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Amylase Production by *Saccharomycopsis fibuligera* A11 in Solid-State Fermentation for Hydrolysis of Cassava Starch

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Abstract The optimization of process parameters for high amylase production by *Saccharomycopsis fibuligera* A11 in solid-state fermentation was carried out using central composite design. Finally, the optimal parameters obtained with the response surface methodology (RSM) were moisture 610.0 ml/kg, inoculum 30.0 ml ($OD_{600\ nm}$=20.0)/kg, the amount ratio of wheat bran to rice husk 0.42, cassava starch concentration 20.0 g/kg, temperature 28 °C, and natural pH. Under the optimized conditions, 4,296 U/g of dry substrate of amylase activity was reached in the solid-state fermentation culture of the yeast strain A11 within 160 h, whereas the predicted maximum amylase activity of 4,222 U/g of dry substrate of amylase activity was derived from the RSM regression. It was found that cassava starch can be actively converted into monosaccharides and oligosaccharides by the crude amylase.

Keywords Amylase · *Saccharomycopsis fibuligera* · Cassava starch · Solid-state fermentation · Response surface methodology · Hydrolysis

Introduction

Cassava (*Manihot esculenta*) is a root crop of tropical American origin and is the fourth most important staple crop in the tropics. It is also cultivated in many provinces in the south of China, and cassava starch is produced on large scale in different regions of China. The cassava plant is extremely robust, is resistant to disease and drought, and can grow in relatively low-quality soils. Therefore, cassava starch is also a good starchy material in food and fermentation industry in China. Cassava starch is composed of unbranched amylose (20±5%) and branched amylopectin (80±5%), both of which can be hydrolyzed

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enzymatically (either with pure enzymes or amylase-producing microorganisms) to release their constituent glucose and maltooligosaccharides [1]. Because of high starch debranching activity, amylases produced by *Saccharomycopsis fibuligera* have been extensively used to hydrolyze cassava starch [2, 3]. α-Amylase and glucoamylase have been purified and characterized from *S. fibuligera* [4]. It was also found that glucoamylase produced by *S. fibuligera* can digest raw starch [5]. The genes encoding α-amylase, glucoamylase, and raw starch digesting glucoamylase have been cloned from *S. fibuligera* and expressed in *Saccharomyces cerevisiae* [5–9]. The α-amylase and glucoamylase can efficiently hydrolyze starch to yield glucose syrup for ethanol production by *S. cerevisiae* [9]. It was found that the trehalose poor-assimilating mutant *S. fibuligera* A11 obtained from *S. fibuligera* sdu by chemical mutagenesis also can efficiently convert corn starch into trehalose in its cells [10, 11]. So far, amylase production by *S. fibuligera* has been only carried out by submerged fermentation [3]. It has been well documented that solid-state fermentation (SSF) offers numerous advantages for the production of bulk chemicals and enzymes due to low cost substrates, simplified downstream and environment-friendly process, reduced energy requirement, reduced waste water produced, high yield of fermentation products, high volumetric productivity, increased product recovery, and simplicity of bioreactor design compared to the submerged fermentation (SmF) [12, 13]. It can be of special interest in those processes where the crude fermented products may be used directly as the enzyme sources for biocatalysis and biotransformation. However, to date amylase production by *S. fibuligera* has not been performed by solid-state fermentation yet.

Therefore, the present study aimed to optimize process parameters for high amylase production by *S. fibuligera* A11 in solid-state fermentation using response surface methodology and hydrolysis of cassava starch with the crude amylases produced by *S. fibuligera* A11.

Materials and Methods

Yeast Strain

S. fibuligera A11 is the trehalose poor-assimilating mutant isolated from *S. fibuligera* sdu [10, 11]. This yeast strain was maintained in yeast peptone dextrose (YPD) medium containing 20.0 g/l glucose, 10.0 g/l yeast extract, and 20.0 g/l polypeptone at 4 °C.

Cassava Starch

The cassava starch used in this study was purchased at the local cassava starch company in Qingdao, China.

Screening of Chemical Parameters Using Central Composite Designs

The central composite design (CCD) for four variables which includes chemical parameters at five levels (+2, +1, 0, −1, −2) (Tables 1 and 2) was used for screening. Among the chemical parameters, moisture, the amount ratio of wheat bran and rice husk, the concentration of cassava starch, and inoculation amount were tested for their significance in the amylase production by the yeast strain A11 used in this study.

