J. Microbiol. Biotechnol. (2008), **18**(4), 695–703

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Coproduction of Thermostable Amylase and β-Galactosidase Enzymes by *Geobacillus stearothermophilus* SAB-40: Application of Plackett-Burman Design to Evaluate Culture Requirements Affecting Enzyme Production

Soliman, Nadia A.*

Mubarak City for Scientific Research and Technology Applications, Genetic Engineering and Biotechnology Research Institute, Bioprocess Development Department, New Burg El-Arab City, Universities and Research Institutes Zone, Post 21934, Alexandria, Egypt

Received: March 29, 2007 / Accepted: July 2, 2007

A locally isolated thermophile, *Geobacillus* sp. SAB-40, producing thermostable extracellular amylase constitutively and an induced intracellular β-galactosidase was characterized and identified based on 16S rRNA sequencing. A phylogenetic analysis then revealed its closeness to *Geobacillus stearothermophilus*. To evaluate the effect of the culture conditions on the coproduction of both enzymes by *G. stearothermophilus* SAB-40, a Plackett-Burman fractional factorial design was applied to determine the impact of twenty variables. Among the tested variables, $CaCl_2$, the incubation time, $MgSO_4 \cdot 7H_2O$, and tryptone were found to be the most significant for encouraging amylase production. Lactose was found to promote β-galactosidase production, whereas starch had a significantly negative effect on lactase production. Based on a statistical analysis, a preoptimized medium attained the maximum production of amylase and β-galactosidase at 23.29 U/ml/min and 12,958 U/mg biomass, respectively, which was 3- and 2-fold higher than the yield of amylase and lactase obtained with the basal medium, respectively.

Keywords: Thermostable amylase, galactosidase, *Geobacillus*, experimental design, optimization

Hydrolases are enzymes with increasing industrial applications, where amylases and galactosidases have received special attention. The main natural substrate of amylases is starch, which is cleaved into branched and unbranched oligosaccharides. Thus, the enzymes work from either the nonreducing end of the chain (exoenzymes), producing low molecular weight products (*e.g.*, β-amylase, glucoamylase, and α-glucosidase), or the interior of the molecule in a random fashion (endoenzymes), producing linear and branched saccharides with various lengths (*e.g.*, α-amylase). The industrial interest in amylolytic enzymes is based on their application in sugar production, starch liquefaction, baking, and more recently detergency processes, which require amylases with a high thermostability and/or high activity [4, 45]. Most industrial starch processes are performed at a high temperature and involve the hydrolysis of starch into glucose, maltose, or oligosaccharide syrups. These syrups are then used as fermentation syrups to produce a variety of chemicals (*e.g.*, ethanol, lysine, and citric acid). A thermostable amylase from a thermophilic microorganism is associated with rapid cell growth and reduces the risk of contamination. Furthermore, the application of a high temperature during the hydrolysis of starch reduces the viscosity of the starch paste [21, 39]. Yet, the biosynthesis of thermostable amylolytic enzymes is rarely observed in natural bacterial strains [38, 40]. Bacteria from the genus *Bacillus* are widely used as producers of commercial thermostable amylases [14, 31, 40]. Many of the reported thermostable α-amylases have been produced from thermophilic bacteria, including *Bacillus* sp. JF [23], *Bacillus licheniformis* [53], *Geobacillus stearothermophilus* [2, 47], *Geobacillus thermoleovorans* YN [3], *Thermus* sp. [46], *Dictyoglomus thermophilum* [16], *Pyrococcus furiosus* [9, 10], and *Thermococcus onnurineus* NA1 [29].

Galactosidase enzymes are classified into α-galactosidases and β-galactosidases according to the substrate used and end product. α-Galactosidases are useful in the oil and gas industries and for sugar beet processing [27]. For example, the raffinose content in sugar beet is 0.15%, yet when processing sugar beet, the raffinose concentration in the beet molasses gradually increases to inhibit sugar crystallization. Thus, using an α-galactosidase enzyme to hydrolyze the raffinose into galactose and sucrose increases the beet sugar yield [51].

*Corresponding author
Phone: 0020-3-4593420; Fax: 0020-3-4593423;
E-mail: nadiastuttgart@yahoo.com

β-Galactosidases (E.C. 3.2.1.23) (or lactase) hydrolyze lactose, the sugar in milk, into its components, glucose and galactose, allowing milk and its derivatives to be treated for consumption by people with lactose intolerance. β-Galactosidases also have other applications, such as the prevention of lactose crystallization in frozen and condensed milk products, reduction of water pollution caused by whey, and increasing the sweetening properties of lactose [17, 41]. In addition to the normal hydrolysis of the glycosidic linkage in lactose, some β-galactosidase enzymes can catalyze the formation of galactooligosaccharides through the transfer of one or more D-galactosyl units into the D-galactose moiety of lactose. This transglycosylation reaction [24] has already been shown to be a characteristic of β-galactosidase enzymes derived from a wide variety of bacterial and fungal species [11, 35, 36, 55]. Nonetheless, even though many β-galactosidases derived from mesophilic microorganisms have been thoroughly investigated, relatively few studies have reported on thermostable β-galactosidases [1, 5, 7, 13, 23, 26, 30, 39, 54].

The use of different statistical designs for medium optimization has recently been employed for many enzymes [6, 8, 15, 19], where such statistical methods have proven to be effective and efficient when compared with the common "one-variable-at-a-time" method.

Accordingly, owing to the broad range of applications for thermostable amylases and galactosidases and limited reported data on their coproduction [23, 26], the present study attempted to optimize a fermentation medium for the coproduction of amylase and galactosidase enzymes. Furthermore, this study would appear to be the first attempt to maximize the coproduction of amylase and galactosidase enzymes using statistically designed experiments.

As such, a thermophilic bacterium producing both types of enzyme (amylase and β-galactosidase) was first isolated and identified. After determining the coproduction of extracellular amylase and intracellular β-galactosidase by *Geobacillus stearothermophilus* SAB-40, a Plackett-Burman experimental design was then applied to evaluate the impact of various culture conditions, including nutritional and physiological variables, on the coproduction of both enzymes.

