by *Bacillus subtilis* DJ5 using solid agroresidual substrates

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Abstract Eight different low cost starchy agroresidues namely Barley (B), Wheat bran (WB), Sattu (S), Rice powder (RP), Corn flour (CF), Rice husk (RH), Yellow peas split (YPS) and arrowroot (A) were used for solid culturing of *Bacillus subtilis* DJ5 for production of novel hyperthermostable β amylase. Various process parameters like initial moisture content, inoculum load, medium pH and incubation temperature affecting enzyme production were optimized to ensure maximum enzyme yield. Only 10 % inoculums load and medium pH of 6.9 was found sufficient to achieve maximum enzyme production in all substrates in a decreasing order, B > WB > S > RP > CF > RH > YPS > A. Optimum β amylase production was highly dependent on initial moisture content of substrate as observed from varying requirement of moisture for different substrates. Only 50 % moisture was sufficient for maximum enzyme production of 84.29 U/gdm in CF. For B, RH, YPS, and A 60 % initial moisture resulted in higher production of 120.34, 35.19, 26.59, and 21.58 U/gdm, respectively, at 37 °C. However, for S and RP higher (70 %) moisture content allowed 113.4 and 85.56 U/gdm enzyme production, respectively. Under optimized conditions, maximum β amylase production was observed after 25 h for A, YPS, RH, RP; 41 h for B, WB, CF, and 45 h for S.

Keywords Agroresidues · Barley · Moisture content · Wheat bran

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Introduction

Advancement of science and technology in the agricultural field has successfully accumulated huge amount of crops and crop residues. Improper preservation and lack of proper management of these residues is posing a serious threat to the environment (Gustavsson et al. 2011). Beside human consumption, large parts are being used as cattle feed, power generation, still huge amount of crop residues are left unutilized (Parfitt et al. 2010). It must be managed in other ways to reduce wastage and environmental pollution. In a view to proceed in that direction, microbiologists have successfully used solid state fermentation (SSF) to convert agroresidues into economically important value added products (Pandey et al. 1999; Pandey and Soccol 2000; Kim and Dale 2004; Haddadin et al. 2001) that not only the helps in waste management (Pandey et al. 2000a, b, c), but also strengthen economy of a country.

History tells us that SSF technology was initiated early with the civilization as the process is less complicated, requires minimum process monitoring. SSF process is carried out on a non-soluble material that acts both as physical support and as source of nutrients in absence of free flowing water (Pandey 2003). SSF offers numerous advantages over submerged fermentation (SmF) in processing of agro-industrial residues as it has low capital investment, lower levels of catabolite repression (González and Torres 2006) and end product inhibition, low wastewater output, better product recovery, and high quality production (Lonsane et al. 1985) and are environmentally friendly.

During the last decade, an increased attention was paid to the use of various agro-industrial wastes for amylase production using SSF (Alva et al. 2007). Amylases being the most important industrial enzymes have found a wide

variety of applications in food, detergent, textile, paper, confectionery, baking, and pharmaceutical industries (Sivaramakrishnan et al. 2006). These enzymes account for about 30 % of the world's enzyme production (van der Maarel et al. 2002).

Several agroresidues like wheat bran, rice husk, oil cakes, etc., have been tested either separately or in combination in SSF for production of alpha amylase (Gangadharan et al. 2006; Balkan and Ertan 2007). In such pursuit of α amylase, β amylase was continuously ignored though it has found wide applications in enzyme industry (Ray and Nanada 1996). Reports on β amylase production using agroresidues by SSF are very scanty that has left an open avenue to exploit the feasibility of β amylase production by SSF.

