Food Sci. Biotechnol. 25(1): 185-192 (2016)
DOI 10.1007/s10068-016-0028-4

Food Science
and
Biotechnology

Optimization of Submerged *Aspergillus oryzae* S2 α-Amylase Production

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Received June 12, 2015
Revised September 6, 2015
Accepted October 25, 2015
Published online February 29, 2016

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pISSN 1226-7708
eISSN 2092-6456

Abstract Use of 4 agro-industrial by products and organic materials as nitrogen sources for production of *Aspergillus oryzae* S2 α-amylase in liquid culture was investigated. The 2 agro-industrial byproducts maltose and saccharose, and also lactose and starch were individually evaluated for use as carbon sources. A Box-Behnken experimental design was used to determine optimal conditions for production of α-amylase. A maximum amylase activity of 750 U/mL was obtained at a temperature of 24°C, a urea concentration of 1 g/L, and a C/N ratio of 2. Laboratory scale application of optimal conditions in a 7 L fermentor produced a final α-amylase activity of 770 U/mL after 3 days of batch cultivation. Addition of 10% starch to the culture medium each 12 h immediately after the stationary phase of cell growth led to a production yield of 1,220 U/mL at the end of fed-batch cultivation.

Keywords: *Aspergillus oryzae* strain S2, Box–Behnken design, 7 L-fermentor, batch, fed-batch

Introduction

Amylases are glycoside hydrolases (GHs) that catalyze hydrolysis of α-(1,4)- and/or β-(1,6)-linkage starch polymers. Commercially important GH enzymes, representing approximately 25-33% of worldwide commercial enzyme production, are classified into different families. While some amylases are grouped into GH family 13 (α-amylase family), β-amylases (EC 3.2.1.2) and gluco-amylases (EC 3.2.1.3) are grouped in GH families 14 and 15, respectively (1). Furthermore, α-amylases (endo-1, 4-α-D-glucan glucanohydrolase EC 3.2.1.1) are extracellular enzymes that randomly cleave α-(1, 4)-linkages between adjacent glucose units of starch polymers and ultimately generate short oligosaccharides and different α-limit dextrins (1). The α-amylase family of enzymes has potential applications in a wide range of industries, including bread making, brewing, starch processing, pharmaceuticals, textiles, and paper. A low-cost medium for α-amylase production is needed to meet increasing industry demands (2). In this respect, varieties of *A. oryzae* have been reported to be valuable sources for production of a large variety of industrial enzymes, including glucoamylases and α-amylases (3). *A. oryzae* varieties are particularly interesting fungi due to ease of cultivation.

Industrial production of enzymes can be achieved using the 2 basic approaches of genetic manipulation and media engineering. In fact, submerged fermentation has been particularly useful for production of industrially important enzymes due to ease of handling and greater control of environmental factors (4). Several studies have shown that submerged amylase production depends on culture medium composition, sources of nitrogen and carbon, pH, temperature, aeration, inoculum age, inhibitors, and activators (2).

The agro-industrial residues of hydrolyzed sago starch (5), brewer's spent grain (6), groundnut oil cake (4), Pearl millet (7), and orange waste powder, have been reported to be valuable starting materials for low cost production of amylase (8). The agro-industrial residues of hydrolyzed sago starch (5), brewer's spent grain (6), groundnut oil cake (4), Pearl millet (7), and orange waste powder, have been reported to be valuable starting materials for low cost production of amylase (8). Other non-conventional substrates, including dextrose (9), lactose, and casitone and fructose (2), have also been used for α-amylase production. While ammonia was described as the best mineral nitrogen source (10), peptone (11), casein hydrolysate, yeast extract, and soyabean flours (2), have been recognized as the best organic nitrogen sources.

Understanding combined effects of all factors and setting of optimal experimental conditions for amylase production are essential in the submerged fermentation process. Response surface methodology (RSM) is a collection of mathematical and statistical techniques for empirical model building. RSM is used for problems where an output parameter is influenced by several input parameters and the objective is to optimize the desired response. It is a simple, time-saving, and reliable tool that requires few experimental trials for evaluation of interactions of several parameters with a large number of factors.

A fed-batch is a batch growth process based on feeding of a

growth limiting nutrient substrate to a culture. The fed-batch strategy is an evolutionary cultivation process designed to replace batch culture and extend the product formation stage, to help reduce medium viscosity, and to eliminate repressive effects of rapidly used carbon sources, including use for amylase filamentous fungi production (12).

