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An Overview of Amylase Production by Solid State Fermantation (SSF) since 2010

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Abstract: Amylases are enzymes acting in glycosidic bonds in amylose and amylopectin polymers. They have broad application areas such as food, textile, paper, pharmaceutical, cosmetics, detergent, etc. taking almost 25% of the total world enzyme market. They are also used clinically and medicinally. While amylases are extensively distributed in nature, microbial enzymes have a broad range of industrial applications. Microbial enzymes are produced mainly with submerged fermentation (SmF) and solid state fermentation (SSF) methods. SmF is very expensive due to contents of synthetic media. Agricultural uses of by-products decrease the cost of the medium and makes SSF highly attractive.

2010 Yılından İtibaren Katı Faz Fermentasyon (SSF) Tekniği İle Amilaz Üretimine Genel Bir Bakış

Anahtar Kelimeler
Fungal Amilazlar, Bakteriyal Amilazlar, Katı Faz Fermentasyonu, Tarımsal Atıklar

Özet: Amilazlar amiloz ve amilopektin polimerlerinde glikozidik bağlar üzerinde etki gösteren enzimlerdir. Gıda, tekstil, kağıt, farmasötik, kozmetik, deterjan gibi birçok alanda kullanılırlar ve dünya enzim pazarının yaklaşık %25'ini oluştururlar. Ayrıca klinik alanlarda ve ilaç sanayinde kullanım alanlarına sahiptirler. Doğada yaygın olarak dağılmış olsada, özellikle mikrobiyal enzimler çok çeşitli endüstriyel uygulamalara sahiptir. Mikrobiyal enzimler, esas olarak batık fermantasyon (SmF) ve katı faz fermantasyon (SSF) yöntemleri ile üretilir. Ancak, sentetik medyanın içeriğinden dolayı SmF oldukça pahalı bir yöntemdir. SSF'de tarımsal yan ürünlerin kullanımı maliyetini düşürür ve bu tekniği oldukça çekici bir hale getirir.

1. Introduction

Amylases are glycoside hydrolases (GHs) acting in glycosidic bonds of amylose and amylopectin polymers. Several starch acting enzymes, α-amylase (EC3.2.1.1), maltogenic amylase (EC3.2.1.133), pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68), belong to this GH family 13 [1].

α-Amylases, endo enzymes, hydrolyse randomly the α-(1-4) glycosidic bonds of the linear starch polymers. They form oligosaccharides and α-limit dextrins having different chain length and containing α-(1-6) bonds [2,3].

Maltogenic amylases release α-maltose by hydrolysing α-(1-4) glycosidic bonds in starch polymers [4].

Isoamylase and pullulanase type I hydrolyse α-(1-6) glucosidic bonds. The greatest difference between isoamylase and pullulanase is the minimum length of the releasable side chain. Isoamylase is a starch debranching enzyme and it just hydrolyses glucose oligomers having α-(1-6) glycosidic bound with a degree of polymerisation of a least three. Pullulan is the substrate of pullulanase. But the enzyme also hydrolyse α-(1-6) glycosidic bounds in amylopectin and β-limit dextrins [5,6].

2. Industrial applications of amylases

The first industial enzyme used in 1984 as to treat digestive disorders was a fungal amylase [7]. After that time researches have continued till date to find potential microorganisms having amylases.

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Amylases have broad application areas such as food, textile, paper, pharmaceutical, cosmetics, detergent, etc. taking almost 25% of the total world enzyme market [3,8,9]. They are also used clinically and medicinally [8,10]. In addition, they are used in baking, starch, juice and brewing industries, pretreatment of animal feed for improving digestibility, desizing of textile fabrics, preparing starch coatings of paints, in manufacturing of cold water detergents, in removing wall paper and bioconversion of solid waste etc. [3,8,9,11-14].

