Kor. J. Appl. Microbiol. Bioeng.
Vol. 16, No. 6, 494—498 (1988)

Culture Conditions for Glucoamylase Production and Ethanol Productivity of Heterologous Transformant of *Saccharomyces cerevisiae* by Glucoamylase Gene of *Saccharomyces diastaticus*

Kim, Young-Ho* and Jung-Hwn Seu

Department of Microbiology, College of Natural Science, Kyungpook National University, Taegu 702-701, Korea

Transformant의 Glucoamylase 생성조건과 Ethanol 발효성

김영호*·서정훈

경북대학교 자연과학대학 미생물학과

The optimum conditions for glucoamylase production, and ethanol productivity of the transformant TSD-14 were investigated as compared with the parental strains. The properties of TSD-14 were comparatively similar to the donor *S. diastaticus* IFO 1046 as regards the conditions of glucoamylase production and ethanol productivity. The soluble starch was the most effective carbon source for the glucoamylase production. While inorganic nitrogen sources did not prompt cell growth and enzyme production, the organic nitrogen sources generally enhanced both cell growth and glucoamylase production. The metal salts such as $FeSO_4$, $MgSO_4$, $MnCl_2$, and $NiSO_4$ were favorable to the enzyme production. And the optium temperature and initial pH for glucoamylase production were 30°C and 5. The transformant TSD-14 produced 8.3%(v/v) ethanol from 15% sucrose medium, 4.8%(v/v) ethanol from 15% soluble starch medium, and 7.5%(v/v) ethanol from 15% liquefied potato starch medium. The corresponding fermentation efficiency were 84%, 45% and 70%, respectively.

We previously reported that the yeast transformation without a foreign vector, using only partially *Bam*HI-digested the chromosomal DNA of *Saccharomyces diastaticus* in an attempt to introduce the capability of starch utilization into *S. cerevisiae,* and obtained the transformants capable of converting soluble starch to ethanol directly(1).

In the present work, the influence of different culture conditions on glucoamylase production of the most desirable transformant TSD-14 was investigated. And the ethanol productivities from 15% sucrose, soluble starch, and potato starch liquefied by treatment with alpha-amylase were compared with those of the parental strains.

Materials and Methods

Strains

The yeast strains used in this work are listed in Table 1.

Table 1. List of strains used

Strain	Genotype	Remark
S. cerevisiae X2180-1A	a SUC2 mal mel gal2	recipient
S. diastaticus IFO 1046	CUP1 wild type	donor
TSD-14		heterologous transformant

Media and Culture conditions

The culture medium for glucoamylase production consisted of 2% glucose, 0.3% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% yeast extract. And the cultivation was carried out in a 100 m*l* Erlenmyer flask containing 20 m*l* of the

Key words: Transformant, glucoamylase production, ethanol productivity
*Corresponding author

Table 2. Effect of carbon source on glucoamylase production

Carbon source	*S. diastaticus* IFO 1046		TSD-14	
	Growth (620nm)	Glucoamylase(U)	Growth (620nm)	Glucoamylase(U)
D-glucose	2.20	0.45	2.46	0.45
Maltose	2.58	0.32	2.16	0.56
Sucrose	2.37	1.27	2.21	1.80
Dextrin	1.46	3.52	1.33	7.15
Soluble starch	1.36	14.95	1.47	17.60

Various carbon sources were added at final concentration of 1%(w/v) to basal medium that consisted of 0.3% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.1% yeast extract. Other culture conditions are in Materials and Methods.

Table 3. Effect of nitrogen source on glucoamylase production

Nitrogen source	*S. diastaticus* IFO 1046		TSD-14	
	Growth (620nm)	Glucoamylase(U)	Growth (620nm)	Glucoamylase(U)
NH_4Cl	0.47	2.89	0.68	7.27
$NH_4H_2PO_4$	0.18	1.67	0.36	1.94
$(NH_4)_2SO_4$	0.49	3.17	0.70	7.87
Casamino acid	0.75	5.56	3.19	9.57
Corn steep liquor	2.44	9.12	2.16	13.89
Meat extract	1.74	8.74	3.72	11.07
Peptone	4.12	21.56	4.01	22.78
Yeast extract	4.03	11.67	3.94	18.92

Various nitrogen sources were added at final concentration of 0.4%(w/v) to basal medium, which consisted of 2% soluble starch, 0.05% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. Other culture conditions were as in Table 2.

medium at 30 °C for 4 days.

In flask experiments, fermentation medium consisted of 0.3% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, 0.2% yeast extract, and 15% carbohydrate such as sucrose, soluble starch, or liquefied potato starch. Fermentation was conducted in 250 m*l* flasks with 200 m*l* medium per flask at 30 °C for 12 days.