Materials and Methods

Isolation, Identification, and Maintenance of Bacterium

The bacterium used in this study was isolated from Egyptian desert soil. The soil sample was suspended in a liquid minimal medium composed of (g/l) glucose, 0.1; KCl, 1; $MgCl_2$, 0.04; $CaCl_2$, 0.02; NH_4NO_3, 1; EDTA (disodium salt), 0.08; $Fe(NH_4)_2(SO_4)_3 \cdot 6H_2O$, 0.025; $MnSO_4 \cdot 4H_2O$, 0.002; $CoSO_4 \cdot 7H_2O$, 0.0002; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0001; and $ZnSO_4 \cdot 7H_2O$, 0.002; supplemented with 0.1% (v/v) yeast extract and incubated at 55°C while shaking at 200 rpm [20]. Subsequent dilutions of the 24-h-old culture were then plated into the same medium for further purification, and the purified isolates qualitatively tested for the coproduction of amylase and β-galactosidase by streaking in a petri dish containing an LB agar supplemented with starch and lactose (0.5:0.5%). A positive starch-hydrolyzing isolate produced a clear halo zone around the growth, whereas a positive lactose-fermenting isolate produced a blue-color growth in the presence of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal, 20 mg/ml). A potent isolate showing binary activities towards starch and lactose was selected and maintained on an LB agar slant composed of (g/l) tryptone, 10; yeast extract, 5; NaCl, 5; and agar, 20. The stock culture was subcultured at regular intervals of one month and stored under refrigeration.

The bacterium was characterized and identified by 16S rRNA gene sequencing using universal primers, with the following forward and reverse primer sequences, respectively: 5'-GAGTTTGATCMTGGCTCAG-3' and 5'-TACGGYACCTTGTTACGACTT-3'. The 16S rRNA sequence was aligned with published sequences from the NCBI database using ClustalX software, and the phylogenetic tree depicted using the TREEVIEW program [37]. The sequence has since been deposited in the GenBank under the accession number EF86524.

Enzyme Assays

The activity of the starch-hydrolyzing enzyme (amylase) was determined using Fuwa's colorimetric method of an iodine-starch color reaction [18]. Fifty μl of the amylase solution in a 2 mM imidazole-HCl buffer (pH 7.0) was mixed with 100 μl of a prewarmed 1.1% soluble-starch solution and incubated at 60°C for 10 min. The reaction was then stopped by adding 250 μl of a stop solution (0.5 N acetic acid:0.5 N HCl=5:1), and a 100-μl aliquot of the reaction mixture mixed with 1 ml of an iodine reagent (0.01% iodine and 0.1% KI). The absorbance at 660 nm was measured after incubating at room temperature for 20 min, where one unit of amylase activity was defined as the amount of enzyme that decreased the absorbance at 660 nm by 0.01 within 1 min. The activity was expressed as U/ml/min.

The galactosidase activity was determined as described by Miller [34] with some modifications. Each frozen pellet was thawed in 1 ml of a Z-buffer containing 1% toluene and vortexed for membrane permealization. Appropriate volumes were then used to determine the enzyme activity at 60°C with *ortho*-nitrophenyl-β-D-galactosidase as the substrate, where one unit of galactosidase activity (Miller unit) was defined as the amount of enzyme hydrolyzing 1 nmol of *o*NPG formed per min. The specific activity was expressed in Miller units/mg biomass. All the assays were performed in triplicate.

Growth and Production Conditions

The bacterium was allowed to grow in a 50-ml aliquot of an LB broth in a 250-ml Erlenmeyer flask, and incubated for 12 h at 55°C and 200 rpm. A 1% inoculum of the overnight culture was then used to inoculate LB broth, LB broth supplemented with lactose (1%), LB broth supplemented with starch (1%), and LB broth supplemented with starch plus lactose (0.5:0.5%), and then incubated at 55°C and 200 rpm. Quantitative estimations of the extracellular and intracellular enzyme production were determined after 24 h of incubation. The enzyme activities were determined in the culture supernatants after clarifying the cultures by centrifugation and cell pellets after resuspension in the same buffer used for the enzyme assays.

Table 1. Variables and levels employed in Plackett-Burman design for screening culture conditions affecting the coproduction of thermostable starch-hydrolyzing (amylase) and lactose-fermenting (β-galactosidase) enzymes produced by *G. stearothermophilus* SAB-40.

Variable code	Variable	Value	
		−1	+1
X_1	Lactose (g/l)	0.00	10
X_2	Tryptone (g/l)	1.00	10
X_3	Yeast extract (g/l)	1.00	5.0
X_4	Casein hydrolysate (g/l)	1.00	5.0
X_5	Glycine (g/l)	0.50	2.0
X_6	Soluble starch (g/l)	1.00	10.0
X_7	Peptone (g/l)	1.00	10.0
X_8	Beef extract (g/l)	0.50	3.00
X_9	Maltose (g/l)	1.00	5.00
X_{10}	Na citrate(g/l)	1.00	4.00
X_{11}	Na glutamate (g/l)	1.00	4.00
X_{12}	$(NH_4)_2SO_4$ (g/l)	1.00	3.00
X_{13}	NH_4Cl (g/l)	1.00	3.00
X_{14}	$NaNO_3$ (g/l)	0.50	2.00
X_{15}	K_2HPO_4 (g/l)	0.25	1.00
X_{16}	$MgSO_4.7H_2O$ (g/l)	0.20	0.50
X_{17}	$NaCl_2$ (g/l)	0.50	5.00
X_{18}	$CaCl_2$ (g/l)	0.10	2.00
X_{19}	Incubation time (h)	16.0	24.0
X_{20}	Incubation temperature (°C)	50.0	60.0

Experimental Design

Fractional Factorial Design. A Plackett-Burman experimental design [43] was applied to investigate the significance of various medium components on the coproduction of the starch-hydrolyzing (amylase) and lactose-fermenting (β-galactosidase) enzymes. Twenty culture variables were tested at two levels, −1 for low and +1 for high, based on a Plackett-Burman matrix design (Table 1), representing a two-level factorial design and allowing the investigation of n-1 variables in at least n-experiments. In this study, the independent variables were screened in 24 combinations according to the matrix shown in Table 2. The main effect of each variable was calculated simply as the difference between the average of measurements taken at the high setting (+1) and the average of measurements taken at the low setting (−1) for that factor.

As such, the Plackett-Burman experimental design was based on the first-order model (Equation 1):

$$\mathbf{Y}=\beta_0+\sum\beta_i\mathbf{x}_i \quad (1)$$

where Y is the predicted response, β_0 and β_i are constant coefficients, and x_i is the coded independent variable estimate or factor.

Data Analysis. The starch-hydrolyzing enzyme(s) and lactose-fermenting enzyme(s) data were subjected to statistical analysis, where Essential Experimental Design free software [48] was used for the data analysis, determining the coefficients, and the polynomial model reduction. The factors with the highest t-value and a confidence level over 95% and 80% were considered to be highly significant for amylase and β-galactosidase production, respectively.

Table 2. Plackett-Burman experimental design for evaluating factors affecting the coproduction of thermostable starch-hydrolyzing (amylase) and lactose-fermenting (β-galactosidase) enzymes produced by *G. stearothermophilus* sp. SAB-40.