Table 1 Range of the factors investigated in the experimental design for the amylase production by the yeast strain A11.

Variables	Code	Levels				
		−2	−1	0	+1	+2
Moisture (%)	A	55.0	58	61.5	65	68.5
The amount ratio of wheat bran to rice husk	B	0.24	0.33	0.42	0.51	0.6
The amount of cassava starch (%, *w*/*w*)	C	0	1.0	2.0	3.0	4.0
Inoculation amount (%, *v*/*w*)	D	1.0	2.0	3.0	4.0	5.0

Inoculation amount was the milliliters of the culture ($OD_{600\ nm}$=20.0) per 100 g of the solid medium

Solid-State Fermentation

Wheat bran and rice husk were used as the solid substrate; 1.5 g of wheat bran was mixed with 3.5 g of rice husk. The substrates were supplemented with 8.0 ml of distilled water and mixed well. The concentration of cassava starch and moisture of the substrates were set at different levels according to Tables 1 and 2, respectively. All substrates were autoclaved at 15 psi for 20 min. Preliminary studies showed that no changes in moisture content of the substrate after autoclaving were detected. One loop of the cells of the yeast strain was transferred to 50.0 ml of YPD medium in 250 ml flask and aerobically cultivated for 24 h. The cell culture ($OD_{600\ nm}$=20.0) was transferred to the solid-state media prepared above. Each flask was inoculated with different volume of the cell suspension ($OD_{600\ nm}$=20.0) and incubated at 28 °C. A 4^5 factorial design was performed to assess the effect of moisture, the amount ratio of wheat bran to rice husk, the concentration of cassava starch, and inoculation amount on the amylase production by the yeast strain A11. A central point was carried out in triplicate plus two axial points for each independent factor for experimental error evaluation and second-order effects estimation, respectively. Table 1 shows the range of the studied factors and the correspondent coded levels.

Extraction of Enzyme

A weighed quantity of the fermented matter was treated with acetate buffer (0.1 M, pH5.0) and mixed thoroughly on a magnetic stirrer for 30 min at room temperature. The whole contents were centrifuged at 9,992×*g* and 4 °C for 10 min, and the supernatant obtained was taken as the crude amylase.

Determination of Amylase Activity

The reaction mixture containing 700 μl of the crude amylase, 100 μl of acetate buffer (0.1 M, pH5.0), and 200 μl of soluble starch (20.0 g/l) was incubated at 55 °C for 40 min. The reaction was inactivated immediately by keeping the reaction mixture at 100 °C for 10 min. The same mixture to which the same amount of the inactivated crude amylase (heated at 100 °C for 10 min) was added before the reaction was used as the control [14]. The mixtures were cooled to the room temperature and were centrifuged at 2,823×*g* and 4 °C for 5 min. After suitable dilution of the supernatant obtained, the amount of reducing sugar in the dilute was assayed by using the methods described by Spiro [15]. One amylase

Table 2 Experiment designs used in RSM by using four independent variables each at five levels showing observed values of amylase production by yeast strain A11.

Runs	*A* %	*B* %	*C* %	*D* %	Amylase activity (U/gds)
1	−1	−1	1	−1	1,502±4.5
2	0	0	0	0	4,221.9±2.1
3	1	1	1	−1	136.2±3.0
4	1	−1	1	−1	276.6±4.4
5	−2	0	0	0	1,638.3±5.6
6	0	−2	0	0	621.38±2.5
7	1	1	1	1	1,003.6±1.7
8	1	−1	−1	−1	1,700±3.6
9	0	0	0	0	4,221.9±3.7
10	−1	1	−1	−1	212±0.4
11	0	0	0	0	4,221.9±2.8
12	1	1	−1	−1	123.1±0.9
13	0	0	0	−2	886.3±1.4
14	−1	−1	−1	1	2,200±2.4
15	1	−1	−1	1	496±1.8
16	0	0	0	0	4,221.9±4.8
17	1	−1	1	1	167±1.7
18	−1	1	−1	1	2,332±3.3
19	0	0	0	2	2,109±6.1
20	1	1	−1	1	1,600±2.8
21	−1	1	1	−1	353.3±0.7
22	−1	1	1	1	2,937±2.7
23	0	0	2	0	1,065.2±1.5
24	2	0	0	0	899±0.5
25	0	0	−2	0	2,185.5±3.7
26	−1	−1	1	1	1,099±0.5
27	0	0	0	0	4,221.9±4.2
28	−1	−1	−1	−1	3,899.1±3.5
29	0	2	0	0	688±1.5
30	0	0	0	0	4,221.9±4.8

