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Optimisation of Composition of Media for the Production of Amylolytic Enzymes by *Thermomyces lanuginosus* ATCC 34626

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Summary

The composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* was optimised in different ways. Effects of various carbon and nitrogen sources were investigated. *Thermomyces lanuginosus* grown on starch, maltodextrin, dextrin, maltose, amylopectin, glucose and dextran substrates showed good α-amylase (92–125 U/mL) and glucoamylase (6–13 U/mL) activities. Among the tested nitrogen sources L-asparagine was the best one. The optimum pH of fermentation medium was found and fixed to 4.9 by using 100 mM citrate buffer for the production of amylolytic enzymes. Response Surface Method (RSM) was applied for searching the optimum concentration of components of media for amylolytic enzyme production. A second-order polynomial model was fitted at significance level 95 % ($P<0.05$) for both α-amylase and glucoamylase. The developed composition of media was checked in respect of the production of amylolytic enzymes. The concentration of L-asparagine was adjusted to 0.75 %. The concentration of starch was set at 6.5 % for α-amylase and 2 % for glucoamylase.

Key words: Thermomyces lanuginosus, glucoamylase, α-amylase, fermentation, Response Surface Method (RSM)

Introduction

Amylolytic enzyme preparations (α-amylase EC 3.2.1.1. and glucoamylase EC 3.2.1.3.) are now commercially produced by *Bacillus*, *Aspergillus* and *Rhizopus* species for processing starch. Barnett and Fergus *(1)* reported that extracellular amylolytic enzymes were produced by *Thermomyces lanuginosus* grown in a starch-based medium. Since then several authors worked on the screening of microorganisms for enzyme production *(2)*, isolation and production of extracellular amylases *(3,4)*, and purification and characterisation of the enzymes *(5,6)*.

The media optimisation is a relevant aspect to be considered in the development of fermentation technology. However, there are only a few reports concerning the comprehensive optimisation of medium composition. Gupta and Maheshwari *(7)* reported on the relationship between growth (dry mass) of *T. lanuginosus* ATCC 44008 and pH of the medium. Arnesen *et al. (8)* stimulated the secretion of α-amylase by adding Tween 80 to the growth medium.

The traditional one-factor at a time approach to optimisation is time-consuming and unsuitable for reaching the true optimum mainly because of interaction of the factors. Response Surface Method (RSM), as an experimental strategy for seeking the optimum conditions of a multivariable system, is a much more efficient methodology for optimisation. It has been successfully ap-

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plied for optimising the media composition and operating conditions in many bioprocesses.

In the present report the effects of pH, nitrogen and carbon sources, K_2HPO_4 and KH_2PO_4 on the production of amylolytic enzymes were studied.

Materials and Methods

Microorganism used

Thermomyces lanuginosus strain ATCC 34626 originated from American Type Culture Collection and kindly supplied by Dr. Bhat (Institute of Food Research, Norwich, UK).

Maintenance of stock culture

The strain was maintained on Yeast-Pepton/Soluble Starch (YPSS) agar medium *(9)* or on Potato Dextrose Agar (PDA), and stored in refrigerator.

Cultivation and production of amylolytic enzymes by Thermomyces lanuginosus

A three-stage cultivation technique was used. In the first stage, the fungus was grown on YPSS or PDA slant agar for 8 to 10 days at 50 °C in a humidified thermostat. In the second stage a suspension of spores was prepared using 0.1 % of Triton X-100 solution. A volume of 5 mL of the suspension was added to 100 mL glucose--asparagine medium (pH=6.0) to initiate the cultivation at 50 °C and 220 rpm in an orbital shaker for 1 to 2 days to obtain a homogeneous mycelium growth. In the third stage, 10 mL of the mycelial suspension was used as inoculum for initiating the production of amylolytic enzymes in 150 mL starch-asparagine medium (soluble starch: 40 g, L-asparagine: 4 g, KH_2PO_4: 3 g, K_2HPO_4: 2 g, $MgSO_4 \cdot 7H_2O$: 0.5 g, Vogel's trace elements solution *(10)*: 1 mL was dissolved in 1 L distilled water). Samples (10 mL) were taken under a laminar box from duplicate flasks at varying times. The samples were filtered and the α-amylase and glucoamylase activities were assayed in the filtrate.

