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Optimization of culture medium for the cultivation of *Actinoplanes* sp. mutant strains and purification of acarbose

Tối ưu hóa điều kiện nuôi cấy và tinh sạch acarbose từ các thể đột biến Actinoplanes sp.

Research article

Nguyen The Dương, Le Thanh Hoang, Do Thi Tuyen*

Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Hanoi, Vietnam

In order to improve the production of acarbose, the fermentation medium of acarbose-producing strain Actinoplanes sp. KCTC 9161 – L14 mutant was optimized in this internship. Fractional factorial design was employ to investigate the influences of glucose, maltose and corn power on acarbose production (by α-glucosidase inhibitory ability). Two significant factors: glucose and maltose have significant and positive effects on acarbose amount. In addition, a model was obtained from the regression results of fractional factorial experiment. Other success, we demonstrated that chromatography by active charcoal column can used to purify acarbose from fermentation broth. Acarbose amount in purification solution was 191.5 g/L and an acarbose - purification process was inducted.

Nhằm mục đích nâng cao khả năng sinh tổng hợp hoạt chất acarbose từ chủng đột biến Actinoplanes *sp. KCTC 9161-L14, môi trường lên men của chủng dùng để sản xuất acarbose đã được tối ưu hóa. Một phần mềm thiết kế đã được thiết lập để khảo sát ảnh hưởng của glucose, maltose và bột ngô đến khả năng sản xuất acarbose (thông qua hoạt tính ức chế α-glucosidase). Kết quả đã cho thấy, hai yếu tố glucose và maltose có ý nghĩa quan trọng và ảnh hưởng trực tiếp đến khả năng sinh tổng hợp acarbose. Một phương trình đã được hình thành từ kết quả tối ưu. Bên cạnh đó, chúng tôi đã chứng minh được cột sắc ký sử dụng than hoạt tính có thể tinh sạch acarbose từ dịch lên men. Hàm lượng acarbose trong dung dịch tinh sạch đạt 191,5 g/l và một quy trình tinh sạch acarbose được đề xuất.*

Keywords: Acarbose, active charcoal, *Actinoplanes sp.,* α-glucosidase inhibitory, purification

1. Introduction

Acarbose is an α-glucosidase inhibitor (Derosa et al., 2011) . It was used as a novel therapeutic agent for treating hyperglycemia in non-insulin-dependent diabetes mellitus (NIDDM) (Inoue et al., 1997). The action of acarbose depend on its structure: a compound of acarviosyl and maltose. Acarviosyl is core, which is involved valienamine and 4-amino-4,6dideoxyglucose by N-glycosidic bond. Therefore, acarbose is seem to be like a pseudo- oligosaccharide. It acts causing a competitive with oligosaccharides, delays the digestion of starch and is used to reduce postprandial plasma glucose and insulin levels in diabetic patients (Baron et al., 1987). Acarbose has high biological activity, inhibitor of intestinal α-glucosidase with IC50 = 11 nM (Chemicalbook, 2010). Caspary and Graf (1979) demonstrated that acarbose has higher affinity – 15,000 fold with intestinal sucrase compared to sucrose and the acarbose binding resulted in a very stable enzyme/substrate complex. Acarbose is only minimally absorbed from the gastrointestinal tract (<2%), and its action is extended throughout the small intestine; then metabolized by colonic bacteria enzymes (Lebovitz, 1998).

Acarbose was the first identified in the fermentation broth of *Actinoplanes* sp. SE50 that was isolated in the form of coffee soil in Kenya last 1960s (Schmidt et al., 1977). The production of acarbose was depend on various sources: maltose (Choi and Shin, 2003), extracts of corn (Wei et al., 2010), monosodium glutamate (Wang et al., 2011). *Actinoplanes* sp. SE50/110 (*Actinoplanes* sp. KCTC 9161) is

* Corresponding author
E-mail: dttuyen@ibt.ac.vn

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other one that can produced 1g/l of acarbose and was used widely for all research after about acarbose synthesis gene (Schwientek, 2012; Klein et al., 2013). Acb-gene cluster is the acarbose production cluster in *Actinoplanes* sp. SE50/110. It consist of 41323 bp, was named Y18523.4 in GenBank (Hemker et al., 2001; Schwientek et al., 2012). The twenty five known acb genes from the producer strain *Actinoplanes* sp. SE50/110 encode various functions, including biosynthesis of a 6-deoxyhexose (6DOH) and a C7Ncyclitol (valienol), intra and extracellular anabolism and catabolism of alpha-glycosides, and uptake and export of (oligo)-saccharides (Rockser and Wehmeier, 2009).