3. Importance of SSF

Conversion of starch to glucose was previously achieved by acid hydrolysis. But this method requires highly acidic environment and elevated temperatures. These drawbacks are overcome by the use of α-amylase. Glucose and fructose syrup are produced from starch with hydrolysis activity of this enzyme. After the starch is converted to glucose, the released glucose is isomerized to fructose by glucose isomerase. High fructose (42%) containing syrups (HFCS) are prepared with this manner [3].

Microbial α-amylases are produced mainly with submerged (SmF) and solid state (SSF) fermentation methods [15].

In SSF, an insoluble solid porous substrate containing carbon and nitrogen sources and minerals serves an anchorage for microorganisms [16]. Microorganisms can produce some enzymes and metabolites more efficiently than in SmF when grown in conditions similar to their natural environment [17].

SmF has been a traditional method to produce many important enzymes because of multiple facilities like simplicity and better control of some factors such as pH, temperature, aeration, and moisture level. But, the process is very expensive due to contents of synthetic media. Also, the concentration of products is low and it requires large volume of water during down-stream processing [18]. In SSF, inexpensive agricultural and food industry waste products are used and the cost of the medium is reduced [12]. The use of agricultural by-products makes this method highly attractive [19].

Solid substrates such as wheat, barley, rice, corn and millet bran, soy bean, rice husk, coconut oil cake are used in SSF [20-22]. Anto et al. (2006) reported that glucoamylase from an *Aspergillus* sp. was produced by using rice flake production wastes [23].

4. Production of Amylases under SSF

When literature is reviewed it is seen that the production of amylases under SSF from many fungal and bacterial sources is quite common. The following section provides some information about the production of some bacterial and fungal amylases with SSF, especially since 2010.

In the text, you will see especially enzyme activities as U, IU and U/mL. One enzyme unit (International Unit, U, IU) is defined as the amount of protein releasing 1 μmol of product per minute. Volume activity (U/mL) of an enzyme is generally calculated as below:

$U/mL = (V_t. A.1000/ \varepsilon.l.V_s.t).d_f$

V_t: Total volume of reaction mixture (mL)

A: Absorbance

ε: molar absorption coefficient (cm^2mol^{-1})

l: path length (cm)

V_s: Volume of enzyme solution added to reaction mixture (mL)

t: Reaction time (minute)

d_f: Dilution factor

4.1. Fungal amylases

α-Amylase was produced from *Aspergillus oryzae* S2 under SSF. AmyC was purified with acetone precipitation and size exclusion chromatography. Optimum pH and temperature of the enzyme was reported as pH 5.6 and 60 °C, respectively. Starch hydrolysis with this enzyme resulted maltose and maltotriose as major end-products. The best appropriate solid substrate was reported as soya bean meal. Enzyme production was optimized with statistical design methodology (central composite design, CCD). The maximum activity was achieved by 76.25% moisture and C/N substrate ratio of 0.62 [23].

α-amylase production of *Monascus sanguineus*, a homothallic fungus, was screened with SSF by using beetroot as substrate. Maximum enzyme production was obtained as 0.0287 U/mL and 0.0284 U/mL by using beetroot peel powder and orange peel, respectively. Minimum activity was obtained as 0.0171 U/mL in the presence of onion peel. Amylase activity was also optimized by response surface methodology (RSM) and the highest optimized experimental activity was 0.014 U/mL at pH 5.0 and 50 °C. The enzyme was partially purified 6.46 fold by using $(NH4)_2SO_4$ precipitation. *M. sanguineus* α-amylase also had high efficiency for the removal of Spinach curry and Chocolate stains by using with a commercial detergent (Surf excel) at 20 °C [24].

α-amylase and glucoamylase from *Aspergillus niger* was produced simultaneously on triticale grains without any nutritive supplements. Triticale is an important industrial crop containing proteins, starch and many of the mineral elements. Triticale with three different particle sizes was used as solid substrate. The enzyme production increased 30% with the reduction of the particle size. Production of enzymes were also increased by the reduction of relative humidity to 30%. *A. niger* amylase cocktail hydrolyzed the raw starch from wheat flour more efficiently than

SAN Super 240L, an amylase cocktail widely used in industry [25].