In our previous study, we have already reported production of hyperthermostable β amylase from *Bacillus subtilis* DJ5 (Poddar et al. 2011a). The enzyme showed remarkable thermostability being fully active at 121 °C for 15 min. Previously, a thermostable β amylase was isolated from *Clostridium thermosulfurogenes* by Shen et al. (1988) that was stable at 80 °C. Since thermostability is the crucial criteria for industrial application (Haki and Rakshit 2003), β amylase of this study has got a leading edge over all previously reported β amylase. Moreover, this enzyme is capable of digesting raw starches (Poddar et al. 2011b). This passively illuminates possibilities of using the organism in SSF technology to secret hyperthermostable β amylase. The importance of enzymatic saccharification of raw starches without thermal gelatinization is gaining popularity as this process saves energy and reduces cost of starch processing (Shiau and Hung 2003) and makes process simple (Achi and Njoku-Obi 1992). With such background, we have used *B. subtilis* DJ5 in SSF and used several agro-waste residues separately to determine best substrate for production of β amylase. The work was initiated on October 2010 in P.G. Department of Microbiology, Bidhannagar College and continued 7 months long. Several process parameters affecting enzyme production were evaluated and optimized at that time to determine best fermentation condition.

Materials and methods

Microorganisms

One mutated strain of *B. subtilis* DJ5 (GenBank Accession Number GU357825) (Poddar et al. 2011b) was used in this study. The organism was isolated from kitchen waste and was subsequently mutated in our laboratory. The organism was maintained on starch peptone agar medium with the following composition (gram per liter): peptone 0.9, $(NH_4)_2HPO_4$ 0.4, KCl 0.1, $MgSO_4 \cdot 7H_2O$ 0.1, $NaH_2PO_4 \cdot 2H_2O$ 0.5, soluble starch (Sigma, USA) 5, agar–agar 15, and pH 6.9.

Inoculum preparation

Initial study indicated that *B. subtilis* DJ5 is producing maximum enzyme only at six and half hour in starch peptone broth during SmF. For SSF study, such highly active cells were harvested by inoculating 6 % of 24 h broth culture in fresh sterile 100-ml starch peptone broth in a rotary shaker at 160 rpm at 37 °C. Active cells were prepared nutrient free by centrifuging the cells at 8,000 rpm for 10 min and dissolving in same amount of sterile 0.1 M phosphate buffer (pH 6.9). It was used as inoculum for SSF. By serial dilution and plating, the number of viable colonies in the inoculum was found to be 3×10^8 CFU/mL.

Selection of substrate

Selection of a proper substrate is a key aspect of SSF. In SSF, solid material is non-soluble that acts both as physical support and as source of nutrients. Solid material could be a naturally occurring solid substrate such as agricultural crops, agro-industrial residues or inert support (Pandey et al. 2000c, d; Pandey et al. 2001; Peralta-Perez et al. 2001; Hoogschagen et al. 2001).

In an attempt to choose a potential substrate for SSF for β amylase production, eight different starchy agricultural products namely barley (B), sattu (S), wheat bran (WB), corn flour (CF), arrowroot (A), yellow peas split (YPS), rice husk (RH), and rice powder (RP) were screened individually.

Solid state fermentation

Solid substrates were purchased from local market and grinded to make fine powder. 5 gm of substrate was taken in 250 mL Erlenmeyer flask and was autoclaved at 121 °C for 20 min. It was then dried in an oven at 80 °C for 24 h. To maintain desired moisture level (% by mass per volume), sterile 0.1 M phosphate buffer (pH 6.9) was added aseptically and mixed thoroughly. Culture equivalent to 6 % inoculum load of SmF were added to it, mixed thoroughly and incubated at 37 °C for 48 h.

Unless it is specified otherwise, 0.5 gm of fermented mass was mixed with 2.5 mL of extraction buffer (0.1 M phosphate buffer pH 6.9 mixed with 0.1 % Tween 80), vortexed well and centrifuged at 10,000 rpm at 15 min at 4 °C. Supernatant was used as a source of crude enzyme for enzyme assay.

Optimization of process parameters

Various process parameters affecting enzyme production during SSF were optimized. Each parameter was optimized independently of the other and subsequently optimal conditions were employed in all experiments.