2. Materials and methods

2.1 Materials

Actinoplanes sp. KCTC 9161 strain was purchased from Korea.

Actinoplanes sp. KCTC 9161 – L14 mutant strain was supplied by the Department of Enzyme Biotechnology at the Institute of Biotechnology, Vietnam Academy of Science and Technology.

2.2 Chemicals

Acarbose standard and N-methyl-N'-nitro-N-nirosoguanidine (NTG) was purchased from Sigma Chemical Co (USA); *p*-nitrophenyl-α-D-glucopyranoside, α-glucosidase, peptone (Biobasic INC, Canada). Charcoal, n-butanol, ethanol, methanol, ethyl acetat, acid fomic, $CaCl_2$, $CaCO_3$, KCl, $FeSO_4.7H_2O$, K_2HPO_4, $KH_2PO_4.3H_2O$, $MgSO_4$ were purchased from AR Co, China.

2.3 Method

2.3.1. Activation of cultured *Actinoplanes* sp. KCTC 9161

Actinoplanes sp. KCTC 9161-L14 from the tubes was maintained on agar slant containing (g/L): glucose 20; peptone 5; KCl 0.5; K_2HPO_4 1.0; $MgSO_4.7H_2O$ 0.5; and agar, 2.0; pH 7 (Wei et al., 2010).

2.3.2. Inoculum *Actinoplanes* sp. KCTC 9161-L14

Actinoplanes sp. KCTC 9161-L14 was culture sinking after the active of propagation medium, at 28°C, 200 rpmin 120 hours in an incubator shaker. The inoculum medium (CPC) was composed (g/L): sucrose 30; peptone 2; KCl 0.5; $MgSO_4.7H_2O$ 0.5; $FeSO_4.7H_2O$ 0.1; $KH_2PO_4.3H_2O$ 1.0; casein hydroxylsate 1.0.

2.3.3. Fermentation *Actinoplanes* sp. KCTC 9161-L14

The MT1 medium (Nguyen TN, *et al.,*2013) - fermentation medium was added 5% of seed varieties of *Actinoplanes* sp. KCTC 9161-L14, cultured sinking at 28°C, 200 rpm in 168 hours shaking. The MT1 medium (g/L): maltose 50; glucose 30; core powder 15; $CaCl_2$ 2.0; $CaCO_3$ 2.5; monosodium glutamate 1.0; $KH_2PO_4.3H_2O$ 1.0. After 168 hours, broth solution was collected and checked by TLC with acarbose standard and measured active enzyme α-glucosidase inhibitors.

2.3.4. Thin layer chromatography (TLC)

The fermentation broth was centrifuged at 12000 rpm, 4ºC for 10 minutes. The supernatant of solution was cleaned by ethanol (1 sample: 4 ethanol (v/v)) in 30 minutes. Centrifugation again at 12000 rpm, 4^0C for 10 minutes and the supernatant after was dropped on TLC silica gel.
Thin-layer chromatography was performed on a Merck silica gel 60 F254, 0.25 mm thick with a solvent system 94% A and 6% B (A contain ethyl acetate: methanol =1:1 and B contain H_2O : acid formic = 5:2), then by the color burst slightly acid (10% H_2SO_4 in ethanol) at 121°C in 15 minutes.

2.3.5. Determination of inhibitory activity of α-glucosidase

p- nitrophenyl α-glucopyranoside (pNP-glucose) was used as the substrate to assay α-glucosidase activity during expression and purification experiments. pNP- glucose is hydrolyzed by α-glucosidase to yield glucose and a p-nitrophenol group. Under basic conditions, the p-nitrophenolate group shows absorption at 405 nm (yellow). After being centrifuged at 12500 rpm at 4°C for 15 min, a 10 µl supernatant of broth sample is was mixed with 40 µl buffer of 0.1 M phosphate pH 6.9 and 100 µl of α-glucosidase 1 µ/ml solution in experienced wells. In the controlled wells, the 10 µl of broth sample was replaced with 10 µl of buffer. This mixture was incubated at 25°C for 10 minutes. Then, 50 µl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside was added and incubated for 5 minutes. OD measures were performed before and after incubations. Ability of inhibition of α-glucosidase (%) was difined as

$$\% \text{ inhibition} = \frac{\Delta A_C - \Delta A_s}{\Delta A_C} \times 100$$

ΔA_C is the changing of OD values of controlled sample before and after incubation

ΔA_s is the changing of OD values of experienced sample before and after incubation

2.3.6 Purification acarbose by charcoal

Based on the absorption capacity of the activated carbon with pigments or various sugars, the weaker adsorbent will be removed with the lower solvent concentration and reverse the strong adsorbent will be ejected with the solvent concentration is higher.