Trial	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}	X_{18}	X_{19}	X_{20}	Amylase (U/ml/min)	β-Galactosidase (Miller unit)
1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	18.35	2,391
2	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	10.93	22
3	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	10.90	516
4	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	21.34	1,165
5	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	18.88	39
6	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	19.73	1,214
7	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	22.99	34
8	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	17.50	12,958
9	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	21.85	573
10	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	07.08	42
11	−1	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	18.01	110
12	+1	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	23.29	48
13	−1	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	19.56	1.4
14	−1	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	21.28	97
15	−1	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	18.54	349
16	−1	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	13.21	232
17	+1	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	21.17	285
18	+1	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	14.75	5,144
19	−1	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	17.69	410
20	−1	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	0.0	564
21	+1	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	-1	−1	−1	0.0	126
22	−1	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	00.54	38
23	−1	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	09.95	192
24	+1	+1	+1	−1	−1	+1	+1	+1	+1	−1	+1	−1	+1	−1	−1	−1	−1	+1	+1	−1	22.51	1,573

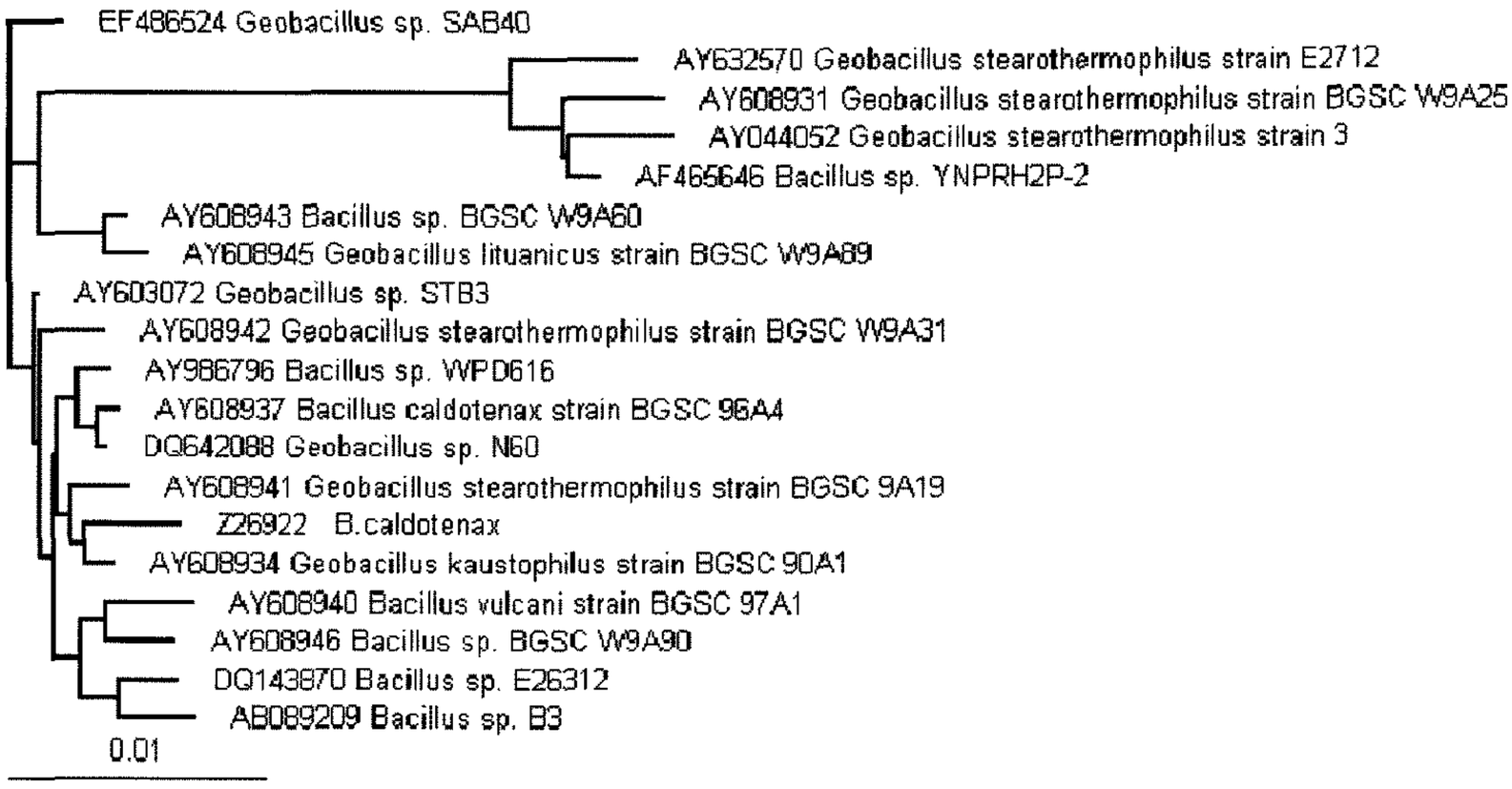

Fig. 1. Phylogenetic tree of SAB-40 isolate obtained by distance matrix analysis showing the position of SAB-40 among selected bacilli and phylogenetically related geobacilli based on 16S rRNA sequence comparisons.
Bar, 0.1 substitutions per nucleotide position.

Results and Discussion

Isolation, Characterization, and Taxonomic Classification of New Isolate SAB-40

In a screening program for the isolation of thermostable-enzyme-producing bacteria, 42 isolates were obtained from soil samples tested from different localities in Egypt. Isolate SAB-40 was found to be able coproduce an amylase and β-galactosidase with a high potency. The morphological and physiological characteristics of this isolate showed that it was rod shaped, spore forming, Gram positive, and a true thermophilic isolate (unable to grow below 40°C).

To investigate the phylogenetic affiliation of this isolate, the 16S rRNA gene was sequenced and deposited in GenBank under the accession number EF86524. When comparing the obtained sequence with 16S rRNA sequences available in the NCBI database, the greatest similarity was found with *G. thermoleovorans* and *G. stearothermophilus* (99%), plus a phylogenetic tree constructed using the ClustalX program (Fig. 1) revealed that isolate SAB-40 was more related to *G. stearothermophilus*.

Coproduction of Thermostable Starch-hydrolyzing and Lactose-fermenting Enzymes by *G. stearothermophilus* SAB-40

In a preliminary study, the effects of four different media on the production of both enzymes were examined, where the media were LB, LB+lactose, LB+starch, and LB+lactose+ starch. The extra- and intracellular enzyme production was determined after incubation for 24 h at 55°C and 200 rpm.

When growing *G. stearothermophilus* SAB-40 on the four tested media, the coproduction of both enzymes varied greatly in potentiality, as shown in Fig. 2.