Imai *et al*. (13) previously studied effects of fed-batch culture on amylase production by *A. oryzae* RIB 642 in a rotary draft tube fermentor (RTF) and reported that fed-batch production was 2x higher than for batch production at a feed rate of 1 g h^{-1}. Starch has generally been used as an induction substrate in *A. oryzae* batch alpha amylase production and has been considered as a sole or major carbon source in fed-batch α-amylase production (14). Maltose has also been used as a sole substrate and showed trends similar to starch in fed-batch *Bacillus amyloliquefaciens* α-amylase production (2). Fed-batch experiments have demonstrated that α-amylase production is enhanced at low starch feed concentrations as a sole carbohydrate source.

Production of *A. oryzae* S2 α-amylase using an Erlenmeyer flask submerged production level of 350 U/mL after 92 h of culture (15) has been reported with an optimized production medium for *A. oryzae* CBS 819.72 α-amylase (16). *A. oryzae* strain S2 produced the 2α-amylases AmyA and AmyB in submerged culture. This study focused on investigation of optimization of *A. oryzae* S2 amylase production using submerged fermentation with a statistical experimental design. Scale-up production in a 7 Laboratory scale fermentor and assay of fed-batch cultivation were also examined.

Materials and Methods

Microorganism *A. oryzae* strain S2 was used in α-amylase production assays under submerged fermentation due to efficiency, ease of cultivation, and non-toxicity in amylase production (15). The strain was isolated from a sweet soy sauce, propagated on PDA (Potato Dextrose Agar, Fluka, France) medium plates at 30°C, and stored at 4°C prior to use.

Substrate origins and characterization

***Nitrogen and carbon sources*:** Unless otherwise specified, all substrates, chemicals, and reagents were of the analytical grade or the highest commercially available purity from Sigma-Aldrich (St. Louis, MO, USA). The 4 agro-industrial byproduct residues tuna powder, wheat gluten meal, soyabean meal, and deactivated yeast were used as organic nitrogen sources. Tuna powder obtained from a local tuna processing factory in June of 2014. Consisted of 78.3% protein, 13.3% sugar, 7.2% lipid, and 1.2% ash (dry matter= 98.7%). Wheat gluten meal obtained from a local factoryin August of 2014 contained 72.1% protein, 26.67% sugar, 0.034% lipid, and 1.2% ash (dry matter=87.6%). Soyabean meal obtained from a local factoryin June of 2014 contained approximately 55.01% protein, 37.65% sugar, 0.13% lipid, and 7.2% ash (dry matter=91%). Deactivated yeast obtained from a local baker yeast factory in July of 2014 consisted of 80.3% protein, 3.4% sugar, 0.12% lipid, 2.1% ash, and a 14.08% water content (dry matter=85.92%).

The 2 agro-industrial byproducts gruel and whey powder, along with maltose, saccharose, lactose, and starch, were evaluated for use as carbon sources for *A. oryzae* S2 α-amylase production. Gruel obtained from a local semolina factory in June of 2014 that processed durum wheat contained starch (60%), other carbohydrates (5%), cellulose (1.5%), gluten (12%), and total nitrogen (2.1%) (16). Whey powder obtained from a local semolina factory in June of 2014 contained13% protein, 70% sugar, 0.1% lipid, 9% ash, and 7.9% water content (dry matter=92.1%). Starch from potatoes (BioChemika; Sigma-Aldrich) was also used as carbon source.

Total carbohydrate contents were determined after total acid hydrolysis. The protein content was determined following the standard AACC method 46-10A (17). The lipid content was determined following AOAC method 920.39 (18). The dry matter content was determined based on oven drying at 105°C to a constant weight, and the ash content was determined based on combustion in a muffle furnace at 550°C for 12 h. All contents were expressed as dry matter weight/weight.

Media The MR optimized *A. oryzae* CBS 819.72 alpha amylase culture medium used in this study was previously described by Kammoun *et al.* (16) and Sahnoun *et al.* (15) for *Aspergillus oryzae* strain S2 alpha amylase submerged culture production. The medium contained (g) gruel (25), urea (12.5), casein acid hydrolysate (12.5), peptone hy-soy (6.25), glycerol (6.25), KH_2PO_4 (5), $(NH_4)_2SO_4$ (2.5), $MgSO_4$ (2.5), and distilled water (1 L). MW medium contained (g) starch (8), yeast extract (1), KH_2PO_4 (2), $MgSO_4$-$7H_2O$ (0.3), $CaCl_2$-$2H_2O$ (0.3), 1 mL of Tween 8, and distilled water (1 L). MZ medium contained (g) starch (8), $(NH_4)_2SO_4$ (5.0), KH_2PO_4 (1.5), $MgSO_4$-$7H_2O$ (1), NaCl (1.0), $CaCl_2$-2 H_2O (0.1), and distilled water (1 L). MW and MZ media were supplemented with 1 mLof the trace elements (g/L) $MgSO_4$ (1.6), $ZnSO_4$ (1.4), $FeSO_4$ (5),and $CaCl_2$ (2). Media contents were thoroughly mixed, and the initial pH was adjusted to 5.5. Flasks used for media preparation were plugged with cotton and autoclaved at 121°C at 15 psi for 20 min.