In a sequential order, tested process parameters were initial moisture content (50, 60, and 70 % by mass per volume), inoculum concentration (10, 20, and 30 % by volume per mass), medium pH (5, 6.9, and 10) and incubation temperature (37 and 45 °C).

Enzyme assay

β Amylolytic activity was measured by the method described by Bernfeld (1955). Assay mixture contained 0.5 mL of 0.1 M phosphate buffer (pH 6.9), 1 mL soluble starch (0.5 %, Sigma, USA), and 0.1 mL of enzyme. Control was prepared as same without adding substrate. The reaction mixture was incubated at 100 °C for 15 min. Enzyme–substrate reaction was then stopped by addition of 1 mL 2 M NaOH. Both the assay mixture and control were then allowed to boil in boiling water bath for 10 min after addition of 0.5 mL of 3,5-dinitrosalisylic acid reagent (Merck, Germany). After cooling the assay mixture at room temperature, absorbance were measured spectrophotometrically (Elico, India) at 540 nm. Amount of maltose released (mg) was measured from standard curve of maltose. One unit (U) of β amylolytic activity was defined as the amount of enzyme releasing 1 µmol of maltose equivalent per minute per mL from soluble starch (Sigma) under the standard assay conditions. Maximum enzyme titer was expressed as units per gram of dry mass of substrate (U/gdm).

Statistical analysis of data

All experiments were repeated thrice independently and representative data are shown as average values of triplicates. Error bars stand for percentage error of results. Error percentage below 5 % was considered significant for this study.

Result and discussion

Effect of initial moisture content of medium on the production of β amylase

Presence of moisture or water activity (a_w) in substrate plays the most critical role in SSF. Microbial activity in solid substrate is severely influenced by moisture exist in absorbed or complex form within solid matrix. The a_w of the medium has been attributed as a fundamental parameter for mass transfer of the water and solutes across the microbial cells. Variation of moisture content can directly influence microbial metabolism. Insufficient moisture may lead to poor accessibility of nutrients, insufficient diffusion of solute and gas resulting in poor microbial growth and so decreases enzyme production (Gervais and Molin 2003; Ramesh and Lonsane 1990). On the other hand, higher moisture content results in decrease of enzyme production as it decreases interparticle space of solid substrate and changes particle structure. This sterically hinder microbial growth, reduce gas volume and decreases rate of diffusion and ultimately affects oxygen transfer (Perez-Guarre et al. 2003). The optimum moisture content in SSF depends on organism and the substrate used for fermentation. In general, moisture level has found to vary between 30 and 85 %. Due to lesser demand of water during growth, only fungi and yeast were termed as suitable microorganisms for SSF. It was thought that due to high water activity requirement, bacterial cultures might not be suitable for SSF. However, experience has shown that bacterial cultures can be well managed and manipulated for SSF processes (Ramesh and Lonsane 1990; Anto et al. 2006; Baysal et al. 2003). Result of the present study indicated (Fig. 1) that optimum initial moisture level of substrates was 50 % for CF, 60 % for B, A, YPS, RH and 70 % for S, WB, RP. After 41 h of incubation at 37 °C, highest enzymatic production were recorded in case of B (120.34 U/gdm) followed by WB (119.79 U/gdm) and S (115.08 U/gdm) in their optimum moisture level. Other substrates showed little production at the same time. Different optimum moisture level indicated variation of moisture holding capacity of the substrates. A similar finding was reported for α amylase production by *B. licheniformis* using SSF (Lonsane et al. 1985) where optimum moisture level was varied between 60 and 75 %. Higher than expected level of moisture content (90 %) was also reported in case of *Thermomyces lanuginosus* ATCC 58157 by SSF on wheat bran (Kunamneni et al. 2005).