In general, the isolate produced the starch-hydrolyzing enzyme extracellularly, whereas the lactose-fermenting enzyme was mainly produced intracellularly, as shown in Figs. 2A and 2B, respectively. Similarly, the *Bacillus* sp. JF strain was also previously found to produce an extracellular thermostable α-amylase and intracellular α-

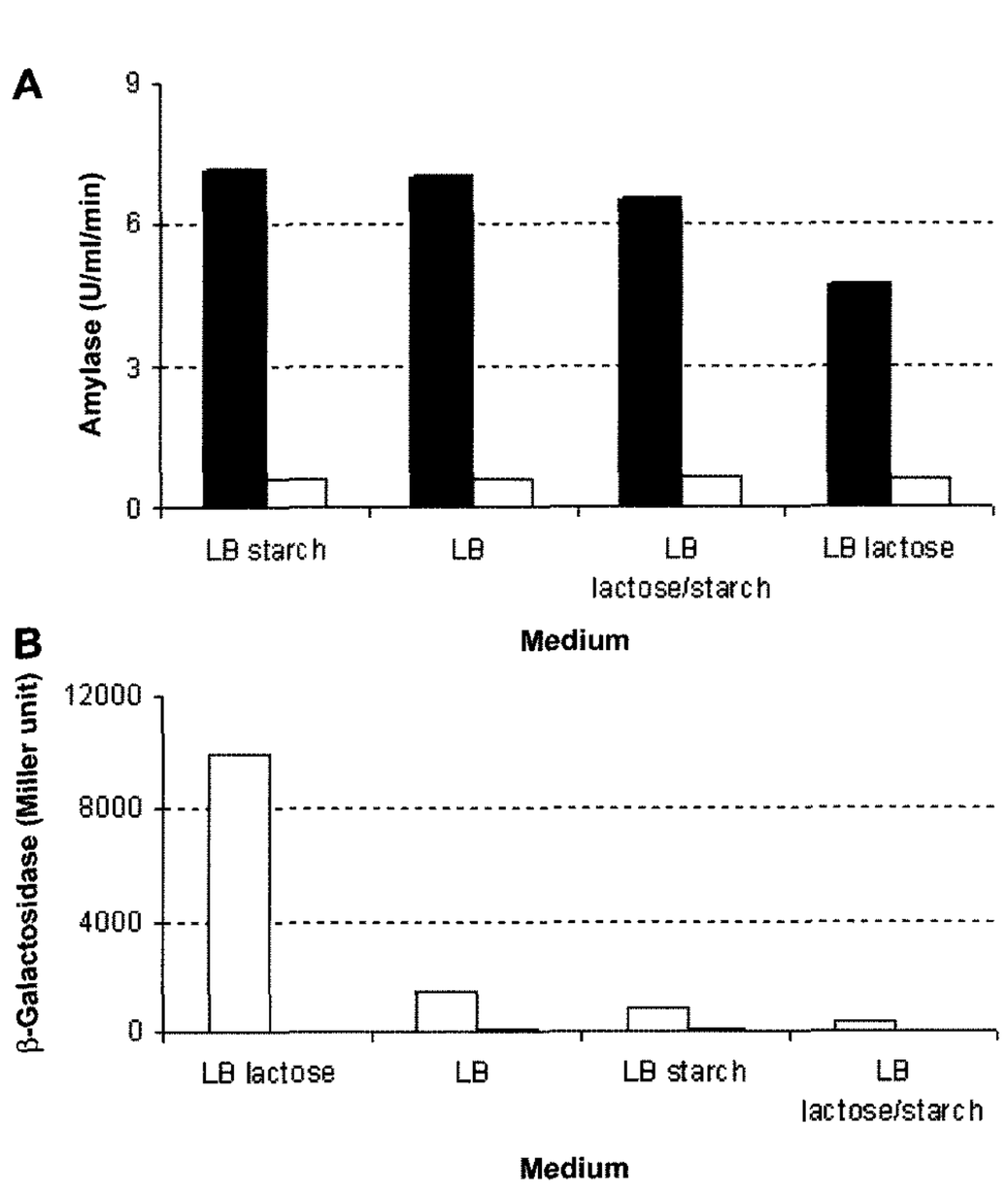

Fig. 2. Extra- ■ and □ intracellular production of thermostable amylase (**A**) and β-galactosidase (**B**) enzymes by *G. stearothermophilus* SAB-40 in different media.

galactosidase simultaneously [23]. The highest level of starch-hydrolyzing enzyme production was measured in the LB+starch medium, followed by the LB and LB+ lactose+starch media, whereas the highest level of lactose-fermenting enzyme production was found in the LB+ lactose medium, indicating that the lactose-fermenting enzyme was induced with lactose, whereas the starch-hydrolyzing enzyme could be produced constitutively. Accordingly, the lactase enzyme was identified as a β-galactosidase. The present findings are also in accordance with previous reports, where *G. thermoleovorans* YN was found to produce α-amylase constitutively [3] and *Thermoanaerobacter* produced β-galactosidases under lactose induction [30, 39].

Evaluation of Factors Affecting Coproduction of Thermostable Starch-hydrolyzing and Lactose-fermenting Enzymes

Normally, there is no general defined medium for specific enzyme production by different microbial strains [40], as every microorganism has its own particular physico-chemical nutritional requirements for either growth or primary and secondary metabolite production. Thus, for the simultaneous production of both amylase and β-galactosidase, a screening strategy for a large number of factors is required.

To evaluate the factors affecting the coproduction of both enzymes by *G. stearothermophilus* SAB-40, a Plackett-Burman statistical design was employed. The settings for the twenty independent variables examined are shown in Table 1, and the experiments were carried out