Determination of dry mass of growing mycelia

A volume of 10 mL of homogeneous mycelium suspension was harvested, washed and, after drying, weighed.

Analytical chemicals were from Sigma, Reanal, Fluka and Merck.

Enzyme assays

α-amylase. A reaction mixture containing 1 mL of 0.1 M sodium-acetate buffer pH=5.0 and 1 mL 0.5 % (w/v) soluble starch solution was mixed and pre-incubated at 50 °C for 10 min before adding 1 mL of appropriately diluted culture filtrate as an enzyme source. After 5 min the reaction was terminated by adding 1 mL of 0.5 M HCl. The unhydrolysed starch in this aliquot was estimated by the iodine method described below. One unit of α-amylase activity was defined as the amount of enzyme that hydrolyses 1 mg soluble starch in 1 min under relevant conditions.

Glucoamylase. A volume of 1 mL of reaction mixture containing 0.25 mL of 0.1 M phosphate buffer pH=4.6 and 0.25 mL of 1 % (w/v) soluble starch solution was pre-incubated at 50 °C for 10 min. A volume of 0.5 mL of appropriately diluted culture filtrate as an enzyme source was added and the incubation continued for further 15 min. The reaction was terminated by placing the tubes in a boiling bath for 30 min. After cooling, the released glucose concentration was estimated by glucose oxidase/peroxidase *(11)* using a standard glucose curve prepared under the same conditions. One unit of glucoamylase activity was defined as the amount of enzyme that releases 1 μmol glucose in 1 min under relevant conditions.

Iodine method for starch estimation

An aliquot of the sample (starch hydrolysate) was mixed with 1 mL of iodine reagent in a total volume of 2 mL (distilled water). This reagent contained 0.02 % (w/v) iodine and 0.2 % (w/v) KI in 0.5 N HCl. To this mixture 5 mL of distilled water was added and the developed colour was measured at 590 nm against blank. The amount of starch was estimated using a standard potato soluble starch (Merck, Darmstadt) curve prepared under the same conditions.

Experimental design

In order to optimise the medium composition for amylolytic enzymes production, various carbon-sources and N-sources modelling industrial fermentation technology were investigated in respect of the amylolytic enzymes. Experimental design of Response Surface Method (RSM) was also applied. At the same time the effect of buffer capacity (KH_2PO_4 and K_2HPO_4) of the medium was checked as well. In all experimets enzyme activities were assayed. A full polynomial model obtained by a multiple regression technique for two factors using SPSS 8.0 for Windows (Copyright © by SPSS Inc., 1994–1997) was used to determine the optimum composition of the medium.

Results and Discussion

Effects of pH on production of enzymes

Based on the preliminary experimental results, it was confirmed that the pH of the fermented broth reached a very high value (pH=8.0–9.5) at the end of the fermentation (after 82 h) and this may cause inactivation of the enzymes. The effect of pH on the productivity of amylolytic enzymes in fermentation was studied. The media of these experiments were prepared with 100 mM of citrate-buffer with different pH values.

Due to the higher buffer capacity of the media a pH control was partly achieved. The experiments were run up to 168 h. Fermentation was followed by α-amylase and glucoamylase assays, and pH measurement. The activities of both amylolytic enzymes (α-amylase and glucoamylase) on all trials were increased and reached maxmum values at about 96th h of fermentation. After that, these activities were decreased (data not shown). The maximum values of activities are presented in Table 1.

From the analysis of the changes of pH values during fermentation it can be concluded that the pH in the reference run increased to a great extent. The application of a buffer system could restrict the increase of pH. The results revealed that the pH has a strong influence on the α-amylase and glucoamylase activities. When the initial pH of the media was adjusted to 4.9 with citrate-buffer, the glucoamylase activity was 3.5 times and the α-amylase activity 1.7 times higher than that of the reference. Reducing the initial pH of the media both amylolytic activities decreased drastically.

In case of the buffered conditions the profile of the dry mass and the amylolytic activities showed inverse function with the initial pH of the medium. In this way it was possible to repress the mycelium growth and favour the enzyme production. It should be noticed that if the initial pH was 3.0 or less, no growth was observed. Further experiments are needed to clear up the function of the pH and glucoamylase activity. Taylor *et al. (12)* reported that *Humicola lanuginosa* (present name is *Thermomyces lanuginosus*) produces two forms of glucoamylase, one with pH optimum of 4.9 and another of 6.6. This demonstrates that the highest activities were measured when the final pH was between 4.8 and 5.7.