Procedure: 2.5 g charcoal was boiling for 1 hour before stuffing column to expel all the air bubbles contained in the carbon particles, 50 ml of fermentation was centrifuged at 4000 rpm for 20 minutes to remove the biomass cell, then adsorbed on the activated carbon in terms of pH 2-3, a slight stirring for 1 hour and keeping at 4 ° C for 12 hours. The mixture was washed with 200 ml water 3 to 5 times. Translate coal containing acarbose was stuffed on a column and eluting with ethanol along concentration gradient from 0% up to 20%. The segment tested by TLC to check the purity acarbose.

2.3.7 Experimental design and statistical analysis

Fractional factorial design was firstly used to identify the medium components that significantly influence acarbose production by *Actinoplanes* sp. KCTC 9161-L14. The results of the fractional factorial design were regressed to obtain a first order polynomial, and the significance of the regression coefficients was checked by Student t-test. Based on the above statistical analysis of fractional factorial design, the significant factors used steepest ascent path to reach the yield plateau. To efficiently explore the best condition obtained by the steepest method, the central composite design was performed, and the optimal concentrations of medium components for the acarbose production by *Actinoplanes* sp. KCTC 9161-L14 were determined by response surface methodology.

All experimental designs and statistical analyses were carried out by using the software Design – Expert 7.

2.3.8 Quantification of acarbose in the broth

Acarbose concentration in fermentation broth was determined by HPLC. Broth sample (1.0 ml) was extracted for 30 min with 4.0 ml of ethanol. The mixture was then filtrated, and the resulting upper aqueous phase was injected into HPLC system.

Acarbose concentration in experimental design was determined by LC/MS 1100 Agilent, Ion sources ESI, column ODS C18, 3.0 x 150 mm, 3.5 µm, mobile phase MeOH : H_2O (80:20, v/v).

3. Results

3.1. Fractional factorial design

Based on the first success results obtained under mono-factor experiments (MT1 medium for *Actinoplanes* sp. KCTC 9161), the following three components of the fermentation medium were optimized for enhancing acarbose production by *Actinoplanes* sp. KCTC 9161-L14 mutant strain. Factor A: glucose, factor B: maltose and factor C: corn power. In the first optimization step, three components above were evaluated by using fractional factorial design, each factors were named with three levels (-1, 0, +1), as show in Table 1. Based on the experimental design by software Design Expert 7, a total number of 20 runs were required in the fractional factorial design (Table 2).

From the results of regression analysis, a model for ability α- glucosidase inhibitor of acarbose could be:

$$AGI\ (\%) = 90.31 + 5.55*A + 2.99*B - 0.92*C - 2.57*A*B + 2.70*B^2 - 3.93*C^2 \quad (1)$$

In equation 1, the factors A, B and C were glucose, maltose and corn power, respectively. The statistical significance of the model was checked by the Student t-test, with Alpha 0.1, the model was high significant (P=0.0013) and R^2 = 0.775.

In equation 1, coefficients of maltose and glucose were significant and positive, it means that increasing the concentrations of maltose and glucose would have positive effects on α- glucosidase inhibitor (AGI) (Fig. 1). Therefore, in order to improve AGI or acarbose yield, the concentrations of maltose and glucose should be increased.

Table 1. Levels and actual values of factors for fractional factorial design

Factors	Levels		
	-1	0	1
A: Glucose (g/l)	20	30	40
B: Maltose (g/l)	10	30	50
C: Corn power (g/l)	10	20	30

Table 2. Experimental design and results of fractional factional design

Run	Factor 1 A: Glucose (g/l)	Factor 2 B: Maltose (g/l)	Factor 3 C: Corn (g/l)	Response 1 AGI %
1	20.00	50.00	30.00	93.65
2	40.00	10.00	30.00	90.48
3	30.00	30.00	20.00	94.44
4	43.16	30.00	20.00	96.03
5	30.00	30.00	20.00	88.89
6	30.00	30.00	20.00	92.06
7	40.00	50.00	10.00	96.03
8	30.00	3.68	20.00	91.27
9	30.00	56.32	20.00	96.83
10	20.00	10.00	30.00	76.98
11	30.00	30.00	20.00	87.30
12	16.84	30.00	20.00	75.40
13	20.00	10.00	10.00	81
14	30.00	30.00	20.00	92.06