Effect of inoculum load on β amylase production

Higher inoculum concentration increases moisture level to a significant extent. This is due to free excess liquid that forms an additional diffusional barrier (Baysal et al. 2003). On the other hand, lower inoculum load delays desired microbial growth that ultimately affects enzymatic production. Several studies indicated that 20–30 % inoculum load was optimum for microbial activity (Pandey et al. 1999). SSF using *B. subtilis* DJ5 indicates that for B, S, WB, CF, A, YPS, RH only 10 % inoculum concentrations is sufficient for maximum enzyme production (Fig. 2). Maximum enzyme production was observed in B

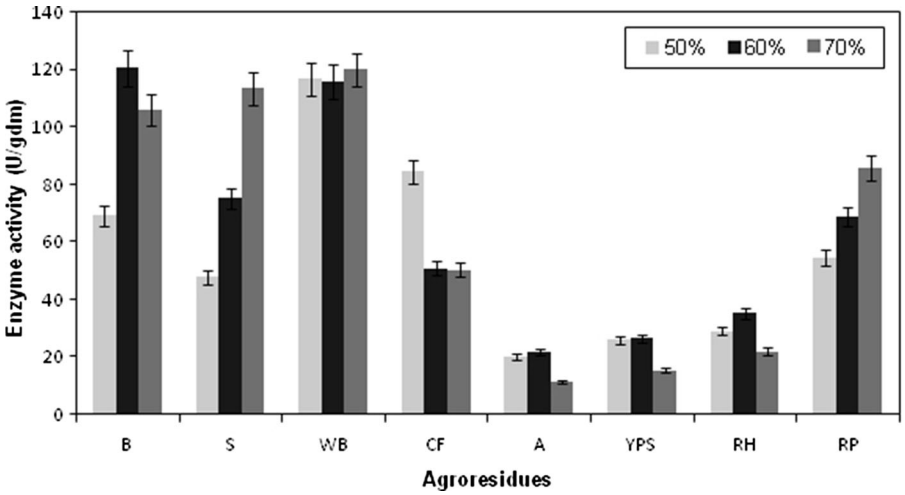

Fig. 1 Effect of initial moisture content (% by mass per volume) on β amylase production in SSF system. Process standard: inoculum load—10 % by volume per mass, incubation time—41 h, incubation temperature—37 °C, media pH—6.9

(120.34 U/gdm) followed by WB (119.79 U/gdm) after 41 h of incubation at 37 °C. Slightly higher inoculum load (20 %) was appropriate for RP (93.75 U/gdm) at the same condition.

Effect of medium pH on β amylase production

Initial pH of the fermentation media induce morphological changes in the organism, affects cellular metabolism (Bellon-Maurel et al. 2003) and also interferes with the stability of extracellular enzyme. pH variation of fermentation medium is performed only when the desired enzyme shows wide range of pH stability. β amylase from *B. subtilis* DJ5 was found to be stable at wide range of pH that allowed evaluation of microbial growth and enzyme production in SSF. Result indicates that at optimum moisture content and inoculum load, maximum enzyme production is obtained at pH 6.9 (Fig. 3). Higher or lower pH adversely affects microbial growth and its metabolism indicating neutrophilic nature of the organism. Similar findings were reported by Haq et al. (2002).

Effect of incubation temperature on β amylase production

Microbial growth is severely influenced by incubation temperature. Higher temperature is detrimental for mesophiles that ultimately affect on the level of enzyme production. In this study (Fig. 4), though the β amylase is hyperthermostable, the organism behaves like truly mesophilic. During SSF using B, S, YPS, WB, CF, RH, and A, such mesophilic property of the strain has been demonstrated by poor enzymatic activity at 45 °C. But interestingly, for RP, enzyme production was maximum at 45 °C (Fig. 4). This is probably due to the shielding effect of this substrate that prevents exposure of microbial cells to high temperature.