Effect of different carbon sources on the production of amylolytic enzymes

The effects of various carbon sources on the production of α-amylase and glucoamylase by *Thermomyces lanuginosus* ATCC 34626 strain are shown in Table 2.

Starch is a generally accepted nutritional component for induction of amylolytic enzymes. This material was applied as reference. To get detailed information about the synthesis of amylolytic enzymes of *Thermomyces lanuginosus* various carbon sources were used. When growing fungus on glucose, maltose, maltodextrin, amylopectin, dextran and dextrin the amylolytic activities were higher than on starch. In case of α-amylase the maltodextrin was found to be the best carbon source. The α-amylase activity was approximately 25 % higher than that of the control (with starch). In the case of glucoamylase the dextrin proved to be the best, giving two times higher activity than starch.

Effects of different nitrogen sources on production of amylolytic enzymes

In the investigation of the effects of various nitrogen sources on amylolytic enzyme production (Table 3) L-asparagine was found to be the most promising one. In case of α-amylase activity yeast extract seemed to be suitable as well, but in case of glucoamylase the enzyme productivity was just half of that on L-asparagine. This result contradicts the observation of Haasum *et al. (4)* who found outstanding glucoamylase activity obtained from fermentation on yeast extract. This difference can be caused by the deviation in pH and the strain applied during the fermentation. When inorganic nitrogen sources were used, the fungi grew excellently, but very poor enzyme activities were achieved.

Experimental design of Response Surface Method

Central composite design (CCD) was adopted as an efficient way to find a culture medium with optimum composition for amylolytic enzymes production. Starch and L-asparagine were selected as carbon and nitrogen sources for independent variables. Based on preliminary

Table 1. Relationship between initial pH of the media, the yield of mycelium and amylolytic enzyme activities

Initial pH of medium	Final pH of fermented broth	*w*(dry mass)	Relative α-amylase activity	Relative glucoamylase activity
		%	%	%
Reference (pH=6.3*)	8.8	0.81	100	100
4.9	5.7	0.57	173	364
4.5	4.8	0.73	124	165
4.0	4.1	0.89	20	41
3.5	3.5	1.28	16	7
3.0	n.g.			
2.5	n.g.			

* prepared with water, n.g.: no growth was detected

Table 2. Effects of various carbon sources on the production of amylolytic enzymes

Carbon source	α-amylase	Glucoamylase	pH
	U/mL	U/mL	
Starch	92.53	6.15	5.53
Glucose	103.76	6.36	5.03
Maltose	110.42	9.92	5.30
Maltodextrin	124.22	10.56	5.30
Amylopectin	92.43	7.76	5.22
Amylose	59.40	4.32	5.23
Dextran	104.50	9.39	5.24
Dextrin	111.16	12.66	5.21

All values in table were measured at 96th h of fermentation

Table 3. Effects of various nitrogen sources on α-amylase and glucoamylase production

Nitrogen source	α-amylase	Glucoamylase	pH
	U/mL	U/mL	
L-asparagine	89.87	6.29	5.16
Yeast extract	72.84	3.18	5.19
CH_3COONH_4	24.21	0.72	5.28
NH_4NO_3	26.55	1.60	4.87
$NaNO_3$	10.54	0.00	5.06
$(NH_4)_2SO_4$	36.11	3.14	4.53
$NH_4H_2PO_4$	28.43	3.27	4.47

All values in the table were measured at 96th h of the fermentation

experiments 2 % concentration change in starch and 0.2 % in L-asparagine have significant effects on enzyme activities. The full second-order polynomial model was used to fit to the dependent variables using the following equation:

$$Y = b_o + b_1 x_1 + b_2 x_2 + b_3 x_1^2 + b_4 x_2^2 + b_5 x_1 x_2$$

where Y (α-amylase or glucoamylase activities that were measured at 96th h of fermentation) is the dependent variable to be modelled, b_i are regression coefficients of model, and x_1 (starch), x_2 (L-asparagine) are independent variables (Table 4).

