Inducing Mechanism of Dextrins with Different DE values on Production of α-amylase by *B. Subtilis* ZJF-1A5

Junliang Sun*, Ruixiang Zhao, Liangbin Hu, Jie Zeng, Benguo Liu
[1]*School of Food Science, Henan Institute of Science and Technology, Xinxiang 453003, Henan province, China.*
sunjunliang-hist@163.com*

(Received on 24th June 2013, accepted in revised form 8th November 2013)

Summary: Alpha-amylase was widely used in food industries, textile technology, paper manufacturing and so on. In this paper, the inducing mechanism of corn dextrins with different DE values (dextrose equivalent value) on production of α-amylase by *Bacillus subtilis (B.subtilis)* ZJF-1A5 was investigated. The results showed that the yield of α-amylase by *B.subtilis* ZJF-1A5 was increased by using dextrin with a certain DE value range as carbon source, which could be attributed to the presence of oligosaccharide in dextrins. By ordinary fermentation with oligosaccharide as carbon source, it was found that the inducing activity of maltopentaose was the strongest. It could be confirmed that the dextrins played important roles during the process of production of α-amylase by *B.subtilis* ZJF-1A5.

Keywords: Dextrin; Inducing mechanism; *B.subtilis*; *α*-amylase.

Introduction

α-Amylase (E.C.3.2.1.1.) breaks down the α-1,4 glucosidic bonding of linear amylose and branching amylopectin which are the major building blocks of starch [1]. It is a key enzyme in the production of starch derivatives and also used in desizing fabrics, baking industry, pharmaceuticals and detergents [2]. Each application of a-amylase requires unique properties with respect to specificity, stability, temperature and pH dependence. Screening of microorganisms with higher a-amylase activities could therefore, facilitate the discovery of novel amylases suitable to new industrial applications [3].

Bacillus is known as the most important producing strain for the production of α-amylase and is used to produce enzyme in SSF (Solid state fermentation) and SMF (Submerged fermentation) form. Salva *et al* [4] investigated the effect of different carbon sources on the production of α-amylase. Syu *et al* [5] also studied the effect of glucose, maltose, xylose and starch, which were taken as carbon source respectively, on the production of α-amylase. Glucose could cause thallus to grow fast and the existence of starch in the medium could obtain higher enzyme activity or certain enzyme activity. Hiller *et al* [6] verified that lactose and nitrogen had impact on α-amylase production by precisely prepared synthetic medium. The result showed that the pattern of cell growth and α-amylase production was similar and had nothing with limited nutrition. In the cultivating process of high yield of α-amylase, ventilation was essential and important, but didn't find that controlling dissolved oxygen could benefit enzyme production. Babu *et al* [7] got the highest yield of α-amylase on the condition of filling with carbon dioxide. Ivanova compared disparate immobilization technologies and found that agar, κ- carrrageenan, polythene oxide colloid, active methanal acrylonitrile/acrylamide film were good for α-amylase production [8].

Although there are lots of research reports about the primary structure and functional theory of α-amylase, the studies about the substrate of fermentation of germ strain efficiently inducing α-amylase and the mechanism are desperately meager. Some researches state that different carbon sources influence α-amylase production. Lactose, glucan and soluble starch are better for producing enzyme [9]. Dissolved oxygen rate is also an important element of α-amylase production and high rate of Dissolved oxygen may generate high yield of enzyme. In our study, we find that α-amylase activity could be increased via fermentation with a certain molecular size, type and number of dextrin as crabon source. If we can explain the fermentation mechanism of this dextrin theoretically and practically, it will be very important to enzyme industries, which is the aim of this study.

Results and Discussion

Effect of Different Dextrin in the Culture on B. subtilis ZJF-1A5 Growth and Soluble Protein Content

According to Fig. 1 (a), CFU (Colony FormingUnits) **of** *B. subtilis* ZJF-1A5 by culture of different carbon sources grew disparately. On the

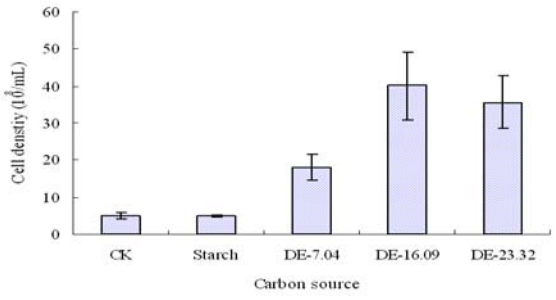
*To whom all correspondence should be addressed.

whole, the medium with high reducing sugar benefited *B. subtilis* ZJF-1A5 growth. Therefore, the greatest density of *B. subtilis* ZJF-1A5 was the one taking dextrin with DE 16.09 and DE 23.32 as carbon source.