Preliminary study and process description of flask, batch, and fed-batch fermentations For maximization of amylase yields, several process production factors were analyzed following a1 variable-at-a-time approach. MR, MW, and MZ media were used for preliminary screenings. The 12 agro-industrial byproducts assayed as nitrogen sources for production of *A. oryzae* S2 α-amylase inliquid culture werethe agro-industrial nitrogen sources tuna powder, wheat gluten meal, deactivated yeast, and soyabean meal, and the organic nitrogen sources beef extract, yeast extract, casaminoacids, and urea, and the mineral nitrogen sources NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, and $NaNO_3$. Several carbon sources were assayed individually, including the 2

agro-industrial byproducts gruel and whey powder at 14 and 11.5 g/L concentrations, respectively, and maltose, saccharose, lactose, and starch, all at a concentration of 8 g/L. In order to investigate the glucose effect on α-amylase synthesis regulation, a glucose concentration of 1-7.5 g/L was gradually added to the medium a substitute for the carbon source. Effects of starch concentrations of 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20 g/L, and aeration in 250, 500, and 1,000 mL Erlenmeyer flasks on α-amylase production were also investigated.

Batch and fed-batch fermentations were performed in a 7 L Infors fermentor (Lab Force AG, Muttenz, Switzerland) under optimal conditions (temperature of 24°C, a urea concentration of 1 g/L, and a C/N substrate ratio of 2). The system was interfaced with Iris software (version 4, Lab Force AG) to register data and control external peripherals. The fermentor was also equipped with a vapor condenser as a heat exchanger ahead of the vessel to ensure that water vapor streams condensed and returned to the broth. Fermentation was carried out at an aeration rate of 1.0 vvm, an agitation speed of 500 rpm, and a dissolved oxygen tension of 20%. The pH was maintained constant at 5.5 using orthophosphoric acid 30% (v/v) and ammonia 30% (v/v). Cultivations were started in batch mode for 60 h in a working volume of 5 L using optimized conditions determined from flask cultivation, and continued in fed-batch mode for 60-220 h. The batch medium contained (g/L) starch(10), KH_2PO_4 (2.5), $MgSO_4$-$7H_2O$ (0.3),$CaCl_2$- $2H_2O$ (0.3), urea (1), and soyabean meal (4.47). The feed-medium (FM) contained 100 g/L starch for fed-batch cultivations.

Inoculum growth assays were performed at 24°C with 7 day old *A. oryzae* strain S2 mycelia harvested from plates and dislodged under aseptic conditions using a production medium. Triplicate amylase fermentations were carried out based on inoculation of 100 mL of aculture medium (starch (10), urea (1), soyabean meal (4), KH_2PO_4 (2), $MgSO_4$-$7H_2O$ (0.3), $CaCl_2$-$2H_2O$ (0.3), 1 mL of Tween 80, and distilled water (L)) in a 500 mL flask with 10% of the inoculum preparation, which was equivalent to 10^6 spores/mL. Optical density, total cell number, and cell dried weight of the strain inoculum preparation were heldat constant values.

Analytical methods

***Amylase recovery and assay*:** Triplicate fermentation medium samples were harvested and cells were separated using refrigerated centrifugation (Rotanta; Hettich, Kirchlengern, Germany) at 8,000×*g* for 20 min. Amylase was assayed using addition of 50 μL of the culture supernatant to 0.5 mL of 1% (w/v) starch dispersed in a 0.1 M acetate buffer (pH 5.6). The reaction mixture was incubated for 30 min at 50°C and liberated reducing sugars were measured as glucose equivalents using the 3,5-dinitrosalicylic acid method (19). A separate blank was made for each medium sample to eliminate non-enzymatic release of sugars. One unit of α-amylase activity corresponded to the amount of enzyme that released reducing sugars equivalent to 1 μmol of glucose per min under standard assay conditions. Results are reported as x±SD, where x refers to the mean value of 3 replications and SD to the standard deviation. Student's *t*-test was used to determine significant differences between means. Data were analyzed using SPSS for Windows (Version 11.0.1, 2001, Lead Technologies, Inc., Charlotte, NC, USA).

***Biomass*:** Biomass was harvested viacentrifugation (Rotanta; Hettich) at 8,000×*g* for 15 min, and then washed with distilled water. Dry biomass was determined based on a constant weight at 105°C with a 0.001 g precision.