The data of Analysis of Variance (ANOVA) table prove that the applied model fits the experimental values (Table 5).

The t-values of the estimated coefficients showed that all coefficients gained by regression analysis have significant ($P<0.05$) effects on both amylolytic activities (Table 6). The increase of the concentration of L-asparagine as nitrogen source has a positive effect on the production of both amylolytic enzymes. In case of starch, the increase of the concentration showed a positive effect on α-amylase production, and a negative effect on glucoamylase production (Fig. 1).

Table 4. Experiment design and results

Trial*	*w*(starch)	*w*(L-asparagine)	α-amylase	Glucoamylase	Final pH
	%	%	U/mL	U/mL	
1	2	0.2	30.12	2.64	5.55
2	2	0.6	91.34	7.33	5.74
3	6	0.2	72.23	4.49	5.15
4	6	0.6	113.19	6.11	5.16
5	1.16	0.4	44.08	3.96	5.86
6	6.84	0.4	104.21	5.89	5.20
7	4	0.116	45.86	2.80	5.15
8	4	0.684	107.43	6.96	5.14
9	4	0.4	89.87	6.29	5.16
10	4	0.4	87.99	6.17	5.16
11	4	0.4	88.48	6.05	5.22
12	4	0.4	87.36	6.15	5.18

* media were prepared with 100 mM citrate buffer (pH=4.9), all enzyme activities and pH values in the table were measured at 96th hour of the fermentation

Table 5. Summarised data of analysis of variance of α-amylase and glucoamylase models

Mode	Sum of squares		Degree of freedom	Mean of squares		F		Significant	
	AA*	GA**		AA	GA	AA	GA	AA	GA
Regression	7769.47	25.84	5	1553.89	5.169	103.19	43.78	0.00	0.00
Residual	90.35	0.71	6	15.06	0.118				
Total	7859.82	26.55	11						

*AA α-amylase activity **GA glucoamylase activity

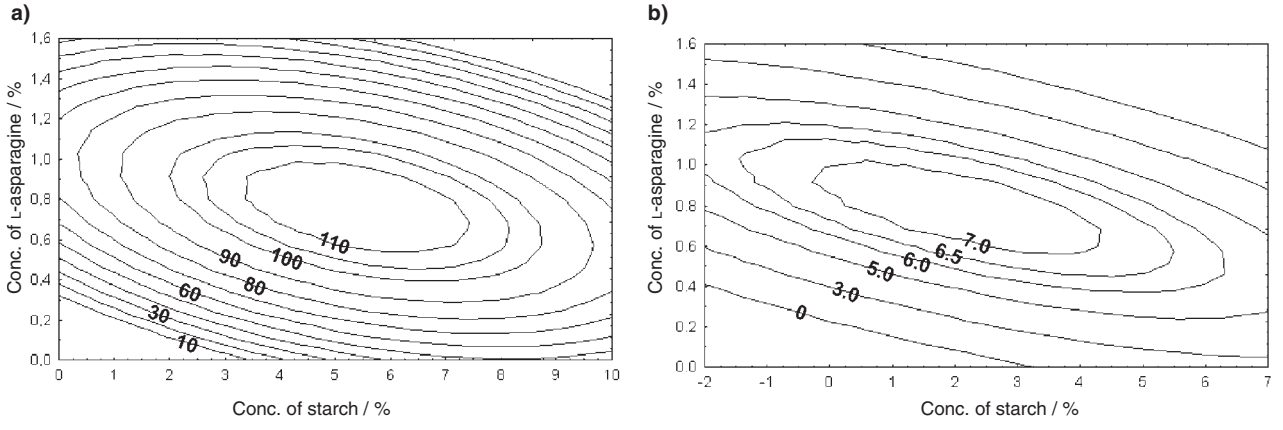

Fig. 1. Contour drawing of fitted second-order polynomial models using SerialLab; (a) α-amylase, (b) glucoamylase

The interaction between the two factors was also significant at 95 % level. According to fitted models, the optimum concentration for production of α-amylase is 5.5 % for starch and 0.75 % for L-asparagine, for production of glucoamylase 2.2 % of starch and 0.75 % of L-asparagine.