Determination of optimum incubation time on β amylase production

The incubation time is governed by substrate availability towards microbial growth and enzyme production (35). Among the different substrates tested in this study, maximum production was recorded after 25 h for A, YPS, RH, RP, 41 h for B, WB, CF and 45 h for S (Fig. 5). Though maximum production was earlier in case of A, YPS, RH, production titer was not very high. RP showed a higher production (93.75 U/gdm) after 25 h of incubation at 45 °C. B and WB showed highest enzyme production of 120.34 U/gdm and 119.79 U/gdm, respectively after 41 h of incubation. S somehow delayed enzyme production. After 45 h of incubation maximum production of 115.08 U/gdm was recorded. Higher enzyme titer obtained after 41 h of incubation in B and WB indicates that it was suitable for growth of *B. subtilis* DJ5. A gradual decrease in enzyme yield was recorded on further extension of fermentation period beyond optimum level. Similar findings have been reported (Sivaramakrishnan et al. 2006; Ramesh and Lonsane 1987) indicating enzyme degradation due to accumulation of toxic and protein denaturing agent in the medium.

Comparison of enzyme production in different substrate

From the Table 1, it is evident that barley is the best substrate for β amylase production followed by wheat bran (WB). Arrowroot (A) was found to be the poorest substrate for SSF.

Susceptibility of substrates towards enzymatic breakdown can be explained in the light of molecular frameworks of the substrate molecule. Scanning electron microscopy has found depressions on surfaces of dehydrated or wet starch granules randomly distributed over granule surfaces (maize, sorghum) or clustered equatorially

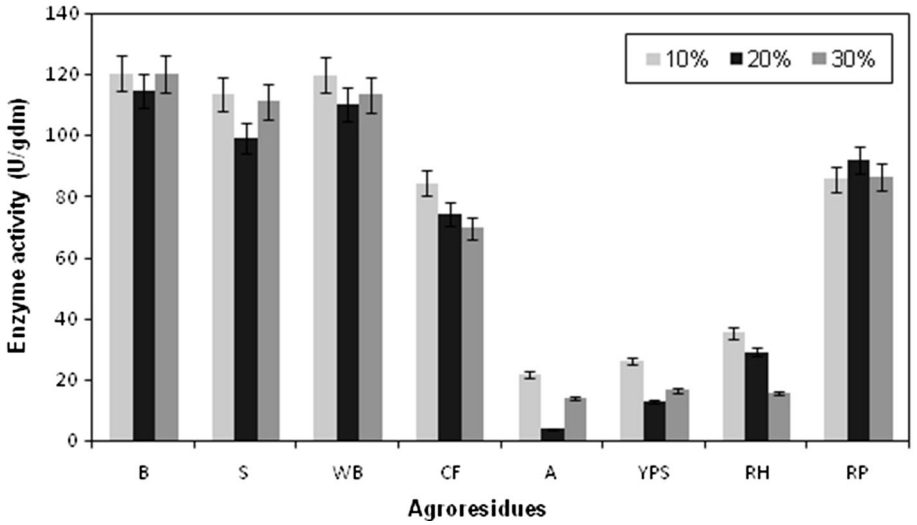

Fig. 2 Effect of inoculums fraction (% by volume per mass) on β amylase production in SSF system. Process standard: initial moisture content—50 % for CF, 60 % for B, A, YPS, RH and 70 % for S, WB, RP, incubation time—41 h, incubation temperature—37 °C, media pH—6.9