Run	Factor 1 A: Glucose (g/l)	Factor 2 B: Maltose (g/l)	Factor 3 C: Corn (g/l)	Response 1 AGI %
15	40.00	10.00	10.00	96.03
16	30.00	30.00	20.00	96.83
17	30.00	30.00	33.16	81
18	40.00	50.00	30.00	93.65
19	20.00	50.00	10.00	88.10
20	30.00	30.00	6.84	84.13

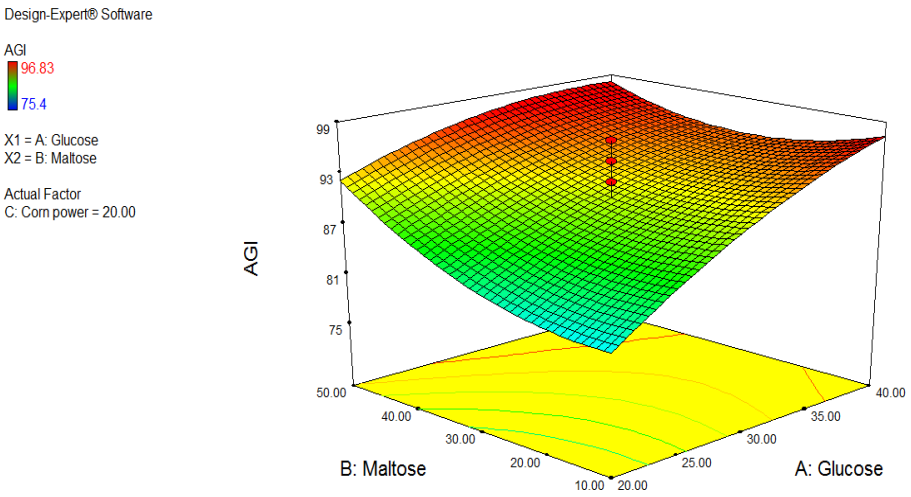

Figure 1. The three-dimensional presentation of the response surface for the concentrations of maltose and glucose on AGI

3.2. Purification of acarbose through active carbon column

Fermentation solutions of *Actinoplanes* sp. KCTC 9161 - L14 mutant were absorbed into active carbon first time. Then, de-absorption by ethanol with concentration gradient from 0 to 20% to harvest acarbose. TLC result (Fig.2) showed that after wash the column by ethanol with different ethanol concentration, contaminants having smear acarbose band and many bands lower than acarbose standard band were collected. After purification through active carbon column second time, in the first several fragments, contaminants were bands higher than acarbose standard band (lane 1 to 6), after that 2 bands including acarbose band with contaminants (lane 7 to 9), finally acarbose substance having one band correlate with standard band was collected (Fig. 3).

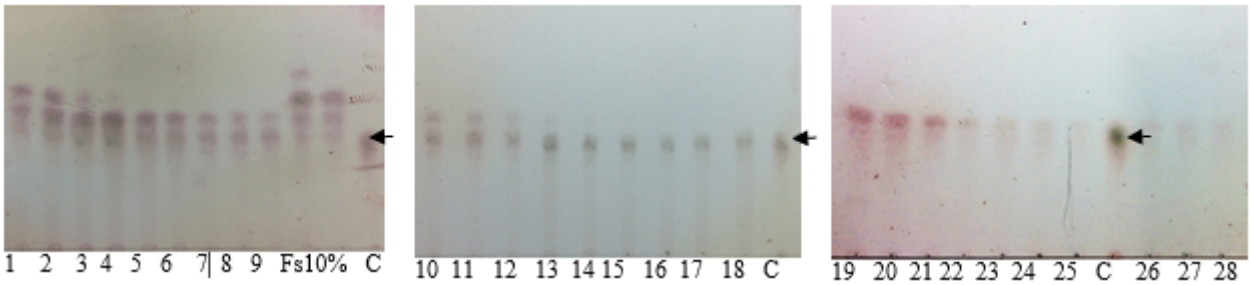

Figure 2. Chromatogram TLC fragments of acarbose purification from fermented solution of *Actinoplanes* sp. KCTC 9161-L14 through active carbon column first time (Fs: Fermented solution; 1-28: fragments; C: standard acarbose)

Thus, after using active carbon column twice, we were preliminary purified acarbose substance from fermentation solution of *Actinoplanes* sp. KCTC 9161 strain. Acarbose amount in fermentation and purification solutions have been determined by HPLC-mass spectrometry system. Chromatograph result (Fig. 4- 5) showed that in all three samples: standard acarbose, fermentation and acarbose purification all have one peak at persistent time point 0.666-minute, molecular weigh 645.6 was acarbose substance. Acarbose amount in fermentation solution was 10.48 g/L in purification solution was 191.5 g/L, higher than 18.3 times compare with fermentation solution.