Data are given as means±SD, $n=3$

A moisture, *B* the amount ratio of wheat bran to rice husk, *C* the amount of cassava starch, *D* inoculation amount, *gds* grams of initial dry substrate

unit (U) was defined as the amount of enzyme that produces 1.0 μmol of reducing sugar per minute under the assay conditions used in this study.

Statistical Analysis of the Data

The statistically planned experimentation is to identify the significant variables and their corresponding coefficients, so that the levels of variables can be managed to obtain a desired output. Hence, the coefficients, sum of squares in percentage (SS%), and coefficient of variation (CV) were analyzed using the experimental results of the amylase activity

produced by the yeast strain A11. Using the design expert (Static Made Easy, Minneapolis, MN, USA; version 7.0.0, 2005), the experimental plan, the analysis, and the results were obtained.

Cassava Starch Hydrolysis

Effect of cassava starch concentration on hydrolysis was studied by varying its concentrations from 1.0% to 40.0% in the reaction mixture containing 1.0 ml of 0.2 M acetate buffer (pH 5.0) and 20 μl of 35 U/ml of the crude amylase by shaking at 150 rpm and 55 °C. To determine the extent of starch hydrolysis, glucose estimation was done after 6 h of incubation as described above. The end products of cassava starch hydrolysis after 6 h of incubation at 55 °C were withdrawn and identified to ascertain the extent of hydrolysis by ascending thin layer chromatography (Silica gel 60, MERCK, Germany) with the solvent system of *n*-butanol–pyridine–water (6:4:3) and a detection reagent comprising 2.0% diphenylamine in acetone–2.0% aniline in acetone–85.0% phosphoric acid (5:5:1, *v*/*v*/*v*) [14].

Effect of the enzyme concentration was examined by varying its concentrations from 25 to 200 U/g of cassava starch in the reaction mixture containing 1.0 ml of 40.0% of cassava starch at 55 °C.

Determination of Total Sugar

The mixture containing 200 μl of the sample and 1.0 ml of 2.0 M HCl was heated at 100 °C for 30 min. After cooling, the mixture was neutralized by adding 1.0 ml of 2.0 M NaOH. The total reducing sugar in the hydrolysate was determined by using the glucose-oxidase kit mentioned above.

Results

Optimization of the Screened Variables

Fermentation process optimization by single dimensional search is usually laborious and time consuming, especially for a large number of variables, and it does not ensure the desirable conditions. In fact, it is practically impossible for the one-dimensional search to achieve an appropriate optimum in a finite number of experiments. Single-variable optimization methods are not only tedious but also can lead to misinterpretation of results especially because the interaction between different factors is overlooked [16]. Recently, response surface methodology (RSM) has been used to optimize solid-state fermentation process by a few researchers [13, 17]. RSM is a model consisting of mathematical and statistical techniques, widely used to study the effect of several variables and to seek the optimum conditions for a multivariable system. In RSM, the number of experimental runs required is very few, leading to saving of time, chemicals, glassware, and manpower [18]. Experimental design and data analysis using appropriate software make the analysis easier as observed in the present study. After fermentation process optimization by single dimensional search (data not shown), the initial moisture content of substrate, inoculum amount, the amount ratio of wheat bran to rice husk, and the amount of cassava starch were identified as most influential among chemical parameters for the production of the amylase by the yeast strain A11 on single-variable optimization methods. A CCD, one of the RSMs, was employed to analyze the interactive effect of these parameters and to arrive at an