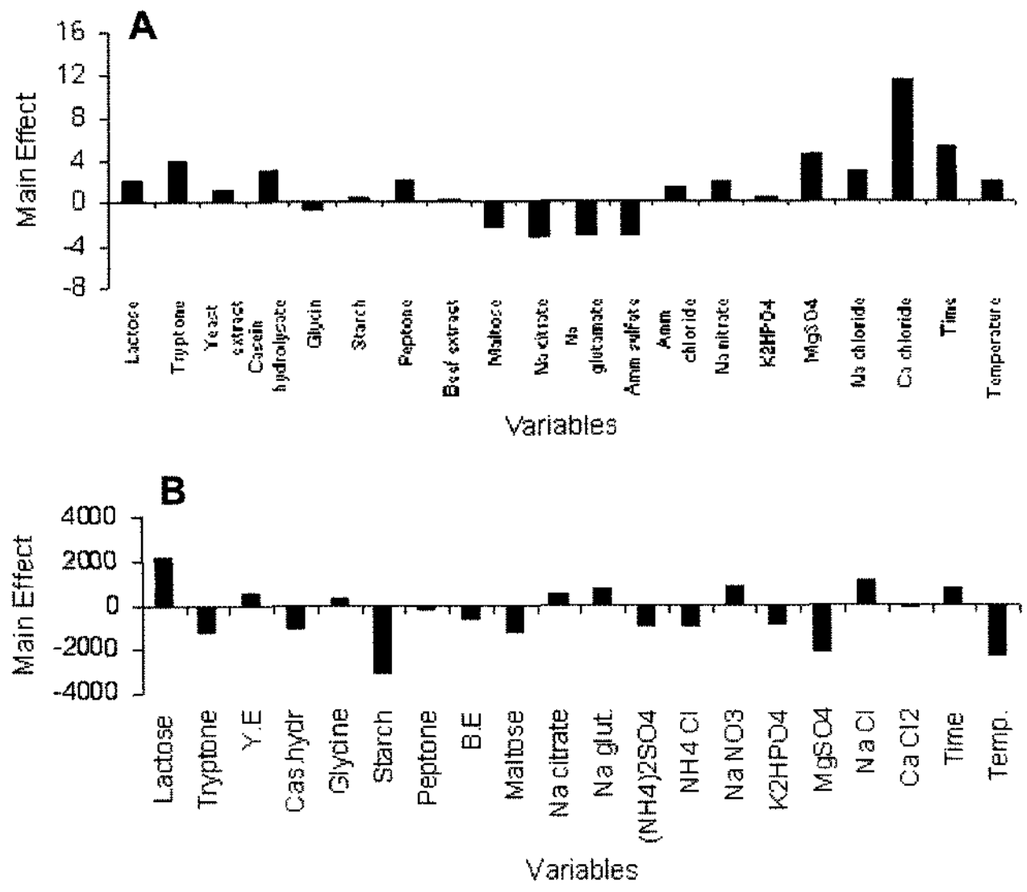

Fig. 3. Effect of culture conditions on starch-hydrolyzing (amylase) (**A**) and lactose-fermenting (β-galactosidase) (**B**) enzymes produced by *G. stearothermophilus* SAB-40 based on Plackett-Burman design results.

according to the experimental matrix presented in Table 2, where the production of the amylase and β-galactosidase enzymes was the measured response. A wide variation was found among the different trials as regards the amylase production results (0.0–23.29 U/ml/min) and β-galactosidase production results (38–12,958 U/mg biomass), reflecting the importance of medium optimization to attain a high

Table 3. Statistical analysis of Plackett-Burman design showing coefficient values, plus t and *P* values, for each variable.

Variable	Amylase (U/ml/min)				β-Galactosidase (Miller unit)			
	Coefficient	*t*-Statistic	*P* value	Confidence level (%)	Coefficient	*t*-Statistic	*P* value	Confidence level (%)
Intercept	15.42				1171.81			
X_1	1.056	1.92	0.1502	84.98	1074.82	1.75	0.1777	82.3
X_2	2.0480	3.62	0.0362	96.38	−627.98	−1.00	0.3928	60.72
X_3	0.598	1.02	0.3819	61.81	304.47	0.47	0.6725	32.75
X_4	1.487	2.44	0.0929	90.71	−533.07	−0.78	0.4910	50.90
X_5	−0.274	−0.45	0.6837	31.63	155.83	0.23	0.8335	16.65
X_6	0.261	0.45	0.6823	31.77	−1543.65	−2.40	0.0960	90.40
X_7	1.098	1.97	0.1435	85.65	−102.75	−0.17	0.8792	12.08
X_8	0.141	0.26	0.8125	87.50	−297.07	−0.49	0.6593	34.07
X_9	−1.132	−2.14	0.1217	87.83	−611.17	−1.04	0.3762	62.38
X_{10}	−1.653	−3.09	0.0538	94.62	227.92	0.38	0.7280	27.20
X_{11}	−1.472	−2.75	0.0707	92.93	363.39	0.61	0.5856	41.44
X_{12}	−1.506	−2.85	0.0652	93.48	−483.92	−0.82	0.4720	52.80
X_{13}	0.614	1.12	0.3431	65.69	−468.68	−0.77	0.4978	50.22
X_{14}	0.936	1.68	0.1916	80.84	420.517	0.68	0.5471	45.29
X_{15}	0.239	0.41	0.7066	29.34	−443.98	−0.69	0.5398	46.02
X_{16}	2.247	3.69	0.0346	96.54	−1055.42	−1.55	0.2184	78.16
X_{17}	1.421	2.33	0.1025	89.75	527.39	0.77	0.4952	50.48
X_{18}	5.735	9.8	0.0023	99.77	−65.17	−0.10	0.9267	7.33
X_{19}	2.581	4.56	0.0197	98.03	386.64	0.61	0.5832	41.68
X_{20}	0.924	1.68	0.1912	80.88	−1134.80	−1.85	0.1611	83.89

yield of the target products. The main effects of the examined factors on amylase and β-galactosidase production were calculated and are presented graphically in Fig. 3. From an analysis of the regression coefficients and t-values for the 20 variables (Table 3), $CaCl_2$, the incubation time, $MgSO_4 \cdot 7H_2O$, and tryptone were the most significant factors increasing amylase production, whereas citrate, $(NH_4)_2SO_4$, glutamate, and maltose were the most significant factors decreasing amylase production. For the β-galactosidase yield, lactose was the most substantial variable enhancing β-galactosidase production, whereas starch, the incubation temperature, and $MgSO_4 \cdot 7H_2O$ were the most significant factors decreasing β-galactosidase production.

The t-test for an individual effect allows an evaluation of the probability of finding the observed effect purely by chance, and several researchers have found confidence levels greater than 70% to be acceptable [49]. Thus, in the current experiments, variables with confidence levels greater than 95% were considered significant for amylase, whereas those with confidence levels greater than 80% were considered significant for β-galactosidase. Moreover, the quality of fit of the polynomial model equation was expressed by the coefficient of the determination of R^2, which was 0.99 and 0.89 for the full model of amylase and β-galactosidase, respectively.

Therefore, when neglecting the insignificant terms (p> 0.05) and (p>0.177) for amylase and β-galactosidase, respectively, the model equations for the enzyme yields could be written as

$$Y_{amylase}=15.418+2.048X_2+2.247X_{16}+5.735X_{18}+2.581X_{19} \quad (2)$$

$$Y_{\beta\text{-galactosidase}}=1171.808+1074.819X_1 \quad (3)$$

where X_1, X_2, X_{16}, X_{18}, and X_{19} are lactose, tryptone, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, and the incubation time, respectively. One of the advantages of the Plackett-Burman design is the ability to rank the effect of different variables on the measured response, independent of their nature (either nutritional or physical factor) or sign (whether it contributes positively or negatively). Fig. 4 shows the ranking of the factor estimates in a Pareto chart, which displays the magnitude of each factor estimate and is a convenient way to view the results of the Plackett-Burman design [50].

Thus, from the confidence level of the variables, it was apparent that $CaCl_2$, the incubation time, $MgSO_4 \cdot 7H_2O$, and tryptone were the most positive significant variables affecting amylase production, whereas lactose was the most positive significant variable affecting lactase production.