***Quantification of reducing sugar contents*:** Reducing sugar concentrations in crude fermentation amylase extracts were measured following the DNS method (19). A reaction was effectuated in boiling water for 5 min, followed by dilution with deionized water, and placement in cool running water to stop the reaction.

Experimental design and statistical analysis The Box-Behnken RSM design has 3 levels of low, medium, and high, usually coded as −1, 0, and +1. Factors screened in a preliminary analytical study included temperature (°C), the C/N substrate ratio defined as the ratio of mass of carbon to mass of nitrogen in substances contained in the medium, and the urea concentration in g/L. The experimental plan consisted of 15 trials performed in triplicate. Mean values (n=3) of alpha amylase production (Y) were taken as responses.

The following general form of a second degree polynomial Eq. (1) was used:

$$Y=a_0+a_1X_1+a_2X_2+a_3X_3+a_{11}X^2+a_{22}X^2+a_{33}X^2+a_{12}X_1X_2+a_{13}X_1X_3+a_{23}X_2X_3,$$

where X_1, X_2, and X_3 are coded factors (Table 1), a_0 is an intercept, a_1, a_2, and a_3 are linear coefficients, a_{11}, a_{22}, and a_{33} are squared coefficients, and a_{12}, a_{13}, and a_{23} are interaction coefficients. Model coefficients were estimated using multilinear regression. Quadratic model equation was expressed by the coefficient of determination (R^2) and its statistical significance was checked by Fischer's test value (F-value) with unequal variance at p<0.05. An analysis of variance (ANOVA) was performed to check compatibility of the proposed model with experimental data. Data were analyzed using the SPSS statistical software package (Version 11.0.1 2001) (Lead Technologies). The response surface was generated using EXCEL (Version 2003, Microsoft Office Inc., Redmond, WA, USA) software. The regression model was determined in accordance with SPSS procedures, which initially considered all factors, then eliminated factors that had no effect in a step-by-step process. Insignificant model terms were, therefore, excluded.

A Levene test was performed to ensure equality of variances. A normal distribution was verified using the P-Plot function. A one-way ANOVA was carried out for analysis of differences between means. Data were then submitted to Duncan's Least Significant Difference (LSD) test for evaluation of critical differences between groups. In all cases, significance was set at (p<0.05).

Results and Discussion

A. oryzae S2 α-amylase production was previously investigated

Table 1. A Box-Behnken experimental design used for optimization of α-amylase production by *A. oryzae* strain S2

Run No.	Temperature (°C)		C/N ratio		Urea (g/L)		α-Amylase activity (U/mL)
	Actual value	Coded value	Actual value	Coded value	Actual value	Coded value	
1	24	(-1)	1.5	(-1)	1.5	(0)	675±0.31
2	24	(-1)	2.5	(+1)	1.5	(0)	640±0.63
3	30	(+1)	1.5	(-1)	1.5	(0)	417±0.75
4	30	(+1)	2.5	(+1)	1.5	(0)	375±0.70
5	27	(0)	1.5	(-1)	1	(-1)	435±0.81
6	27	(0)	1.5	(-1)	2	(+1)	473±0.57
7	27	(0)	2.5	(+1)	1	(-1)	416±0.44
8	27	(0)	2.5	(+1)	2	(+1)	465±0.18
9	24	(-1)	2	(0)	1	(-1)	725±0.25
10	30	(+1)	2	(0)	1	(-1)	263±0.87
11	24	(-1)	2	(0)	2	(+1)	635±0.45
12	30	(+1)	2	(0)	2	(+1)	380±0.97
13	27	(0)	2	(0)	1.5	(0)	415±0.55
14	27	(0)	2	(0)	1.5	(0)	408±0.34
15	27	(0)	2	(0)	1.5	(0)	586±0.72

Activity was expressed as: means±SD of 3 trials

under submerged fermentation in Erlenmeyer flasks using an optimized production medium consisting of the 2 relatively costly nitrogen sources of casein acid hydrolysate peptone, and hy-soy (16). This study investigated use of agro-industrial residues and wastes as substitutes for expensive MR nitrogen sources for amylase production. Values for interval factors used in preliminary and optimization testing in this study was based on previous preliminary testing and experimental designs reported for *A. oryzae* CBS 819.72 α-amylase production under submerged fermentation (16). Values and deviation numbers of different medium components and parameters used in the Plakett-Burman design were determined based on previous study and previous work on *A. oryzae* α-amylase production under submerged fermentation (16).