The predicted maximum enzyme activities from the models are 119 U/mL and 7.5 U/mL in case of α-amylase and glucoamylase, respectively. The set of experiments with media containing different starch concentrations was carried out to check how the real data fit the data predicted by modelling. At 6.5 % starch concentration the maximum of α-amylase activity was reached with the value of 151 U/mL and the maximum activity of glucoamylase was 8.4 U/mL at 2 % of starch concentration (Fig. 2). The measured values of amylolytic activities were still within the confidence interval (95 %) of predicted values according to SPSS. The fitting of the experimental and predicted data was verified and approved. The enzyme activities were increased by approximately 50 % in case of α-amylase and by 30 % in case of glucoamylase when using the newly developed media.

Table 6. Coefficients of regression analysis for the prediction of α-amylase and glucoamylase production

Model	Coefficients		t		Significant (P)	
	AA*	GA**	AA	GA	AA	GA
B0	–65.520	–5.34	–5.565	–5.12	0.001	0.002
B1	27.925	2.09	7.590	6.43	0.000	0.001
B2	279.518	26.90	7.579	8.26	0.000	0.000
B3	–1.696	–0.14	–4.447	–4.14	0.004	0.006
B4	–138.558	–14.52	–3.634	–4.30	0.011	0.005
B5	–12.662	–1.92	–2.610	–4.47	0.040	0.004

AA α-amylase activity **GA glucoamylase activity

Effects of the concentrations of KH_2PO_4 and K_2HPO_4 on production of amylolytic enzymes

To improve the amylolytic enzyme production the effect of the concentration of KH_2PO_4 and K_2HPO_4 was investigated at different times as well. Based on the previous results the effect of inorganic phosphates on the production of α-amylase and glucoamylase was separately investigated. The results at 94th h of fermentation are presented in Table 7.

The reduction of concentration of KH_2PO_4 and KH_2PO_4 leads to improvement of productivity of both amylolytic enzymes. The maximum activities were reached at 0.15 % of KH_2PO_4 and 0.1 % of K_2HPO_4.

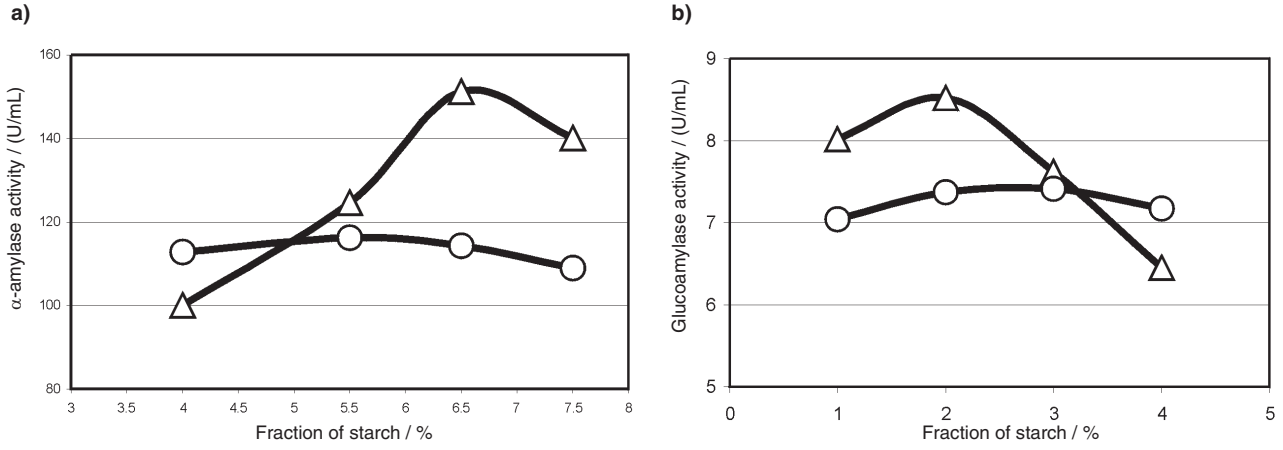

Fig. 2. Effects of starch on production of amylolytic (α-amylase and glucoamylase) enzymes by *Thermomyces lanuginosus* ATCC 34626; (a) α-amylase, (b) glucoamylase; the medium contained 0.75 % (w/v) L-asparagine, 0.3 % K_2HPO_4, 0.2 % (w/v) KH_2PO_4, 0.05 % (w/v) $MgSO_4 \cdot 7H_2O$ and 1 mL Vogel's trace-element solution
-Δ- experimental values; -O- predicted values