The divalent cation Ca^{2+} is known to play a vital role in enzyme activation, stabilization, and protection. Yet, whereas the amylase excreted from the investigated bacterium (*G. stearothermophilus* SAB-40) was calcium dependent, the intracellular β-galactosidase was calcium independent.

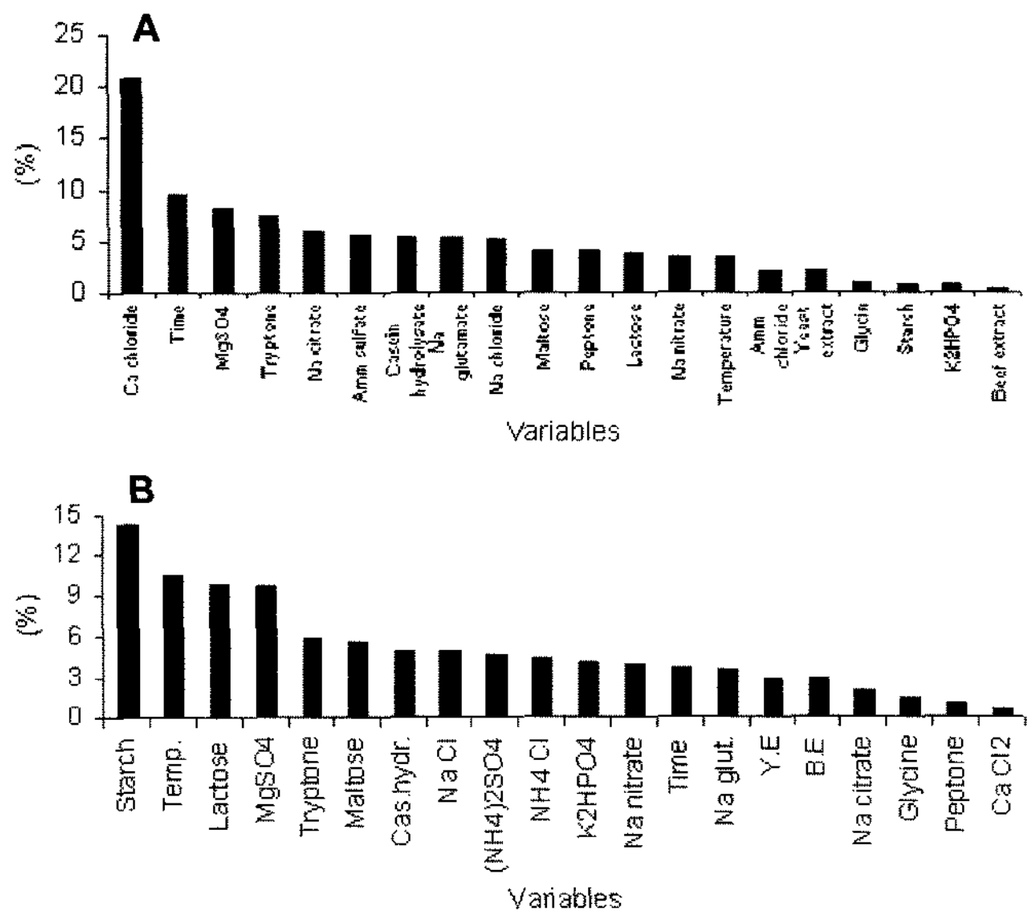

Fig. 4. Pareto plot for Plackett-Burman parameter estimates of starch-hyrolyzing (amylase) (**A**) and lactose-fermenting (β-galactosidase) (**B**) enzymes produced by *G. stearothermophilus* SAB-40.

Thus, although Ca^{2+} was significantly important for the extracellular amylase, it was insignificant for the intracellular β-galactosidase, possibly because Ca^{2+} stabilizes and protects enzymes from direct harsh environmental excretion conditions, whereas the β-galactosidase was only produced inside the cell under less harsh conditions. The present finding is in accordance with previous reports that ascribed the dependency of α-amylase production by *Bacillus subtilis* and *Bacillus stearothermophilus* on the presence of calcium chloride in the growth medium [25, 26, 47]. In contrast, other reports have found that the α-galactosidase of *Bacillus* sp. JF and α-amylase of *Bacillus thermoleovorans* NP54 were calcium chloride independent [28, 32].

The divalent cation Mg^{2+} has also been found to positively affect the formation of amylase, as described in [47], while inhibiting β-galactosidase production. However, based on the present data, among the organic and inorganic nitrogen sources tested on the coproduction of thermostable amylase and β-galactosidase, tryptone was found to favor the production of amylase, whereas $(NH_4)_2SO_4$ reduced its production. Thus, an organic nitrogen source rich in minerals, vitamins, amino acids, and cofactors would be expected to enhance the biomass production significantly and thereby the enzyme production, as previously reported [12, 25, 42].

Among the carbon sources tested, citrate, glutamate, and maltose all reduced the production of amylase. Several authors have also reported that the utilization of simple carbon sources causes catabolite repression with a direct negative effect on enzyme production [3, 47]. Furthermore, soluble starch was found to decrease the β-galactosidase

production and did not increase the amylase production. Moreover, the only carbon source that contributed positively to the β-galactosidase production and increased the enzyme yield was lactose. Therefore, these results verified the constitutive and induced nature for amylase and β-galactosidase, respectively, as described previously in the preliminary experiment. Nonetheless, other researchers have suggested that soluble starch is the most suitable substrate for the production of both enzymes [26].

The incubation temperature had an inverse effect on the amylase and β-galactosidase production, as it had no significant effect on the amylase (although positive), yet a highly significant negative effect on the β-galactosidase production. It is also worth mentioning that the incubation temperature and starch constrained the β-galactosidase production, due to a direct relation and interactive effect between the availability of starch in the medium and the temperature, as the starch distribution increased when increasing the incubation temperature. Furthermore, the inverse effect of the incubation temperature on the amylase and β-galactosidase production was ascribed to the absence of any significant effect of divalent cations on β-galactosidase production, since these cations, especially Ca^{2+}, play a protective role as regards inactivation when enzymes are exposed to various temperatures [47].

In the present study, the incubation time had a highly significant effect on the amylase production by *Geobacillus* sp. SAB-40, where higher enzyme levels were measured after 24 h. The mesophilic *B. licheniformis* and *B. amyloliquefaciens*, which are the main sources of commercial amylase at present, have been reported to require a batch time of 48–72 h for optimum production [24, 44, 52]. Amylase production by thermophiles is growth associated [33], which is of special interest owing to the fast growth of thermophiles at a high temperature, allowing the enzymes to be harvested earlier.

Nonetheless, after evaluating the culture conditions affecting the coproduction of the investigated enzymes, each enzyme was found to have its own variables affecting production, and the two enzymes were not concomitant, meaning it is impossible to produce both enzymes in one form and each enzyme should be studied individually for yield maximization.