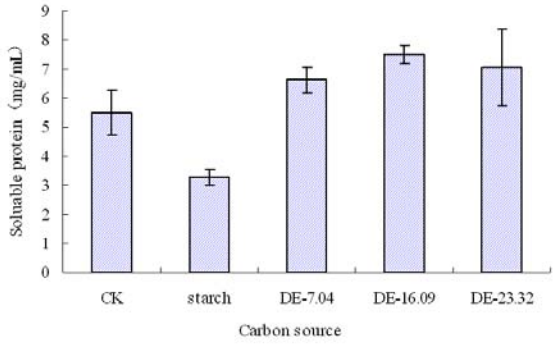
Fig.1 (b) showed that different carbon sources produced a slight effect on soluble protein content by *B. subtilis* ZJF-1A5. Among these when carbon source was starch, soluble protein content was the lowest. While yield of soluble protein was nearly equal and slightly higher than reference averagely when three kinds of dextrin were taken as carbon source.

Effect of different carbon source in the culture on the protease and amylase activity produced by B. subtilis ZJF-1A5

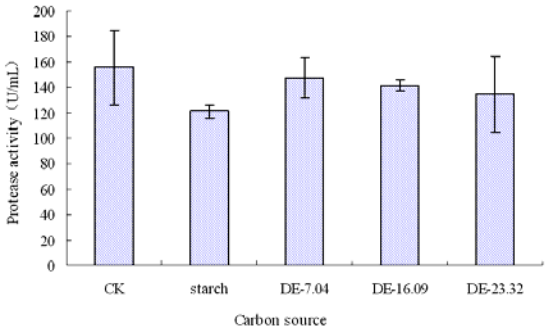
Fig.1 (c) showed that different carbon sources had slight influence on proteolytic enzyme activity by *B. subtilis* ZJF-1A5, but proteolytic enzyme activity by starch as carbon source was lowest in general. However, different carbon sources had significant impact on α-amylase activity by *B. subtilis* ZJF-1A5. The greatest α-amylase activity was the one by dextrin with 16.09 DE value as carbon source (Fig.1 d).



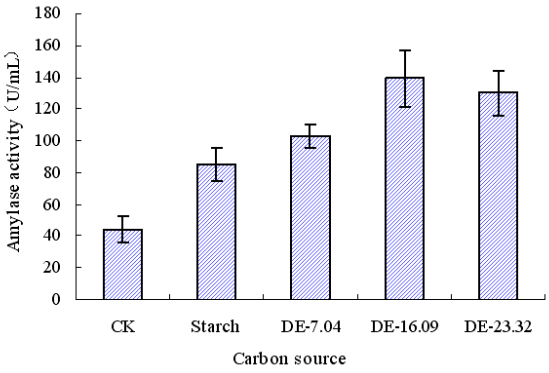
(a)



(b)



(c)



(d)

Fig. 1: a, Density of *B.subtilis* ZJF-1A5; b, The content of soluble protein; c, Protease activity; d, α-amylase activity.

Effect of different carbon source in the culture on the isozymogram produced by B. subtilis ZJF-1A5

According to the previous research, we studied α-amylase produced by *B. subtilis* ZJF-1A5 concludes a neutral amylase with lower activity and an acidic amylase with higher activity. In Fig.2, the neutral amylase became strong noticeably when starch and other three dextrins were taken as carbon source. In addition, when dextrin was carbon source the activity of acidic amylase was stronger than the amylase activity by using starch as carbon source.



Fig. 2: Effect of different carbon source in the culture on the iso-zymogram of α-amylase secreted by *B. subtilis* ZJF-1A5.

Determination of the increased yield of α-amylase produced by dextrin via the renaturation technology of SDS-PAGE gel (elimination of dependency of allosteric effect)

Fig. 3 showed that the α-amylase activity was increased apparently when dextrin was carbon source on the condition of renaturation after SDS denaturation, and the α-amylase activity by dextrin with higher DE was higher than by dextrin with lower DE. Due to SDS denaturation and the same renaturation, enzyme activity on glues tape could eliminate original conformation differences to a certain extent. Therefore, enzyme activity merely depended on enzyme content.