optimum. The base points for the design were selected from a single-parameter study (data not shown). A summary of the variables and their variation levels is given in Table 1. SSF was carried out according to the design (Table 2) for 160 h. The crude amylase in the fermented samples was extracted and assayed for amylase activity as mentioned in "Materials and methods." The results were analyzed on a PC running under Windows OS, using Design expert 7.0.0 statistical software, and the response surface was generated using STATISTICA (StatSoft Inc., Tulsa, USA). The design and results (amylase activity) of the experiments carried out with the CCD are given in Table 2. The analysis of variance (ANOVA) was employed (Table 3) for the determination of significant parameters. ANOVA consists of classifying and cross classifying statistical results and testing whether the means of a specified classification differ significantly. This was carried by Fisher's statistical test for square due to regression to the mean square due to error and indicates the influence (significance) of each controlled factor on the tested model. The results obtained are submitted to ANOVA on SAS package, and the regression model is given as Eq. 1:

$$\begin{aligned} R1 = {} & +4,221.9 - 437.9 \times A - 104.6 \times B - 305.3 \times C + 253.2 \times D + 193.1 \times A \\ & \times B + 26.0 \times A \times C - 98.2 \times A \times D + 338.4 \times B \times C + 654.0 \times B \times D \\ & + 140.3 \times C \times D - 739.9 \times A^2 - 893.4 \times B^2 - 650.7 \times C^2 - 682.6 \times D^2 \end{aligned} \quad (1)$$

where the R1 was amylase yield, A was moisture, B was the amount ratio of wheat bran to rice bran, C was the amount of added cassava starch, and D was the inoculum.

Table 3 Analysis of variance for regression.

Source	Sum of squares	*df*	Mean square	*F* value	*p* value Prob>*F*
Model	61,747,315	14	4,410,523	24.1	<0.0001
A	4,603,031	1	4,603,031	25.1	0.0002
B	262,327.4	1	262,327.4	1.41	0.2500
C	2,237,470	1	2,237,470	12.2	0.0033
D	1,539,068	1	1,539,068	8.4	0.0110
AB	596,368.1	1	596,368.1	3.3	0.0913
AC	10,815.74	1	10,815.74	0.1	0.8113
AD	154,226.1	1	154,226.1	0.8	0.3734
BC	1,831,648	1	1,831,648	10.0	0.0064
BD	6,842,926	1	6,842,926	37.4	<0.0001
CD	314,887.9	1	314,887.9	1.7	0.2095
A^2	15,014,493	1	15,014,493	82.0	<0.0001
B^2	21,890,591	1	21,890,591	119.5	<0.0001
C^2	11,613,175	1	11,613,175	63.4	<0.0001
D^2	12,780,868	1	12,780,868	69.8	<0.0001
Residual	2,747,650	15	183,176.7		
Lack of Fit	2,747,650	10	274,765		
Pure Error	0	5	0		
Cor Total	64,494,965	29			

Table 4 Test of significance for regression square.

Std. Dev.	428.0
Mean	1,848.7
C.V. %	23.2
PRESS	15,826,467
R^2	0.96
Adj R^2	0.92
Pred R^2	0.75
Adeq precision	14.77

The ANOVA of the quadratic regression model demonstrates that Eq. 1 was a highly significant model, as was evident from the Fisher's F test with a very low probability value [F value=24.1] (Table 3). Values of "Prob>F" less than 0.0001(Table 3) indicate that model terms are significant. The model F value of 24.1 implies that the model is significant. There is only a 0.01% chance that a "model F value" this large could occur due to noise. The goodness of fit of the model was checked by determination coefficient (R^2) (Table 4). In this case, the value of the determination coefficient (R^2=0.96) (Table 4) indicates that only 4.0%