Glucoamylase assay

Quantitative assay for glucoamylase activity was performed by a modification of the method of Somogyi-Nelson as reported previously(1).

Liquefaction of potato starch

As the substrate for the ethanol fermentation, potato starch was liquefied. A 15% (w/v) suspension of potato starch (Hayashi) was prepared in distilled water. When the temperature reached 50 °C, 0.08% (v/w, to starch content) of Thermamyl (alpha-amylase, Novo) was added and the temperature was raised to 90 °C with continuous stirring for 20 min. After liquefaction, the liquefied potato starch was used for preparing the fermentation medium.

Ethanol fermentation

Ethanol fermentation was carried out in 250 m*l* flask with 200 m*l* medium per flask. The flask was equipped with air restrictor containing sulfuric acid as described by Ueda *et al*(2). For quantitative assay of ethanol productivity, the flask was incubated at 30 °C for 12 days and measured the loss in weight resulting from carbon dioxide production. The result was converted to ethanol equivalent.

Results

Effect of carbon source on glucoamylase production

We examined the effect of various carbon sources on the production of glucoamylase. As shown in Table 2, soluble starch was the most effective carbon source. Glucose, maltose, and sucrose were good for growth but not for the glucoamylase production.

Effect of nitrogen source on glucoamylase production

The effect of nitrogen source was investigated by adding various kinds of organic or inorganic nitrogen sources. Each of the indicated nitrogen sources was added to the basal medium containing 2% soluble starch, 0.5% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. As shown in Table 3, inorganic nitrogen sources did not prompt cell growth and enzyme production. The organic nitrogen sources tested generally enhanced both growth and enzyme production. In particular, the addition of peptone or yeast extract were favorable for cell growth and glucoamylase production.

Table 4. Effect of metal salt on glucoamylase production

Metal salt (2×10^{-4}M)	*S. diastaticus* IFO 1046		TSD-14	
	Growth (620nm)	Glucoamylase(U)	Growth (620nm)	Glucoamylase(U)
None	2.42	14.44	2.56	15.23
$BaCl_2$	2.35	9.39	2.37	15.00
$CaCl_2\cdot 2H_2O$	2.20	11.76	2.50	15.63
$CoCl_2$	0.66	4.74	0.71	6.25
$CuSO_4\cdot 5H_2O$	0.89	10.14	2.43	14.07
$FeSO_4\cdot 7H_2O$	2.68	19.28	2.92	21.07
$HgCl_2$	0.09	0	0.06	0
$LiSO_4$	0.76	5.74	2.12	14.80
$MgSO_4\cdot 7H_2O$	2.38	15.96	2.60	18.24
$MnCl_2\cdot 4H_2O$	2.35	15.60	2.41	16.44
$NiSO_4\cdot 7H_2O$	2.48	16.48	2.59	15.20
$PbCl_2$	0.62	5.35	0.65	4.93

Various mineral compounds were added at the indicated concentrations to a medium consisting of 2% soluble starch, 0.3% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% $MgSO_4\cdot 7H_2O$, and 0.1% yeast extract.

Effect of metal ions on glucoamylase production

The effect of metal ions at a concentration of 2×10^{-4}M was examined. As shown in Table 4, $FeSO_4$, $MgSO_4$, $MnCl_2$ and $NiSO_4$ prompted the cell growth and enzyme production. But $CoCl_2$, $HgCl_2$ and $PbCl_2$ markedly inhibited both cell growth and glucoamylase production.

Effect of initial pH on glucoamylase production

The effect of the initial pH of culture medium on the glucoamylase production was investigated as shown in Fig. 1. When the initial pH of the medium was 5.0, the enzyme production and cell growth were markedly increased, and outside the pH of 5.0 those were not favorable.

Effect of temperature on glucoamylase production

To examine the optimum temperature for the glucoamylase production, the cultivation was carried out at temperature range of 20°C to 50°C. As shown in Fig. 2, cell growth and glucoamylase production reached maximum level at 30°C. But cell growth and glucoamylase production were severely decreased above 40°C.

Ethanol productivity

Ethanol productivities of the transformants and

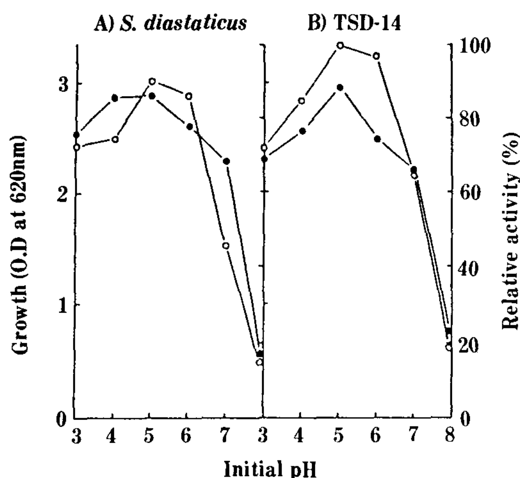

Fig. 1. Effect of initial pH on glucoamylase production. The medium consisted of 2% soluble starch, 0.3% $(NH_4)_2SO_4$, 0.05% KH_2PO_4, 0.05% $MgSO_4\cdot 7H_2O$, and 0.1% yeast extract, and the initial pH of the medium was adjusted at 3.0 to 8.0 by addition of 0.5N NaOH or HCl. Symbols: ●, cell growth; ○, relative activity.