Fig. 3: Determination of α-amylase produced by *B. subtilis* ZJF-1A5 by SDS-PAGE

Proposition and detection of inducing effect and growing balance hypothesis

In Fig. 4 at lower DE value enzyme activity produced by the mixture of starch and glucose as carbon source was nearly equal to dextrin with same DE value, but when DE value was higher the former activity was lower than the later significantly. Besides that, when glucose ratio went up the activity by *B. subtilis* ZJF-1A5 lowered apparently.

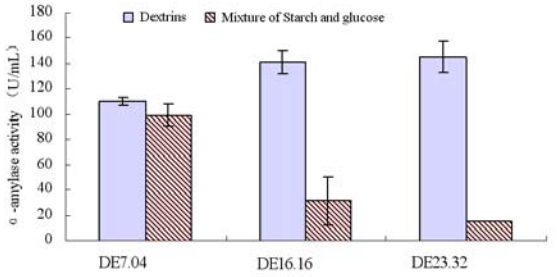


Fig. 4: Production of α-amylase in *B. subtilis* ZJF-1A5 culture mediated by dextrins or starch- glucose-mixture with the same DE values to the tested dextrins.

Determination of reducing active component in dextrin

As Fig. 5 showed, it had induction on α-amylase production of *B. subtilis* ZJF-1A5 by adding different malto-oligosaccharides. And the more the degree of polymerization increased, the clearer induction was. Induction of α-amylase rose to the top when it came to maltopentaose, then induction was on a modest decline while the degree of polymerization increased.



Fig. 5: lane 1.Control; lane 2, maltose; lane 3, maltotriose; lane 4, maltotetraose; lane 5, maltopentaose; lane 6, maltohexaose; lane 7, maltoheptaose. Lane8, dextrins (DE16.16).

In this study, protease activity produced by *B. subtilis* ZJF-1A5 cultivated with different carbon sources has great difference. The samples with higher DE dextrin produced higher soluble protein content. To some extent, that maybe cause to higher activity of α- amylase.

Generally speaking, Protease activity is the negative regulatory factors to the alteration of enzyme activity. Howerer, in our study, the difference of protease activity produced by different carbon sources is not significant, and the protease activity by the dextrins which produce higher amylase activity is higher than the control samples which have lower amylase activity. Thereby protease was not the reason of increase of amylase activity.

By renaturation after denaturation, it is found that the α-amylase activity with higher DE dextrin as carbon source was still higher than that of lower DE dextrin. These results illustrated that the quantity of α-amylase produced by higher DE dextrin was indeed more than that by other carbon sources. Because of different carbon sources cultivating, the density of *B. subtilis* ZJF-1A5 and the total quantity were different, but it didn't mean that the producing efficiency of α-amylase by *B. subtilis* ZJF-1A5 could be increased, that is, there exist in some inductive substance to improve α-amylase by *B. subtilis* ZJF-1A5. Our results stated clearly that the medium with higher content of reducing sugar was benefit for

the growth of *B. subtilis* ZJF-1A5 and the density was maximum when used dextrin DE 16.09 and DE 23.32 as carbon sources. For this reason, the reducing sugar had some effect to high yield of α-amylase by *B. subtilis* ZJF-1A5 when dextrin DE 16.09 and DE 23.32 were used as carbon sources. Because the growth of *B.subtilis* ZJF-1A5 was lower when starch was taken as carbon source while its α-amylase activity was apparently higher than the control. Therefor, it was verdicted that starch had certain reducing effect to α-amylase. According to the judgement above, we proposed hypothesis that malto-glycan with high polymerization had reducing effect, maltose with strong reducibility had growth-promoting effect. when in a certain amount of dextrin DE value reached a point (12 ~ 25), inducing effect and growth get to balance and the α-amylase activity was the highest. Later study by blending glucose and starch and making the DE value of this mixture equal with that of dextrin, we took this mixture as carbon source to ferment, and the result showed that when DE value is lower, amylase activity by this mixture as carbon source was mostly equal to that of dextrin with same DE value, but if the DE value was higher, amylase activity by the mixture was notabley lower than that of dextrin with same DE value. The above findings negated our hypothesis. In addition, the fact indicated that dextrin included some special constituents which were able to induce *B. subtilis* ZJF-1A5 to produce α-amylase greatly. Furthermore, these findings also demonstrated that during the growth of *B. subtilis* ZJF-1A5 there was an effect of repression of glucose metabolism [10], which was common for other *Bacillus* strains and the yield of α-amylase decreased because of repression.