Table 7. The effect of the fraction of KH_2PO_4 and KH_2PO_4 of the growth media on amylolytic activities

γ(KH_2PO_4)	γ(K_2HPO_4)	α-amylase			Glucoamylase		
		w(starch)	Activity	pH	*w*(starch)	Activity	pH
g/L	g/L	%	U/mL		%	U/mL	
3.00	2.00	6.5	184.72	5.73	2.0	17.48	5.30
3.00	0.00	6.5	66.48	4.99	2.0	12.30	5.72
0.00	2.00	6.5	241.23	6.11	2.0	9.48	5.88
1.50	1.00	6.5	259.75	5.64	2.0	22.43	5.67
0.75	0.50	6.5	233.15	5.41	2.0	18.57	5.83
0.00	5.75	6.5	35.85	6.90	2.0	7.16	5.98
4.50	0.00	6.5	32.77	5.02	2.0	8.46	5.90
0.00	3.90	6.5	104.23	6.58	2.0	5.36	5.77
6.10	0.00	6.5	27.07	4.85	2.0	7.70	5.19

Conclusions

Adjusting the initial pH of the medium with 100 mM of citrate-buffer an improvement was achieved in the productivity of the amylolytic enzymes. In the investigated range pH=4.9 was found to be the most suitable one.

L-asparagine was the most promising among 5 investigated nitrogen sources. Yeast extract would be beneficial for α-amylase as well, but for glucoamylase the enzyme activity was decreased to half of that gained on L-asparagine.

The fungus grown on glucose and all tested glucose polymers showed considerable amylolytic activities. The production of amylolytic enzymes on glucose as a sole carbon source indicates a constitutive synthesis of the α-amylase and glucoamylase.

Response Surface Method was used to find optimum concentration of medium components. Second-order polynomial models were applied. All estimated parameters had significant level higher than 95 % and some higher than 99 %. The predicted values were verified experimentally.

Based on the results of the optimisation experiments the proposed compositions of the fermentation media (w/v) are in case of α-amylase production: soluble starch 6.5 %, L-asparagine 0.75 %, KH_2PO_4 0.15 %, K_2HPO_4 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, Vogel's trace elements 0.1 mL and in case of glucoamylase production soluble starch 2.0 %, L-asparagine 0.75 %, KH_2PO_4 0.15 %, K_2HPO_4 0.1 %, $MgSO_4 \cdot 7H_2O$ 0.05 %, Vogel's trace elements 0.1 mL. Both media are prepared with 100 mM citrate biffer (pH = 4.9).

To reach the maximum amylolytic activitities, 96-hour fermentation time is needed.

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Optimiranje sastava podloge za proizvodnju amilolitičkih enzima iz *Thermomyces lanuginosus* ATCC 34626

Sažetak

Sastav podloge za proizvodnju amilolitičkih enzima iz *T. lanuginosus* optimiran je na nekoliko načina. Ispitan je utjecaj različitih izvora ugljika i dušika. *T. lanuginosus* uzgojen na škorbu, maltodekstrinu, dekstrinu, maltozi, amilopektinu, glukozi i dekstranu uvjetovao je dobru aktivnost α-amilaze (92–125 U/mL) i glukoamilaze (6–13 U/mL). Od ispitivanih izvora dušika L-asparagin je bio najbolji. Optimalni pH u podlozi za fermentaciju utvrđen je i održavan pri 4,9, a koristeći citratni pufer (100 mM) za proizvodnju amilolitičkih enzima. Metoda površinskog odziva (Response Surface Method) primijenjena je pri izboru optimalne koncentracije sastojaka podloge za proizvodnju amilolitičkih enzima. Polinomski model drugoga reda usklađen je s eksperimentalnim podacima na razini značajnosti od 95 % ($P<0,05$) za α-amilazu i glukoamilazu. Podloga s izmijenjenim sastavom bila je ispitana s obzirom na proizvodnju amilolitičkih enzima. Udjel L-asparagina iznosio je 0,75 %, udjel škorba 6,5 % za α-amilazu, a 2 % za glukoamilazu.