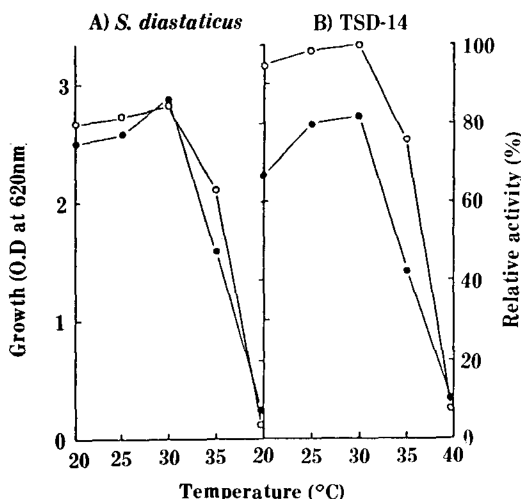

Fig. 2. Effect of temperature on glucoamylase production.
S. diastaticus IFO 1046 and TSD-14 were cultured in the medium described in Fig. 1 at various temperatures. Symbols: ●, cell growth: ○, relative activity.

the parental strains from sucrose, soluble starch, or liquefied potato starch were investigated. Fig. 1 (A) shows the results of ethanol yields of the strains in 15% sucrose medium. After 12 days fermentation at 30°C, the recipient *S. cerevisiae* X2180-1A and the donor *S. diastaticus* IFO 1046 produced 9.3% (v/v) and 7.8% (v/v) of ethanol, respectively,

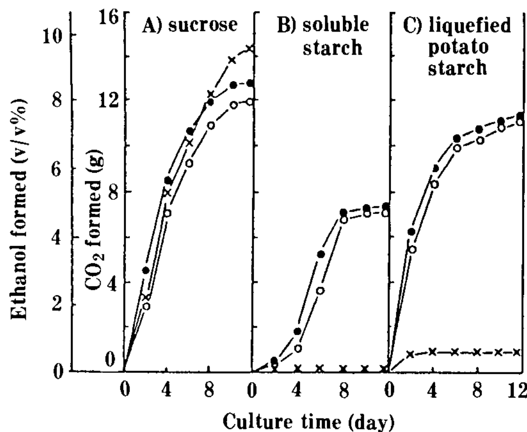

Fig. 3. Ethanol productivity from 15% sucrose, soluble starch, and liquefied potato starch.

Ethanol fermentation was carried out in 250 m*l* flask with 200 m*l* medium per flask, which was equipped with air restrictor containing sulfuric acid, as described in Materials and Methods. Symbols: ●, TSD-14; ○, *S. diastaticus* IFO 1046; x, *S. cerevisiae* X2180-1A.

whereas the transformant TSD-14 produced 8.3% (v/v) of ethanol. The ethanol productivity of TSD-14 was not only better than that of *S. diasaticus* IFO 1046, but also comparable to that of *S. cerevisiae* X2180-1A.

Fig. 2(B) shows the ethanol productivity of the transformant from 15% soluble starch medium compared with those of the parental strains. The transformant TSD-14 produced 4.83% (v/v) of ethanol, while *S. diastaticus* IFO1046 produced 4.77% (v/v) of ethanol. In contrast, *S. cerevisiae* X2180-1A was unable to grow and ferment soluble starch at all.

By using 15% liquefied potato starch medium, we examined the application possibility of the transformant TSD-14 for elimination of the separate saccharification step from the industrial process of ethanol fermentation composed of gelatinization of raw starch, liquefaction, saccharification, and fermentation. As shown in Fig. 3 (C), the ethanol yields of the transformant TSD-14 and the donor *S. diastaticus* IFO 1046 in liquefied potato starch medium were enhanced to 7.48% (v/v) and 7.35% (v/v), respectively, compared with those in soluble starch medium. However, the recipient *S. cerevisiae* X2180-1A produced very little ethanol.

Discussion

In previous study, we descrived transformation of the intact Yeast cells by partially *Bam*HI-digested chromosomal DNA of *S. diastaticus* to develop a new brewing yeast that produces glucoamylase and efficiently ferments soluble starch(1). Among the transformants, TSD-14 seemed the most promising strain as regards glucoamylase production and ethanol formation.