Welker et al [11] had proposed that maltodextrin could induce *Bacillus stearothermophilus* to produce α-amylase, and in their studies they found that the combining rate of α-amylase increased 1.2, 1.6, 3.0, 2.3 and 1.9 times respectively along with taking maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose as carbon sources. While among these carbon sources maltotetraose had the strongest induction. In addition, the content of malto-oligose would increase gradually along with promotion of the value of dextrin. For this reason, we could deduce that the increasing yield of α-amylase by *B. subtilis* ZJF-1A5 via induction of dextrin was probably due to the induction of oligose involved in dextrin. While if the DE value of dextrin was too high, the increasing content of monose went against the production of α-amylase even oligose content in dextrin with DE 16.09 and DE 23.32 for test was indeed higher than that with DE 7.04. Therefore, it didn't mean induction was stronger when the DE value was higher. By cultivating basic medium and adding malto-oligose with different polymerization the result demonstrated that the yield of α-amylase by *B. subtilis* ZJF-1A5 was creased when the addition went from maltose to maltoheptaose. This verified that during the induction of *B. subtilis* ZJF-1A5 to produce α-amylase malto-oligose played an important role on the increasing yield of α-amylase and maltopentaose had the strongest induction. From the above findings, it could be concluded the main inducing component in dextrin probably was malto-oligose and maltopentaose had the strongest induction.

Experimental

Preparation of Dextrin

Corn starch (50 g) was mixed with 150 mL of distilled water and stirred for 10 min. This solution was mixed with 0.1 mol / L NaOH to pH 6.0 and 0.1 % (w/v) $CaCl_2$, respectively. Then, common neutral α-amylase and thermostable α-amylase were added into the above solution to hydrolysis starch. After enzyme denaturing by high pressure (Shanghai Tocan Science and Technology Co. Ltd) and cooling, the DE-value of the product was determined. The dextrins with different DE value were obtained under the following conditions: reaction temperature, 90 °C; hydrolysis time, 3, 15, 18 min (obtained dextrins with DE 7.04, 16.09, 23.32, respectively); addition of common neutral α-amylase, 4 U/g starch; and addition of thermostable α-amylase, 7 U /g starch. Obtained products were centrifuged, freeze-dried and used to evaluate for characteristics.

Organism and Cultivating Condition

B. subtilis ZJF-1A5 was obtained from School of Food Science, Henan Institute of Science and Technology:

- Plate solid medium: Yeast extract 0.5%, peptone 1 %, 0.5% NaCl, 2% agar, pH natural.
- Seed culture medium: Yeast extract 0.5%, peptone 1%, NaCl 0.5%, pH natural;
- Fermentation shake medium: soybean cake powder 5.5%, $Na_2HPO_4 \cdot 12H_2O$ 0.8%, $(NH_4)_2SO_4$ 0.4%, $CaCl_2$ 0.2%, then respectively add different carbon sources (starch 5.5%; DE 7.04 dextrin 5.5%; DE 16.09 dextrin 5.5%; DE 23.32 dextrin 5.5%) and fermented. CK didn't add any carbon source.

Production of Crude enzyme cultivated via different carbon sources

B. subtilis ZJF-1A5 cultivated via different carbon sources fermented 48 hours by shake

cultivation, centrifuged thallus and undissolved substances at speed of 10,000rpm, and then preserved as crude enzyme at 4°C.

Determination of B. subtilis ZJF-1A5 growth under culture conditions of different carbon sources

We determined CFU (cell density) of *B. subtilis* ZJF-1A5 under culture conditions of different carbon sources by plate-dilution colony counting method. Beef extract and peptone medium(NM) ratio: peptone 10g, beef extract 3g, NaCl 5g, agar 15 g, distilled water 1 000 mL, pH 7.2 ~ 7.4. After serial dilution sample was coated on NM medium, cultivated upside down at 36°C and counted bacterial colonies after 24h.

Content determination of soluble protein secreted from B. subtilis ZJF-1A5 under culture conditions of different carbon sources.

Brad-ford method [12] and Coomassie brilliant blue G-250 staining method were used to determine the soluble protein content of crude enzyme cultivated via different carbon sources.

Determination of proteinase and amylase activity produced by B. subtilis ZJF-1A5 under culture conditions of different carbon sources

Determination of proteinase activity was carried out on the basis of taking casein as substrate and the change in concentration of protein solution was determined through Coomassie brilliant blue G-250 reagent [13].