Screening of media, and carbon and nitrogen source effects A previously used MR submerged medium consisting of the 2 expensive nitrogen sources of casein acid hydrolysate, and peptone hy-soy (15) was used as a control medium in this study. MZ medium containing $(NH_4)_2SO_4$ as a sole nitrogen source was also used as a basic medium to reduce production costs. In addition, slightly modified MW medium as a basic medium for amylase production (20) containing yeast extract as a sole nitrogen source, was used for α-amylase production under submerged fermentation. A maximum amylase production yield of 400 U/mL was obtained using MW medium, whereas the lowest production yield of 296 U/mL was achieved using MZ medium after 4 days of culture. Accordingly, MW was subsequently used as a basic medium.

Yeast extracts were efficient nitrogen sources for submerged α-amylase production by *A. oryzae* strain S2, in agreement with previous reports (21,22). Tween-80 had positive effects on the α-amylase productivity of *A. oryzae* S2 as addition of Tween-80 to the culture medium induced a significant (p<0.05) 1.1x increasein α-amylase production, compared with controls, indicating a useful role for permeabilization of cells to improve enzyme secretion.

Individual effects of different carbon sources in MW medium were investigated, including the 2 agro-industrial byproducts gruel and whey powder at concentrations of 14 and 11.5 g/L, respectively, and maltose, saccharose, lactose, and starch, all at a concentration of 8 g/L (Fig. 1A). Starch yielded the highest rate of enzyme activity of 400 U/mL after 4 days of culture for carbon sources. A decrease in enzyme activity induced by gruel was attributed to either a high content of other poorly digestible carbohydrates that could increase the viscosity of the medium and decrease oxygen transfer and mixing and, hence, α-amylase formation, or because starch plays a role as an inducer and stabilizer, particularly in *Aspergillus* amylase production (14). Based on expense and availability, starch was used as a carbon source in subsequent analyses.

For improvement of enzyme production and minimization of production costs, 12 inexpensive mineral and organic agro-industrial by-products were assayed as potential nitrogen source substitutes for the expensive yeast extract nitrogen source in MW medium, taking into consideration equivalent nitrogen input values. Organic nitrogen sources yielded higher levels of amylase activity than inorganic sources (Fig. 1B). The highest level of enzyme activity were obtained when soyabean meal was used as a nitrogen source (470 U/mL), followed by urea (460 U/mL). Soyabean meal, therefore, is a potential substitute for yeast extract, in agreement with a previous report (11) that soybean meal and urea had positive effects on α-amylase production in submerged fermentation.

Starch and glucose concentration optimization Starch was assayed at concentrations ranging between 2.5 and 20 g/L. Use of starch at a concentration of up to 10 g/L (450 U/mL) induced a significant (p<0.05) increase in *A. oryzae* S2 α-amylase production, compared