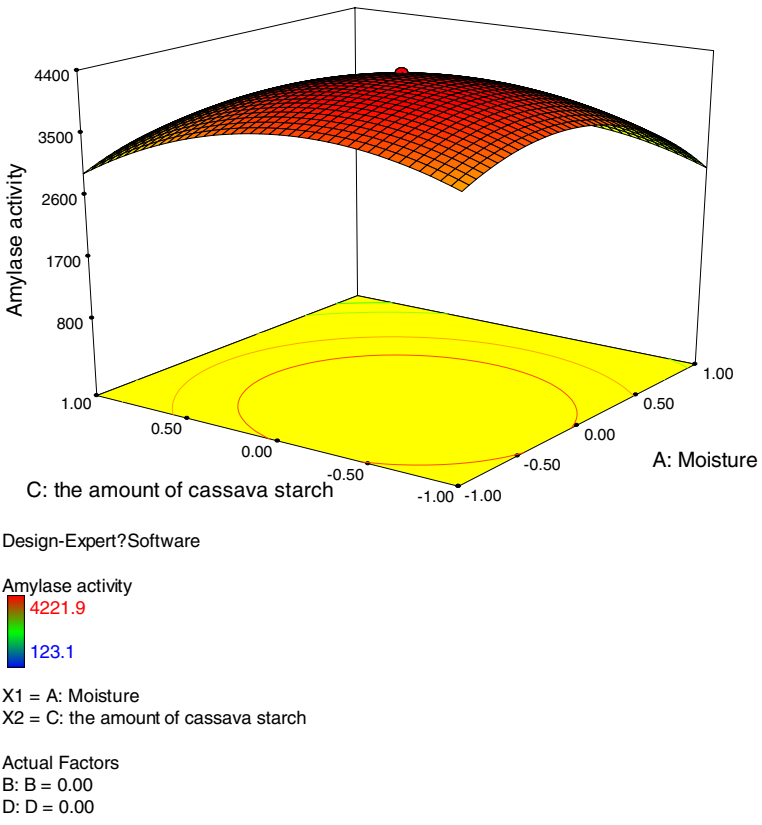

Fig. 1 Response surface of the amylase production by *S. fibuligera* A11 during the solid-state fermentation. **a** Moisture; **b** the amount ratio of wheat bran to rice husk; **c** the amount of cassava starch; **d** inoculation amount

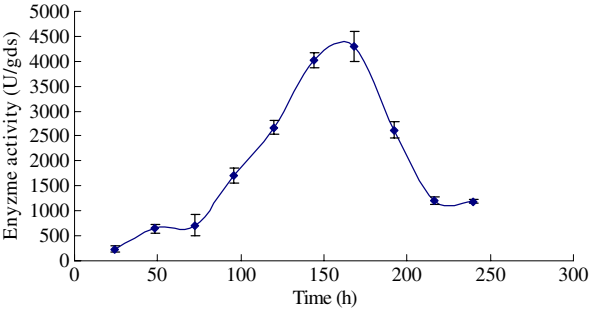

Fig. 2 Time course of the amylase production by *S. fibuligera* A11 during the solid-state fermentation. Data are given as means±SD, n=3

of the total variations was not explained by the model. The value of the adjusted determination coefficient (Adj R^2=0.92) was also very high to advocate for a high significance of the model (Table 4). The "predicted R^2" value of 0.75 for the amylase production by the yeast strain A11 was a reasonable agreement with the "adjusted R^2" values of 0.92 (Table 4). At the same time, a relatively lower value of the coefficient of variation (CV=23.2%) indicates a better precision and reliability of the experiments carried out (Table 4). Among model terms, moisture (*A*), the amount of added cassava starch (*C*), and inoculation amount (*D*) were very significant with a probability of over 99% (Table 3), while the amount ratio of wheat bran to rice bran (*B*) had no obvious effects on the amylase production by the yeast strain A11. Table 3 also indicates that the interactions between *A* and *B*, *A* and *D*, *B* and *C*, *B* and *D*, *A* and *A*, *B* and *B*, *C* and *C*, and *D* and *D* had very significant influence on the amylase yield by the yeast strain A11. However, the interactions between *A* and *C*, *A* and *D*, and *C* and *D* had no clear influence on the amylase yield by the yeast strain A11 (Table 3). The fitted response for the above regression model was plotted in Fig. 1. Three-dimensional graphs were generated for the pair-wise combination of the four variables while keeping the other one at their optimum levels for the amylase production by the yeast strain A11. Graphs were given here to highlight the roles played by the various variables.

The predicted maximum amylase activity (4,222 U/g of initial dry substrate) derived from RSM regression was obtained when the initial moisture, inoculum, the amount ratio of wheat bran to rice bran, and the amount of added cassava starch were 610.0 ml/kg, 30.0 ml ($OD_{600\ nm}$=20.0)/kg, 0.42, and 30.0 g/kg, respectively (Fig. 1).

Table 5 Comparison of the amylase activity before and after the optimization.