Zymogram determination of isoenzyme of enzyme produced by B. subtilis ZJF-1A5 under culture conditions of different carbon sources

Discontinuous polyacrylamide gel electrophoresis (PAGE) was used to determinate crude enzyme of α-amylase secreted by ZJF-1A5 for analysis of isoenzyme. The above samples were going to accept electrophoresis after being concocted to be equal protein concentration.

Determination of α-amylase activity by renaturation technology of SDS-PAGE variance gel

A-amylase, secreted from ZJF-1A5, dealt with electrophoresis with discontinuous polyacrylamide variance gel containing SDS. Production of PAGE gel stated in Table-1. Production of PAGE gel stated as follows. Added nothing as substrate starch in gel.

Table-1: Composition of separating, spacer and stacking gels.

Composition	Stacking gels	Separation gel
30%gel storing liquid (mL)	0.5	3.6
Separation gel buffer solution (mL)	/	2.5
Stacking gel buffer solution (mL)	0.7	/
Double distilled water (mL)	1.8	4
10%SDS (μL)	30	100
TEMED (μL)	15	15
10% ammonium persulfate (μL)	30	25

After electrophoresis, steeped PAGE gel in 2M of Tris for renaturation at 60min, next used water to wash away remnant Tris, then steeped in starch liquor with 2mmol/L $CaCl_2$ taking 1% for reaction at 20min, last placed it in I_2-KI staining fluid for color development and watched the change in 10min.

Condition of α-amylase activity by B. subtilis ZJF-1A5 mediated from mixture of starch and glucose containing equal DE with dextrin

By mixing starch and glucose in varying proportions and the sugar contents in mixture was 7.04%, 16.09% and 23.32%, respectively. These mixtures were used as carbon sources to produce enzyme according to method mentioned above, then compared these amylase activity with that produced from fermentation liquid in which dextrin was used as carbon source and the DE value of dextrin was 7.04, 16.09 and 23.32.

Determination of inducing active component in dextrin

Chose minimal medium, took glucose as carbon source and cultivate 24h, then poured medium into culture bottles, added 5.5% maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and DE value 16.09 of dextrin respectively and continued cultivating 8h. After that we used renaturation technology of SDS-PAGE gel to determine corresponding α-amylase activity.

Conclusion

Dextrins with certain DE value could induce *B. subtilis* ZJF-1A5 to increase the yield of α-amylase. The reasons of high yield of α-amylase includes the following points: 1) the content of outwards-secreting protein of strain goes up, 2) density of strain increases, 3) one kind of isoenzyme is induced to produce, 4) dextrin itself can induce *B. subtilis* ZJF-1A5 to produce α-amylase greatly. The result showed that the main inducing component in dextrin probably was malto-oligose and maltopentaose had the strongest induction.

Acknowledgements

This study was funded by Natural Science Foundation of China (31171641/C200101) and Science and Technology Projects in Henan Province (102102210192).

References

1. S. R. Park, S. D. Ha and J. H. Yoon. *Food Control*, **20**, 946 (2009).
2. I. C. C. Nóbrega, J. A. P. Pereira and J. E. Paiva. *Food Chemistry*, **117**, 693 (2009).
3. M. Asgher, M. J. Asad, S. U.Rahman and R. L. Legge. *Journal of Food Engineering,* **79**, 950 (2007).
4. T. J. G. Salva and I. O. Moraes. *Revista Argentina de Microbilogia*, **26**, 46 (1995).
5. M. J. Syu and Y. H.Chen. *Chemical Engineering Journal*, **65**, 237 (1997).
6. P. Hiller, D. A. J. Wase and A. N. Emery. *Biotechnology letters*, **8**, 795 (1996).
7. K. R. Babu, T. Satyanarayana. *Folia Microbiologica*, **38**, 77 (1993).
8. V. Ivanova, E. Dobreva and M. D. Legoy. *Acta Biotechnology*, **18**, 339 (1998).
9. A. Linden, O. Mayans, W. Meyer Klaucke, G. Antranikian and M. Wilmanns. *Journal of Biological Chemistry*, **278**, 9875 (2003).
10. X. Liu, H. W. Taber. *Journal of Bacteriology*, **180**, 6154 (1998).
11. N. E. Welker and L. L. Campbell. *Journal of Bacteriology*, **86**, 687 (1963).
12. M. M. Bradford. *Analitical Biochemistry*, **72**, 248 (1976).
13. H. Zhou, Z. B. Lu, J.Qi and H. Li. *Journal of Biological Chemistry*, **5**, 630 (1994).