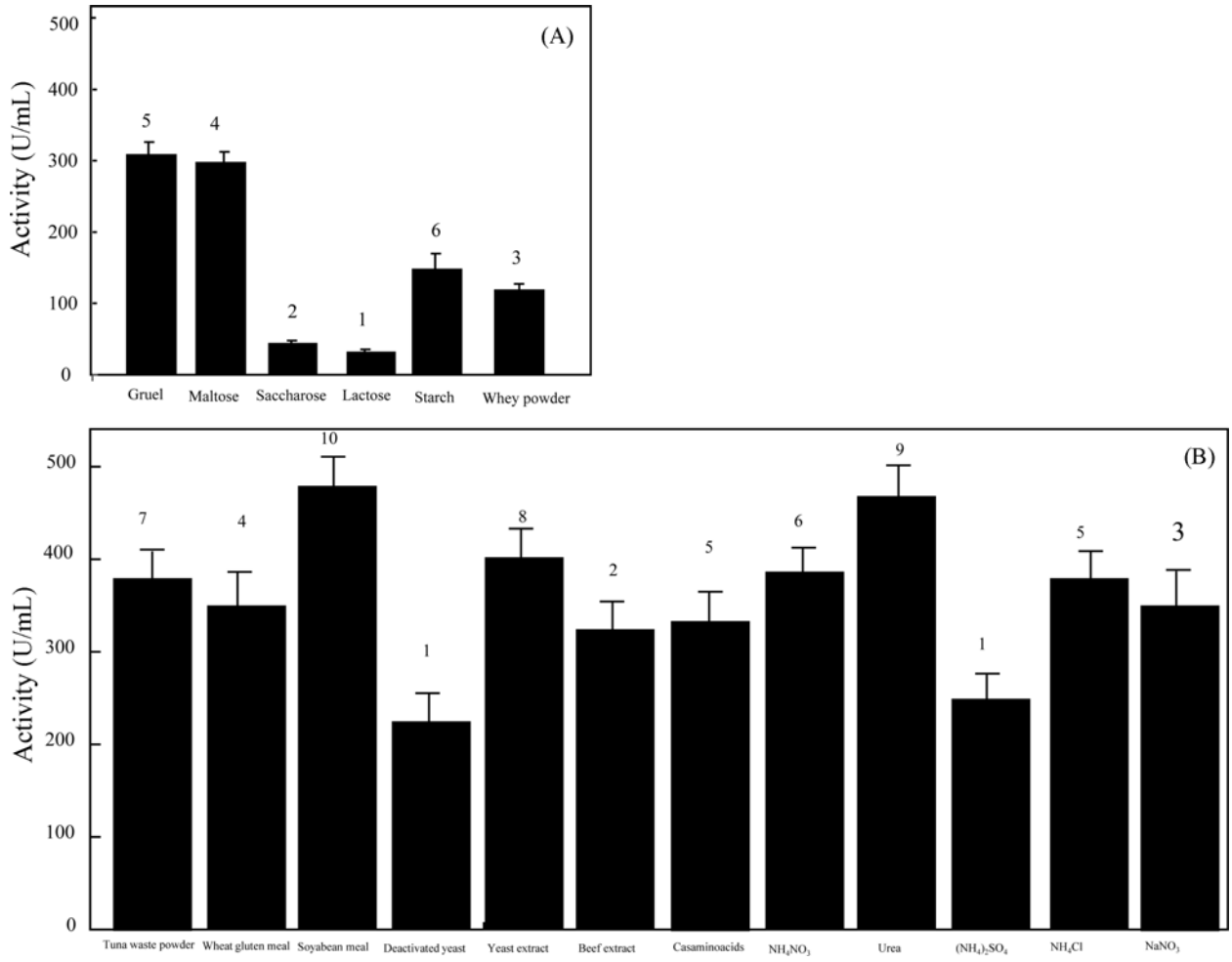

Fig. 1. Screening of carbon (A) and nitrogen (B) source effects on amylase production.

with controls. When used at concentration higher than 10 g/L, starch induced a decrease in α-amylase production. When starch was used at a concentration of 20 g/L, the production yield decreased to 43 U/mL, attributed to accumulation of the glucose hydrolysis product that is responsible for catabolic repression during production.

Effects of glucose addition in a range of 1 to 7.5 g/L on *A. oryzae* S2 α-amylase production were, therefore, evaluated and α-amylase production yields decreased with addition of glucose at concentrations from 5 to 300 U/mL. Thus, *A. oryzae* strain S2 amylase production apparently suffered glucose catabolic repression, a phenomenon that was previously observed for *A. oryzae* (23), *A. awamori* (24), and other fungi (25).

Aeration effects Effects of aeration on amylase under submerged production were investigated. The same inoculum size of *A. oryzae* strain S2 (10^6 spores/mL) was inoculated at a fixed culture volume of 100 mL in flasks of different volume. Based on the ratio of medium volume to container volume, changes in the amount of oxygen availability could have effects on mycelial growth. Appropriate aeration rates related to the ratio of medium volume to container volume induced maximum levels of α-amylase activity of 500 mL (400 U/mL)>250 mL (360 U/mL)>1 L (320 U/mL). Thus, the optimum free air volume was approximately 5x the culture medium volume.

Response surface methodology (RSM) This study used MW medium as a basic medium with starch at a concentration of 10 g/L and urea and soyabean meal as nitrogen sources. The 3 variables of C/N, urea concentration, and temperature (°C), were analyzed using a Box-Behnken design with 15 experiments at 3 levels of −1, 0, 1. Experimental responses are shown in Table 1. Other variables were held at constant levels. An *F*-test (ANOVA) was used to identify significance of the second-order model. The regression model for α-amylase production was highly significant (p<0.01), with a satisfactory value of the coefficient of determination (R^2=0.93), indicating that 93% of variability in the response could be explained by the second order polynomial equation:

$Y=7841.67857-444.778381*X_1-903*X_3+6.32142857*X_1*X_1+34.5*X_1*X_3$,

where Y is activity (U/mL), X_1 is temperature (°C), X_2 is C/N, and X_3 is urea (g/L).