Aspergillus niger ML-17 was grown on wheat bran at pH 5.0 and 30 °C for 96 h. Amylase production was increased to 4.4 ± 0.042 IU upon adding maltose, yeast extract, $NaNO_3$, $MgSO_4$, NaCl, Tween-80 and asparagine to the fermentation media at the concentration of 0.25%, 0.25%, 0.25%, 0.2%, 0.5%, 0.001% and 0.0001%, respectively. *Rhizopus oligosporus* ML-10 was also grown by using wheat bran. The optimum cultural conditions for the amylase production were pH 6.0, 35 °C and 96 h incubation. Addition of maltose (25%), yeast extract (0.25%), NH_4NO_3 (0.25%), $MgSO_4$ (0.2%), NaCl (0.75%), soluble starch (0.001%) and asparagine (0.0001%) to the fermentation medium enhanced the enzyme activity to 3.2 IU [26].

Highest amylase production (141.18 U/g of dry substrate) produced from *Fusarium solani* isolate SY7 was achieved under SSF using wheat bran after 3 days incubation. Optimum enzyme yield was obtained at pH 8.0, 25 °C and 70% moisture level [27].

Aspergillus oryzae LS1 amylase was produced by SSF in the presence of wheat bran as a substrate. Enzyme production was highest after 120 h incubation period, 54.5% initial moisture content of and in the presence of sucrose (1 g/100g WB) at 30 °C. Crude enzyme hydrolyzed soluble starch, corn starch, dextrin and potato starch. The enzyme had maximum activity at 55 °C. Also, the enzyme hydrolyzed wheat flour under optimized conditions with efficiency of 89% [28].

α-amylase from *Penicillium camemberti* PL21 was produced using orange waste. Ammonium sulfate precipitation, then column chromatography with sephadex G-100 and DEAE-Sepharose CL-6B were made and α-amylase was purified 38.5 folds. Optimum pH and optimum temperature was determined as pH 6.0 and 30 °C, respectively [29].

α-amylase from *Trichothecium roseum* was produced under SSF by using corncob leaf, wheat bran, rye straw, rice husk and sunflower oil meal. Optimum production conditions was achieved by using wheat bran, pH 7.0 phosphate buffer, 85% (w/v) initial moisture content, 8 days incubation period, 30 °C incubation temperature, 1,000 μm particle size, lactose and urea (1%, w/w) as additives. Adding of 0.1M $CaCl_2$ to wheat bran increased the production of α-amylase [30].

Aspergillus terreus NCFT 4269.10 α-amylase was produced by using residues of pearl millet as solid substrate. The maximum enzyme production was achieved at 30 °C after 96 h incubation. The enzyme was characterized after purified. Optimum pH and temperature was reported as 5.0 and 60 °C, respectively. It was found that the enzyme was highly compatible with some commercial detergents. Also, it was highly stable in the presence of some metal ions [31].

Streptomyces erumpens MTCC 7317 α- amylase was produced under SSF by using cassava fibrous residue. Optimum incubation period, moisture level and temperature were determined as 60 h, 60% and 50 °C, respectively with Response surface methodology (RSM). Beef extract was the best nitrogen sources supplemented. Soluble starch and cassava starch was hydrolyzed 85% and 70% by crude enzyme, respectively [32].

In another study, α-amylase from *Rhizoctonia solani* AG4 strain ZB-34 was cheaply produced by SSF using corn bran at the moisture level of 100% (v/w) after 6 days incubation at 28 °C. α-amylase production by *R. solani* AG4 ZB-34 increased significantly when the corn bran was supplemented with 1% soluble starch [33].

4.2. Bacterial amylases

Bacillus licheniformis AT70 α-amylase was produced with various wastes of agriculture and kitchen. The maximum enzyme yield was achieved using wheat bran and date waste. Amylase had the maximum activity in the presence of 1.5 M NaCl. Shooma, a local detergent, increased the activity approximately 34%. The enzyme also had remarkable hydrolytic activity of raw corn starch at 55 °C and 14-20% (w/v) concentration [34].