Variables	Levels before the optimization	Levels after the optimization	Amylase activity (U/g of dry weight)		
			Before the optimization	After the optimization	
				Predicted	Experimental
Inoculation amount (*v*/*w* %)	2	3			
Moisture (*v*/*w* %)	61.5	61			
Amount of added cassava starch(*w*/*w* %)	3	2	2,860	4,222	4,296
The amount ratio of wheat bran to rice husk	0.25	0.42			

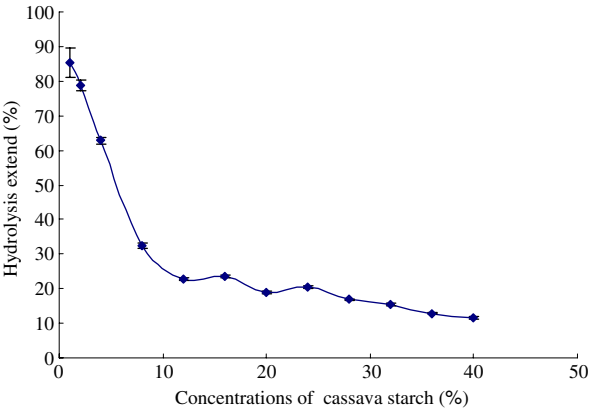

Fig. 3 Effects of different cassava starch concentrations on hydrolysis extent. Data are given as means±SD, n=3. Temperature, 28°C; buffer, 0.1 M acetate buffer (pH5.5); shaking speed, 180 rpm; enzyme concentration, 35 U/ml; reaction time, 6 h

Validation of the Experimental Model

The time course of the amylase production by the yeast strain A11 was examined during SSF under the optimal conditions obtained from RSM. The results in Fig. 2 show that the highest amylase activity (4,296 U/g of dry substrate) was reached within 160 h of the solid-state fermentation. The results also suggest that the actual amylase activity (4,296 U/g of dry substrate) in the optimized medium from three replications was close to the predicted value (4,222 U/g of dry weight), and the model was proven again to be adequate (Fig. 2 and Table 5).

Cassava Starch Hydrolysis

The potential application of the crude amylase produced by the yeast strain A11 in cassava starch hydrolysis was evaluated by studying the extent of hydrolysis of cassava starch at 55°C for 6 h. It can be seen from the results in Fig. 3 that hydrolysis extent of cassava starch by the crude amylase was decreased from 85.0% to 23.0% when the concentration of cassava starch was increased from 1.0% to 12.0% within 6 h. However, the hydrolysis extent was decreased from 23.0% to 12.0% when the concentration of cassava starch was increased from 12.0% to 40.0%. This means that the hydrolysis extent was almost constant when concentration of cassava starch was higher than 12.0%.

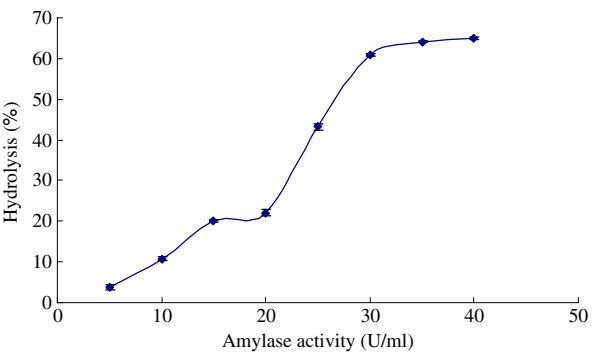

Fig. 4 Effects of different concentrations of the amylase on cassava starch hydrolysis extent. Data are given as means±SD, n=3. The cassava starch concentration was 20.0% (w/v); temperature, 28°C; buffer, 0.1 M acetate buffer (pH5.5); shaking speed, 180 rpm; reaction time, 6 h

Fig. 5 Thin-layer chromatogram (TLC) of the end products of starch after hydrolysis with the crude amylase from the yeast strain A11. *Lane 1* glucose, *lane 2* maltose, *lane 3* unhydrolyzed soluble starch, *lane 4* unhydrolyzed cassava starch, *lane 5* hydrolyzed soluble starch, *lane 6* hydrolyzed cassava starch, *lane 7* hydrolyzed cassava starch. The end products of starch hydrolysis was analyzed by using TLC plate (Silica gel 60, MERCK, Germany) with the solvent system *n*-butanol–pyridine–water (6:4:3) and a detection reagent comprising 2.0% diphenylamine in acetone–2.0% aniline in acetone–85.0% phosphoric acid (5:5:1 by volume)