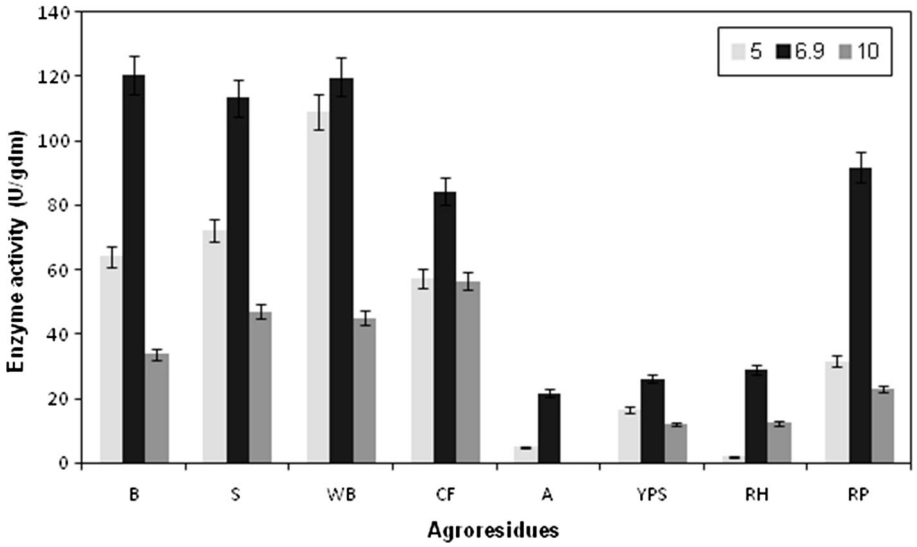

Fig. 3 Effect of pH on β amylase production by SSF system. Process standard: initial moisture content—50 % for CF, 60 % for B, A, YPS, RH and 70 % for S, WB, RP, inoculums load—10 % for B, S, WB, CF, A, YPS, RH and 20 % for RP, incubation time—41 h, incubation temperature—37 °C, media pH—6.9

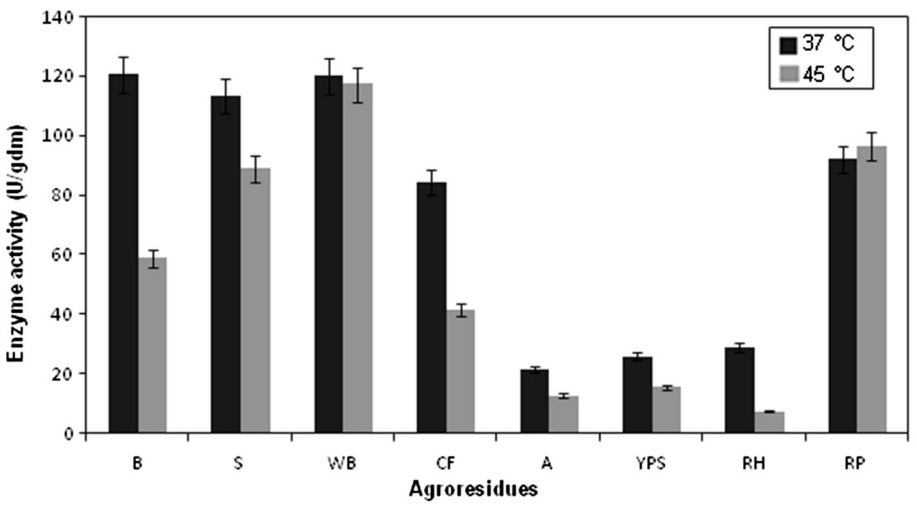

Fig. 4 Effect of incubation temperature on β amylase production by SSF system. Process standard: initial moisture content—50 % for CF, 60 % for B, A, YPS, RH and 70 % for S, WB, RP, inoculums load—10 % for B, S, WB, CF, A, YPS, RH and 20 % for RP, incubation time—41 h, media pH—6.9