Bacillus subtilis was grown on wheat bran as a substrate for 48 h at 37 °C. After extraction, the enzyme was precipitated with ammonium sulphate precipitation and partially purified. The specific activity of partially purified amylase was reported as 13.14 μmol/mg/min at 40 °C. Maximum enzyme activity was achieved at pH 7.1 (8.74 μmol/mg/mL) at 40 °C [35].

α-amylase of *Bacillus* sp. KR-8104 was produced under SSF using wheat bran. Effects of some factors on enzyme production were analyzed by response surface methodology (RSM) based on a Box–Behnken design. Maximum enzyme production was achieved at 37 °C, 72% (w/w) initial substrate moisture, and 0.15 L/min aeration [36].

Bacillus cereus MTCC 7524 and *Bacillus licheniformis* MTCC 7445 was grown by using dairy sludge as substrate under SSF and *B. cereus* caused the higher amylase production than *B. licheniformis.* The highest α-amylase was produced by utilizing dairy sludge with sucrose and ammonium chloride as additives at 48 h at 45 °C and pH of 6.5, and inoculum level of 20 % (w/v) [37].

Bacillus circulans ATCC 4516 α-amylase was produced under SSF by using rice bran as solid substrate. Optimum enzyme production (2716.9±35.9 U/mg) was obtained with inoculum level 25%, initial pH 7.5 at 37 °C for 48 h with supplementation of ammonium chloride [38].

To produce an extracellular α-amylase from *Bacillus subtilis* RSKK96 by SSF, different agro residues were

used as substrate. Maximum enzyme yield was achieved with cotton stalk substrate as 1016.3 ± 32.6 U $g^{-1}\cdot10^{-3}$. Optimized conditions were incubation time of 72 h, incubation temperature of 37 °C, agitation speed of 150 rpm, inoculum size of 35%, initial moisture content of 30%, initial pH of 7.0. Ammonium nitrate at the 1% concentration increased the production of α-amylase to 1483.1±32.5 U $g^{-1}\cdot10^{-3}$ [39].

An amylase by *Bacillus subtilis* was produced under SSF using banana waste. Incubation time, substrate concentration, pH and temperature were optimized as 24 h, 50 g, pH 7.0 and 35 °C, respectively. Amylase had maximum activity in presence $MgSO_4.7H_2O$ (0.02%), $CaCl_2.2H_2O$ (0.04%) and KH_2PO_4 (0.4%) [40].

A thermostable *Bacillus* sp. Iranian S2 α-amylase was produced using wheat bran under solid-state fermentation. Maximum α-amylase production was achieved at 55 °C and pH 5.5 after 72 h incubation. Optimum substrate: moisture ratio was 1:1. Adding of some nitrogen sources (0.02 g/g) decreased the α-amylase production [41].

Bacillus sp. α-amylase having activity of 5400 U/g was produced at 1:3 moisture content and 20% inoculum under SSF using Mustard Oil seed cake after 72 h of incubation. Optimum activity was obtained at 50 °C and pH 6.0. It was highly thermostable at 70 °C for 2 h. α-amylase activity increased in the presence of Na^+, Ca^{2+}, Co^{2+} and Mg^{2+} [42].

Bacillus subtilis MAFE 118079 was used to produce amylase enzyme in SSF using rice bran. The enzyme had maximum activity (2311.1 U/g) at pH 7.0 and 37 °C. Addition of soluble starch and glucose in the concentration of 1% enhanced enzyme production (2311.1 U/g). Nitrogen sources also increased the enzyme synthesis [43].

Production of *Bacillus licheniformis* strain RT7YC α-amylase was maximum at 20% wheat bran under SSF. It was found that corn steep liquor was the most appropriate nitrogen source for enzyme production. The enzyme was stable at 60 °C for 10 min [44].