The present study investigated the significant factors affecting the coproduction of thermostable amylase and β-galactosidase in a batch by the thermophilic strain *G. stearothermophilus* SAB-40. The statistically designed experiment based on a Plackett-Burman matrix allowed factors from different categories to be ranked to enable a better understanding of the effect of the medium. The Plackett-Burman design provides an effective and fast screening procedure and can mathematically compute the significance of a large number of factors in one experiment, thereby saving time and maintaining convincing information on each component. However, the priority of the screening program in the present study was not to examine the interaction between large numbers of variables, as only the most effective factors with positive significance needed to be selected for further optimization. Therefore, at this point, other suitable optimization statistical designs need to be applied to further optimize the significant variables determined in the present study to attain the maximum yield of each enzyme.

Acknowledgment

The author would like to thank Dr. Yasser R. Abdel-Fattah, Bioprocess Development Department, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and Technology Applications, for his valuable suggestions and useful discussions.

REFERENCES

1. Batra, N. and J. Singh. 2002. Production and characterization of a thermostable β-galactosidase from *Bacillus coagulans* RCS3. *Biotechnol. Appl. Biochem.* **36:** 1–6.
2. Ben Ali, M., S. Mhiri, M. Mezghani, and S. Bejar. 2001. Purification and sequence analysis of a typical maltohexaose-forming α-amylase of *B. stearothermophilus* US100. *Enzyme Microb. Technol.* **28:** 537–542.
3. Bereaka, M. M., N. A. Soliman, and Y. R. Abdel-Fattah. Production, partial characterization and cloning of thermostable α-amylase of a thermophile *Geobacillus thermoleovorans* YN. *Biotechnology* **42:** 1090–1100.
4. Bessler, C., J. Schmitt, K.-H. Maurer, and R. Schmid. 2003. Directed evolution of a bacterial α-amylase: Toward enhanced pH-performance and higher specific activity. *Protein Sci.* **12:** 2141–2149.
5. Brady, D. and R. E. Marchant. 1995. Isolation and partial characterization of β-galactosidase activity produced by a thermostable strain of *Kluyveromyces maximas* during growth on lactose containing media. *Enzyme Microb. Technol.* **17:** 696–699.
6. Chang, Y.-N., J.-C. Huang, C.-C. Lee, I.-L. Shih, and Y.-M. Tzeng. 2002. Use of response surface methodology to optimize culture medium for production of lovastatin by *Monascus rubber*. *Enzyme Microb. Technol.* **30:** 889–894.
7. Chang, B. S. and R. Mahoney. 1994. Thermal denaturation of β-galactosidase from *Streptococcus thermophilus* and its stabilization by bovine serum albumin. An electrolytic study. *Biotechnol. Biochem.* **19:** 169–178.
8. Chen, Q. H., G. Q. He, and M. A. M. Ali. 2002. Optimization of medium composition for the production of elastase by *Bacillus* sp. EL31410 with response surface methodology. *Enzyme Microb. Technol.* **30:** 667–672.
9. Dong, G., C. Vielle, A. Savchenko, and J. G. Zeikus. 1997. Cloning, sequencing, and expression of the gene encoding extracellular α-amylase from *Pyrococcus furiosus* and biochemical