Analysis of the quadratic model showed that variables with largest

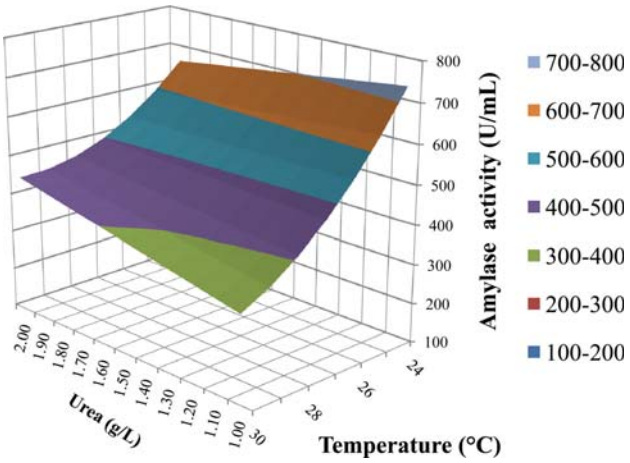

Fig. 2. Response surface plot of amylase activity showing interactions among urea (g/L) and temperature (°C) using a Box-Behnken experimental design with the substrate ratio (C/N) set at the central point.

effects were the linear terms of temperature and urea concentration, the quadratic term of temperature, and the interaction term for urea concentration and temperature. The interaction between urea (g/L) and temperature (°C) was visualized using a 3D response surface graph (Fig. 2). Accordingly, the response surface was obtained based on varying urea (g/L) and temperature values while holding the third factor constant at an intermediate level of 2. The elliptical contour plot of the response surface showed that the interaction between urea (g/L) and temperature (°C) was significant (p<0.05). A high degree of amylase activity was obtained at a low concentration of urea and a low temperature. Thus, *A. oryzae* S2 required a low nitrogen level for production of alpha amylase, in agreement with a previous study of *A. oryzae* (10).

Validation of the model For validation of predicted results, fermentation experiments were performed in 2 tests. The quadratic model showed that maximum rates of α-amylase production could be attained when cells were grown at a temperature of 24°C, a urea concentration of 1 g/L, and a C/N substrate ratio of 2. Under these optimized culture conditions, the maximum rate of α-amylase production attained was 750 U/mL with a yield of 84,269.66 U/g of dried substrate, equivalent to a productivity value of 10.41 U/mL/h. The composition of the optimized medium after RSM analysis was (g): starch (10), urea (1), soyabean meal (4), KH_2PO_4 (2), $MgSO_4$-$7H_2O$ (0.3), $CaCl_2$-$2H_2O$ (0.3), 1 mL of Tween-80, and distilled water (L). The *A. oryzae* S2 yield was 2x higher than the yield obtained using MR medium in submerged culture with the relatively expensive sources (casein acid hydrolysate, and peptone hy-soy) (15). The submerged production rate was higher than rates described for *A. oryzae* submerged production using the byproduct nutrients of orange waste water (8), gruel (16), and starch processing waste water (26). The rate was also higher than other alpha amylase production rates obtained using other *Aspergillus niger* (27) with brewery and meat waste water as substrates, respectively.

Scale-up production studies Validated culture conditions were applied in a 7 L laboratory scale fermentor with a working volume of 5 L. Maximum amylase production occurred under batch fermentation when the cell population reached a peak (Fig. 3A). The amylase activity reached 770 U/mL after 3 days of batch cultivation, which was 2.26x higher than activity previously described for *A. oryzae* CBS 819.72 under the same laboratory-scale batch fermentation conditions (28). The amylase activity was also better than for *Trichoderma reesei* batch fermentation using a substrate of waste cardboard in a 30 L laboratory fermentor (29), and for other *Bacillus amyloliquefaciens* batch fermentations (30).

Fed-batch cultivation was started in batch mode and, once the stationary phase had been reached (48 h), first addition was performed using 100 mL of starch (10%). Four other additions were then performed every 12 h and the production returned to the batch mode until the endof 220 h. The duration of the biomass stationary phase increased from 60 to 180 h after changing the culture mode from batch to fed-batch (Fig. 3A). At this stage, the biomass maintained the same cell concentration, and concordance of a stable maintain of enzyme activity was observed. Starch has high reactivity and adhesion properties that were previously reported to be mediated via surface proteins, including surface proteins of microorganisms (31). Thus, starch apparently plays a supportive role for maintenance and immobilization of both the enzyme and cells in fed-batch culture.

The α-amylase activity level increased to a maximum of 1,220 U/mL in fed-batch culture, which was equivalent to 24.55 FAU/mL, higher than a value previously described for alpha amylase production in fed-batch culture of *A. oryzae* strain IF0 4177 (12). This production yield was also higher than a value reported for an expression system of heterologous α-amylase using *P. Pastoris* with methanol as a sole carbon source (32), and higher than for α-amylase production yields in fed-batch culture of *Escherichia coli* induced using a pulsed electric field technique in a M-100 Jar fermentor (33) and continuous shake flask *Bacillus caldolyticus* submerged production (34). The α-amylase production yield obtained in this study was also higher than a value achieved for continuous heterologous *A. oryzae* α-amylase production using *Saccharomyces kluyveri* as a host for extensive expression (25). Starch hydrolysis was investigated via reducing sugar quantification (Fig. 3B). An increase in the extract crude reducing sugar content at the beginning of batch fermentation was presumably due to an increase in levels of enzyme-released products. Variation in the reducing sugar content was observed after 48 h of culture, ascribed to a compromise between amylase hydrolysis and cell consumption. Under fed-batch fermentation, reducing sugar levels were constant, presumably due to a faster starch hydrolysis rate than the reducing sugar consumption rate.