A thermostable α-amylase from *Paenibacillus amylolyticus* was produced by using wheat bran as solid substrate moistened with a solution containing (%, w/v) nutrient broth (1.0), soluble starch (1.0), lactose (0.5), NaCl (0.5) and $CaCl_2$ (0.2). The best enzyme production was achieved with the initial pH of 8.0 and inoculum level of 1.5 mL [45].

An extracellular amylase from *Bacillus cereus* was produced in solid state fermentation using black gram husk, rice bran, sugarcane bagasse, wheat bran, and green gram husk. The most suitable solid substrate, pH, temperature, inoculum level and fermentation time were observed as sugarcane bagasse, pH 7.0, 40.0 °C, 2.0% and 48 h, respectively [46].

Production of *Bacillus cereus* MTCC 1305 α-amylase was investigated under SSF. Optimum inoculum size and wheat bran:moisture ratio were as 10% (v/m) and 1:1, respectively. While glucose supplementation enhanced enzyme production, different nitrogen sources decreased the enzyme production. Optimum activity was determined at 55 °C and pH 5.0 [47].

Amylase was produced from *Bacillus cereus* IND4 under SSF using cow dung. Optimum enzyme production was achieved with 100% moisture, 0.1% fructose and 0.01% ammonium sulphate. Enyzme had maximum activity at pH 8.0 and 50 °C [48].

Table 1. A summary for some of the SSF techniques given in this article

Microorganism	Optimum SSF conditions (solid substrate, moisture level, pH, temperature, incubation period etc.)	Optimization method	Reference
Aspergillus oryzae S2	Soya bean meal, 76.25% moisture	Central composite design (CCD)	23
Monascus sanguineus	Beetroot peel powder, pH 5.0, 50 °C	Central composite design (CCD)	24
Aspergillus niger ML-17	Wheat bran, pH 5.0, 30 °C, 96 h incubation	-	26
Trichothecium roseum	Wheat bran, pH 7.0, 85% (w/v) initial moisture, 8 days incubation, 30 °C	-	30
Streptomyces erumpens MTCC 7317	Cassava, 60 h incubation, 60% moisture, 50 °C	Response surface methodology (RSM)	32
Rhizoctonia solani AG4 strain ZB-34	Corn bran, moisture level of 100% (v/w), 6 days incubation, 28 °C	-	33
Bacillus subtilis	Wheat bran, 48 h incubation, 37 °C	-	35

Bacillus sp. KR-8104	Wheat bran, 37 °C, 72% (w/w) initial moisture, 0.15 L/min aeration	Box–Behnken design	36
Bacillus subtilis RSKK96	Cotton stalk substrate incubation time of 72 h, 37 °C, 30% moisture, pH 7.0	-	39
Bacillus sp. Iranian S2	Wheat bran, 55 °C , pH 5.5, 72 h incubation	-	41
Paenibacillus amylolyticus	Wheat bran, pH 8.0	-	45
Microbacterium foliorum GA2	Bagasse, pH 8.0, 20 °C, 5 days incubation	Plackett–Burman design	49

The production of *Microbacterium foliorum* GA2, a cold-adapted microorganism, α-amylase under SSF was optimized by Plackett–Burman design and response surface methodology (RSM). It was determined from the result of Plackett–Burman design that bagasse, lactose and pH had significant effects (p-value≤0.05) on enzyme production. Optimum enzyme production (6610 units) was achieved through RSM in medium containing 40% bagasse, 0.003 M lactose, and pH 8.0 at 20 °C after 5 days [49].

Maximum α-amylase from *Bacillus* sp. KR-8104 under SSF was produced using wheat bran moistened with tap water (1:1.5) and adding NH_4NO_3 (1%) and lactose (1%) after 48 h incubation at 37 °C [50].

Table 1 provides a summary for some of the SSF techniques given in this article.

5. Conclusion

SSF is an alternative technique allowing the reuse of cheap agro-industrial wastes such as wheat, barley, rice and corn bran for the production of high valuable enzymes. Amylases have variable industrial applications. From this point of view it is essential to reduce the cost of the enzyme production and meet consumer demands in the industrial areas being used amylases.

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