It also can be clearly observed from the results in Fig. 4 that when the units of the amylase activity per gram of cassava starch was increased from 25 to 150 in the mixture containing 20.0% of cassava starch, the hydrolysis extent was increased from 4.0% to 61.0%. However, when the units of amylase activity per gram of cassava starch were increased from 150 to 200 in the mixture containing 20.0% of cassava starch, the hydrolysis extent was increased from 61% to 65%. These results suggest that the hydrolysis extent was almost constant when the units of amylase activity per gram of cassava starch were higher than 150 and the optimal units of the crude amylase per gram of cassava starch were in the range of 150 to 175.

The results in Fig. 5 show that a large amount of monosaccharides and oligosaccharides were detected in the hydrolysate of cassava starch by action of the crude amylase. The results in Fig. 6 indeed demonstrate that much more reducing sugar was released from cassava starch than from corn starch and potato starch by action of the crude amylase.

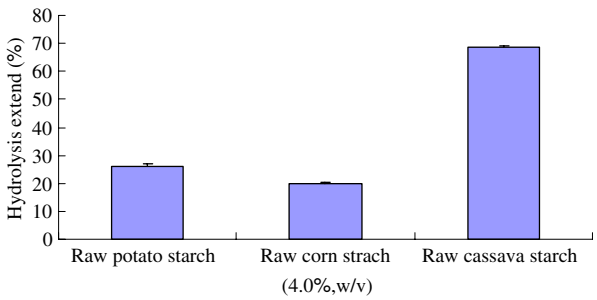

Fig. 6 Hydrolysis of different starch by the crude amylase

Discussion

It has been reported that cassava starch can be used as raw materials to produce ethanol, single cell protein, single cell oil, and other products [19]. However, cassava starch is composed of unbranched amylose (20±5%) and branched amylopectin (80±5%) [1]. Because of high starch debranching activity, amylases produced by *S. fibuligera* have been extensively used to hydrolyze cassava starch [1, 3]. Therefore, it is very important to produce the amylase preparation with high activity using *S. fibuligera*. After optimization of medium and conditions for amylase production by *S. fibuligera* A11, the optimal parameters of the solid-state fermentation were found to be moisture 610.0 ml/kg, inoculum 30.0 ml ($OD_{600\ nm}$=20.0)/kg, the amount ratio of wheat bran to rice husk 0.42, cassava starch concentration 20.0 g/kg, temperature 28 °C, and natural pH. Under the optimized conditions, 4,296 U/g of dry substrate of amylase activity was reached in the solid-state fermentation culture of the yeast strain A11 within 160 h (Fig. 2). The amylase preparation obtained was found to actively hydrolyze cassava starch (Fig. 6), suggesting that cassava starch can be more easily hydrolyzed by the amylase than corn starch and potato starch. This is due to the known fact that potato and corn starch contain less α-1,6 bonds than cassava starch. These results in Fig. 5 suggest that the amylase produced by the yeast strain A11 had cleavage activity on both α-1,4 and α-1,6 glycoside linkages in the starch molecules used in this study, and *S. fibuligera* A11 produces both glucoamylase and α-amylase during the solid-state cultivation. It has been reported that cleavage activity on both α-1,4 and α-1,6 glycoside linkages in starch molecules is a feature of several fungal amylases and has also been demonstrated for the amylases of the yeasts *Candida tsukubaensis*, *Aureobasidium pullulans*, *Schwanniomyces alluvius*, *Schwanniomyces castellii*, and *Filobasidium capsuligenum* [14, 20–24]. As mentioned above, cassava starch contains much higher amount of amylopectin than amylose [1]. Therefore, the crude amylase with high cleavage activity on both α-1,4 and α-1,6 glycoside linkages in starch molecules has highly potential applications in cassava starch hydrolysis.

As mentioned above, solid-state fermentation has many advantages over other fermentations and has widely been used for enzyme and other bioproduct production by bacteria, fungi, and yeasts [12]. The results obtained in this study demonstrate that SSF also can be used for amylase production by *S. fibuligera* A11.

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