Acknowledgments This work was supported in part by a grant from the Tunisian Ministry of Higher Education and Scientific

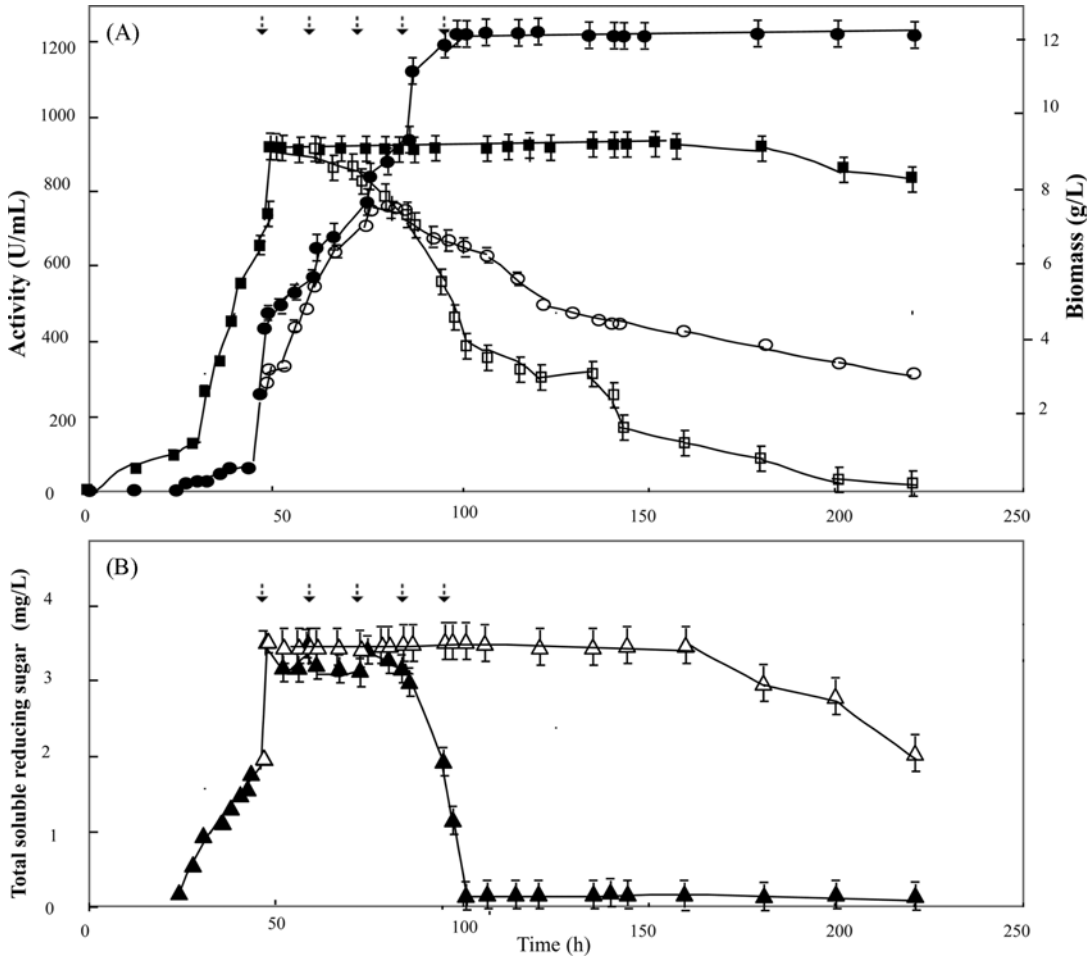

Fig. 3. Time course of α-amylase activity (U/mL) in batch (●) and fed-batch mode (○); biomass in batch (■) and fed-batch mode (□) (A) reducing sugar content in batch (▲) and fed-batch mode (△) (B) One hundred mL of a 10% starch concentration was added at 48 h and subsequently every 12 h during the culture period. Arrow (⇢) indicates the time of feeding.

Research contract program CBS-LMBEE/code: LR15CBS06_2015-2018. Prof. A. Smaoui and Prof. H. Ben Salem of the English Department at the Sfax Faculty of Science (Sfax, Tunisia) provided constructive proofreading and language polishing services.

Disclosure The authors declare no conflict of interest.

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