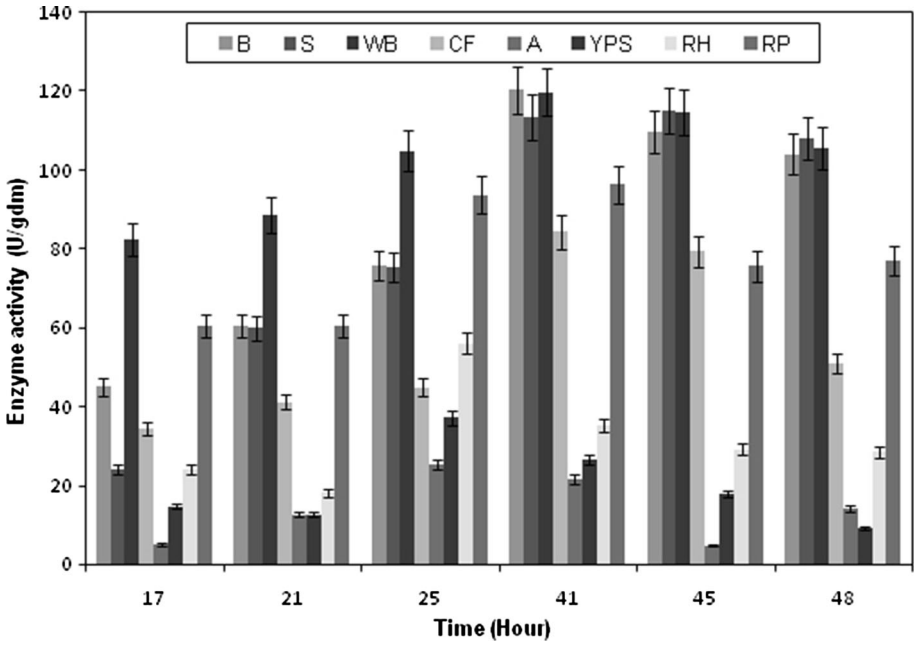

Fig. 5 Effect of incubation time on β amylase production by SSF system. Process standard: initial moisture content—50 % for CF, 60 % for B, A, YPS, RH and 70 % for S, WB, RP, inoculums load—10 % for B, S, WB, CF, A, YPS, RH and 20 % for RP, incubation time—41 h, incubation temperature—37 °C, media pH—6.9

Table 1 Comparison of β amylase production by *Bacillus subtilis* DJ5 using different agroresidues under SSF

Substrate	Moisture content (%)	Inoculum load (%)	Optimum incubation time (h)	Medium pH	Incubation temperature (°C)	Maximum Production (U/gdm)
S	70	10	45	6.9	37	115.08
B	60	10	41	6.9	37	120.34
WB	70	10	41	6.9	37	119.79
CF	50	10	41	6.9	37	84.29
A	60	10	25	6.9	37	25.15
YPS	60	10	25	6.9	37	37.11
RH	60	10	25	6.9	37	56.05
RP	70	20	25	6.9	45	93.75

(wheat, barley) and are not seen on arrowroot starch granules (Robertson et al. 2006). The depressions may be architecturally enzyme-susceptible regions. For digestion of raw starch granules, enzymatic attack is primarily focused to equatorial regions. Relatively mild amylase activity was suggested to be the result of poor presentation of surface molecules to the enzyme and to the α-D (1–4) bond specificity. Poor surface presentation of A and rice resulted in lower production of maltose, hence showed lower enzymatic activity.

Barley is the fourth largest cereal grain crop produced worldwide (after wheat, rice, and corn) and is used as rice substitute in many countries. Literature survey indicates that barley is the most underutilized cereal grain in terms of human consumption (Bhatty 1993). This results in huge wastage of this crop worldwide. On the other hand, WB is obtained while processing of wheat. Since it is not a product of human consumption, it is also accumulated in large quantities. This will be a gentleman's idea to use these in SSF that will minimize wastage.

This study has indicated barley is the most promising substrate for *B. subtilis* DJ5 followed by WB for β amylase production using SSF. Both substrates result in nearly similar productivity of β amylase. So utilization of this culture in SSF using B and WB as substrate will be beneficial not only from industrial point of view, but it will also serve to lower wastage and environmental pollution.

Conclusion

The use of SSF for the production of hyperthermostable β amylase using *B. subtilis* DJ5 is an economical and environment friendly process and is an alternative method of management of agroresidues or agro-wastes. Barley and/or wheat bran is the best substrate for the production of enzyme.

Maximum production can be achieved in a very short time (41 h) using 10 % inoculum load and incubating at 37 °C.

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Nomenclature

B	Barley
S	Sattu
WB	Wheat bran
YPS	Yellow peas split
CF	Corn flour
A	Arrowroot
RH	Rice husk
RP	Rice powder
SSF	Solid state fermentation
SmF	Submerged fermentation

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