characterization of the recombinant enzyme. *Appl. Environ. Microbiol.* **63:** 3569–3576.
10. Dong, G., C. Vielle, and J. G Zeikus. 1997. Cloning, sequencing, and expression of the gene encoding amylopullulanase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. *Appl. Environ. Microbiol.* **63:** 3577–3584.
11. Dumortier, V., C. Brassart, and S. Bouquelet. 1994. Purification and properties of a β-D-galactosidase from *Bifidobacterium bifidum* exhibiting transgalactosylation reaction. *Biotechnol. Appl. Biochem.* **19:** 341–354.
12. El-Helow, E. R., Y. R. Abdel-Fattah, K. M. Ghanem, and E. A. Mohamad. 2000. Application of the response surface methodology for optimizing the activity of an *aprE*-driven gene expression system in *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* **54:** 515–520.
13. Fischer, L. and C. Scheckermann. 1995. Purification and characterization of a thermotolerant β-galactosidase from *Thermomyces lanuginosus*. *Appl. Environ. Microbiol.* **61:** 1497–1501.
14. Fogarty, W. M. and C. T. Kelly. 1990. *In* W. M. Fogarty and C. T. Kelly (eds.), *Microbial Enzyme and Biotechnology*, pp. 7–132, 2th Ed. Elsevier Science, London.
15. Francis, F., A. Sabu, K. Madhavan Nampoothiri, S. Ramachandran, S. Ghosh, G. Szakacs, and A. Pandey. 2003. Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*. *Biochem. Eng. J.* **15:** 107–115.
16. Fukusumi, S. A., A. Kamizono, S. Horinouchi, and T. Beppu. 1988. Cloning and nucleotide sequence of a heat stable amylase gene from an anaerobic thermophile, *Dictyoglomus thermophilum*. *Eur. J. Biochem.* **174:** 25–21.
17. Furlan, S. and A. Schneider. 2000. Formulation of a lactose-free low cost medium for the production of β-galactosidase by *Kluyveromyces marxiamus*. *Biotechnol. Lett.* **22:** 589–593.
18. Fuwa, H. 1954. A new method for microdetermination of amylase activity by the use of amylose as the substrate. *J. Biochem. (Tokyo)* **41:** 583–603.
19. Ghanem, N. B., H. H. Yusef, and H. K. Mahrouse. 2000. Production of *Aspergillus terreus* xylanase in solid-state cultures: Application of the Plackett-Burman experimental design to evaluate nutritional requirements. *Bioresour. Technol.* **73:** 113–121.
20. Gowland, P., M. Kernick, and T. K. Sundaram. 1987. Thermophilic bacterial isolates producing lipase. *FEMS Microbiol. Lett.* **48:** 339–343.
21. Grueniger, B. and B. Sonnleitner. 1984. Bacterial diversity in thermophilic aerobic sewage sludge. *Appl. Microbiol. Biotechnol.* **19:** 414–421.
22. Huber, R. E., G. Kurz, and K. Wallenfels. 1976. A quantitation of the factors which affect the hydrolase and transgalactosylase activities of β-galactosidase (*E. coli*) on lactose. *Biochemistry* **15:** 1994–2001.
23. Jin, F., Y. Li, C. Zhang, and H. Yu. 2001. Thermostable α-amylase and α-galactosidase production from the thermophilic and aerobic *Bacillus* sp. JF strain. *Process Biochem.* **36:** 559–564.
24. Kochhar, S. and R. D. Dua. 1990. Thermostable liquefying α-amylase from *Bacillus amyloliquefaciens*. *Biotechnol. Lett.* **12:** 393–396.
25. Konsoula, Z. and M. Liakopoulou-Kyriakides. 2004. Hydrolysis of starches by the action of α-amylase from *Bacillus subtilis*. *Process Biochem.* **39:** 1745–1749.
26. Konsoula, Z. and M. Liakopoulou-Kyriakides. 2007. Co-production of α-amylase and β-galactosidase by *Bacillus subtilis* in complex organic substrates. *Bioresour. Technol.* **98:** 150–157.
27. Liebl, W., B. Wagner, and J. Schellhase. 1998. Properties of a α-galactosidase, and structure of its gene *galA*, within α- and β-galactoside utilization gene cluster of the hyperthermophilic bacterium *Thermotoga maritina*. *Syst. Appl. Microbiol.* **21:** 1–11.
28. Li, X., L. Yang, F. Zuo, and F. Jin. 1997. Factors regulating production of α-galactosidase from *Bacillus* sp. JF. *Lett. Appl. Microbiol.* **24:** 1–5.
29. Lim, J. K., H. S. Lee, Y. J. Kim, S. S. Bae, J. H. Jeon, S. G. Kang, and J.-H. Lee. 2007. Critical factors to high thermostability of an α-amylase from hyperthermophilic archaeon *Thermococcus onnurineus* NA1. *J. Microbiol. Biotechnol.* **17:** 1242–1248.
30. Lind, D. L. and D. A. Daniel. 1989. β-Galactosidase from a strain of the anaerobic *Thermoanaerobacter*. *Enzyme Microb. Technol.* **11:** 180–186.
31. Lonsane, B. K. and M. V. Ramech. 1990. *In* S. A. Neidleman (ed.). *Advances in Applied Microbiology*, Vol. 35, pp. 1–56. Academic Press, New York.
32. Malhotra, R., S. M. Noorwez, and T. Satyanarayana. 2000. Production and partial characterization of thermostable and calcium independent α-amylase of an extreme thermophile *Bacillus thermoleovorans* NP54. *Lett. Appl. Microbiol.* **31:** 378–384.
33. Mamo, G., B. A. Gashe, and A. Gessesse. 1999. A highly thermostable amylase from a newly isolated thermophilic *Bacillus* sp. WN11. *J. Appl. Microbiol.* **86:** 557–560.
34. Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. Nakao, M., M. Harada, Y. Kodama, T. Nakayama, Y. Shibano, and T. Amachi. 1994. Purification and characterization of a thermostable β-galactosidase with high transgalactosylation activity from *Saccharopolyspora rectivirgula*. *Appl. Microbiol. Biotechnol.* **40:** 657–663.
36. Onishi, N., A. Yamashiro, and K. Yokozeki. 1995. Production of galactooligosaccharide from lactose by *Sterigmatomyces eiviae* CBS8119. *Appl. Environ. Microbiol.* **61:** 4022–4025.
37. Page, R. D. M. 1986. TREEVIEW: An application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12:** 357–358.
38. Pandey, A. 1992. Recent developments in process solid-state fermentation. *Process Biochem.* **27:** 109–117.
39. Pandey, A. and P. Nigam. 2000. Advances in microbial amylases. *Biotechnol. Appl. Biochem.* **31:** 135–152.
40. Pandey, A., P. Nigam, C. R. Soccol, C. T. Soccol, D. Singh, and R. Mohan. 2000. Advances in microbial amylases. *Biotechnol. Appl. Biochem.* **31:** 135–152.
41. Patel, G. B. and C. R. Mackenzie. 1985. Properties and potential advantages of β-galactosidase from *Bacteriodes polyprogmatus*. *Appl. Microbiol. Biotechnol.* **22:** 114–120.
42. Pedersen, H. and J. Nielsen. 2000. The influence of nitrogen sources on α-amylase productivity of *Aspergillus oryzae* in continuous cultures. *Appl. Microbiol. Biotechnol.* **53:** 278–281.

43. Plackett, R. L. and J. P. Burman. 1946. The design of optimum multifactorial experiments. *Biometrika* **33:** 305–325.
44. Roychudhury, S., S. J. Parulekar, and W. A. Weigand. 1988. Cell growth and α-amylase production characteristics of *Bacillus amyloliquefaciens*. *Biotechnol. Bioeng.* **33:** 197–206.
45. Shaw, A., R. Bott, and A. G. Day. 1999. Protein engineering of α-amylase for low pH performance. *Curr. Opin. Biotechnol.* **10:** 349–352.
46. Shaw, J.-F., F.-P. Lin, S.-C. Chen, and H.-C. Chen. 1995. Purification and properties of an extracellular α-amylase from *Thermus* sp. *Bot. Bull. Acad. Sin.* **36:** 195–200.
47. Srivastava, R. A. K. and J. N. Baruah. 1986. Culture conditions for production of thermostable amylase by *Bacillus stearothermophilus*. *Appl. Environ. Microbiol.* **52:** 179–184.
48. Steppan, D., J. Werner, and B. Yeater. 1999. Essential regression and experimental design in MS Excel-free, user friendly software package for doing multiple linear regression, step-wise regression, polynomial regression, model adequacy checking and experimental design in MS Excel. Available at: http://www.geocities.com/Silicon Valley/Network/1032/.
49. Stow, R. A. and R. P. Mayer. 1966. Efficient screening of process variables. *Ind. Eng. Chem.* **58:** 36–40.
50. Strobel, R. J. and G. R. Sullivan. 1999. Experimental design for improvement of fermentations, pp. 80–93. *In* A. L. Demain and J. E. Davies (eds.), *Manual of Industrial Microbiology and Biotechnology*. ASM Press, Washington.
51. Suzuki, H., Y. Ozawa, H. Oota, and H. Yoshida. 1969. Studied on the decomposition of raffinose by α-galactosidase of mold, Part I. α-Galactosidase formation and hydrolysis of raffinose by the enzyme preparation. *Agric. Biol. Chem.* **33:** 506–513.
52. Tonkova, A., R. Manolov, and E. Dobreva. 1993. Thermostable α-amylase from derepressed *Bacillus licheniformis* produced in high yields from glucose. *Process Biochem.* **28:** 539–542.
53. Tsurikova, N. V., L. I. Nefedova, E. V. Kostyleva, V. I. Zvenigorodskii, V. G. Yasinovskii, T. A. Voeikova, and A. P. Sinitsyn. 2002. Selection of a potent *Bacillus licheniformis* strain producing thermostable amylase. *Appl. Biochem. Microbiol.* **38:** 427–432.
54. Vasiljevic, T. and P. Jelen. 2001. Production of β-galactosidase for lactose hydrolysis in milk and dairy products using thermophilic lactic acid bacteria. *Innovative Food Sci. Emerg. Technol.* **2:** 75–85.
55. Yoon, J. I. T. and K. Ajisaka. 1996. The synthesis of galactopyranosyl derivatives with β-galactosidases of different origins. *Carbohydr. Res.* **292:** 153–163.