Thus, in this study, we examined that the culture conditions for glucoamylase production, and ethanol productivities from various media. The glucoamylase productions of *S. diastaticus* and TSD-14 were considerably dependent on the culture conditions. As shown in Fig. 1, soluble starch and dextrin were favorable for the glucoamylase production. Glucose, maltose, and sucrose did not result in much production of the glucoamylase. The decrease in glucoamylase activity seen with the addition of glucose, maltose or sucrose suggests that the glucoamylase is regulated through a glucose catabolite repression mechanism. The organic nitrogen sources tested generally enhanced both cell growth and enzyme production, but inorganic nitrogen sources did not. The peptone and yeast extract were most effective. In the production of microbial enzymes, metal salts have been known to play important role. In this work, $FeSO_4$, $MgSO_4$, $MnCl_2$ and $NiSO_4$ had favorable effects, but $CoCl_2$, $HgCl_2$, and $PbCl_2$ repressed severely cell growth and the glucoamylase production. The optimum temperature for the glucoamylase production was 30 °C, whereas that for enzyme activity was 50 °C (1). And pH 5.0 was most favorable initial pH for cell growth and enzyme production. These results are similar to Laluces's investigation(3).

To investigate the ethanol productivity, 15% sucrose, soluble starch, and liquefied potato starch, were used. The transformant TSD-14 produced 8.3% (v/v) of ethanol from 15% sucrose medium, 4.8% (v/v) of ethanol from 15% soluble starch medium, and 7.5% (v/v) of ethanol from 15% liquefied potato starch medium, and the corresponding fermentation efficiency were 84%, 45%, 70%, respectively. These results were interpreted as evidence that the conversion from starch to glucose was the most limiting step in the starch fermentation, and the debranching activity of the *S. diastaticus* glucoamylase was very weak compared to alpha 1-4 glucosidic bond activity(4).

On the other hand, the transformant TSD-14 did not produce phenolic off-flavor in contrast to

the donor *S. diastaticus*(5) (data not shown). The properties of transformant TSD-14, presented in this work, suggest that it could be developed for the ethanol production from starch materials by further strain improvements, such as gene manipulation or protoplast fusion techniques.

요 약

Starch로부터 직접적으로 ethanol을 발효 생산할 수 있는 새로운 효모균주의 개발을 목적으로 *S. diastaticus*의 glucoamylase gene을 cloning vector를 사용하지 않고 *S. cerevisiae*에 transformation시켜, soluble starch를 직접 발효 할 수 있는 transformants를 얻는데 성공하였으며 이들 중 glucoamylase 생성능이 가장 우수한 균주인 TSD-14를 전보에서 선별하였다.

Transformant TSD-14의 glucoamylase 생성조건과 ethanol productivity를 parent strain과 비교 검토한 결과 이들 성질에 있어서 TSD-14는 donor인 *S. diastaticus*와 거의 유사하였다. 즉, 탄소원으로는 soluble starch가 균의 생육 및 효소생성에 가장 효과적이었으며 glucose, maltose 등은 균의 생육에는 효과적이었으나 효소생성은 저해하였다. 질소원으로 무기태 질소원에 비하여 유기태 질소원이 좋은 효과를 나타냈으며 특히, peptone과 yeast extract가 효과적이었다. 또한 금속염으로는 $FeSO_4$, $MgSO_4$, $MnCl_2$, $NiSO_4$ 등이 효과적이었으며 $CoCl_2$, $HgCl_2$, $PbCl_2$ 등은 오히려 균의 생육 뿐만 아니라 효소생성을 크게 저해하였다. 한편, TSD-14의 ethanol productivity를 조사한 결과, 15% sucrose, soluble starch, liquefied potato starch 등에서 8.3%(v/v), 4.8%(v/v), 7.5%(v/v)의 ethanol을 생성하여 각각 총당에 대해 84%, 45%, 70%의 발효율을 나타냈다.

Acknowledgements

This work is supported by a research grant from the Korea Science and Engineering Foundation (1985).

References

1. in press.
2. Ueda, S., C.T. Zenin, P.A. Monterio and Y.K. Park: *Biotechnol. Bioeng.*, **23**, 291 (1981).
3. Laluce, C. and J.R. Mattoon: *Appl. Environ. Microbiol.*, **48**, 17 (1984).
4. Stewart, G.G.: *Devel. Indus. Microbiol.*, **25**, 183 (1983).
5. Russell, I., I.F. Hancock and G.G. Stewart: *J. Am. Soc. Brew. Chem.*, **41**, 45 (1983).

(Received October 13, 1988)