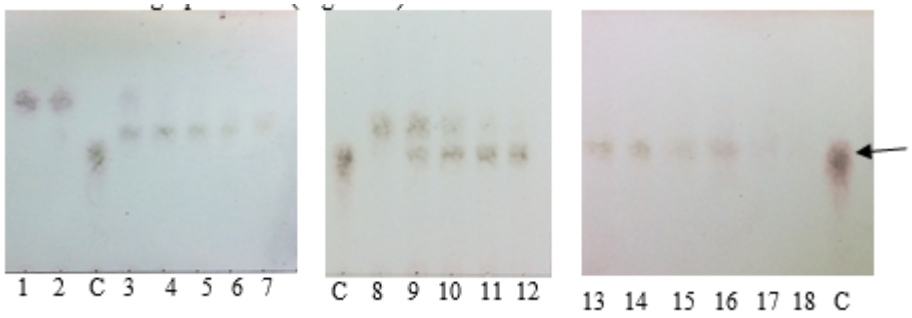

Figure 3. Chromatogram TLC fragments of acarbose purification through active carbon column second time (1-18: fragments; C: standard acarbose)

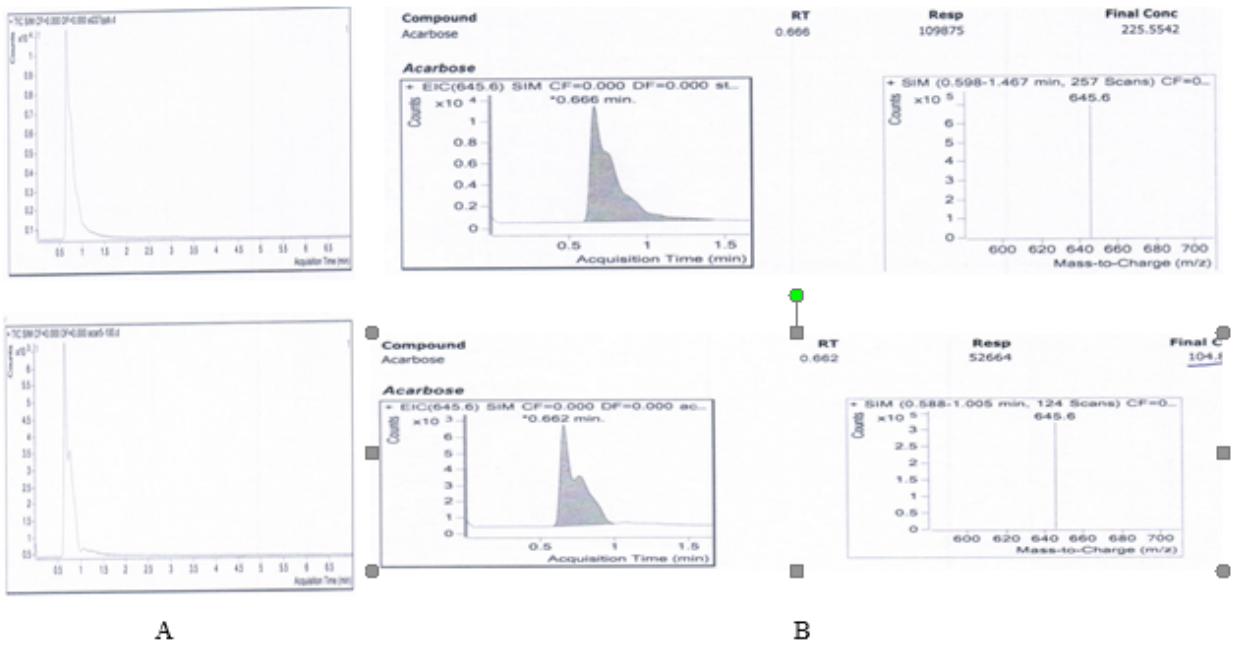

Figure 4. HPLC mass spectrometry chromatograph of acarbose substance from fermentation solution *Actinoplanes* sp. KCTC 9161-L14 mutant

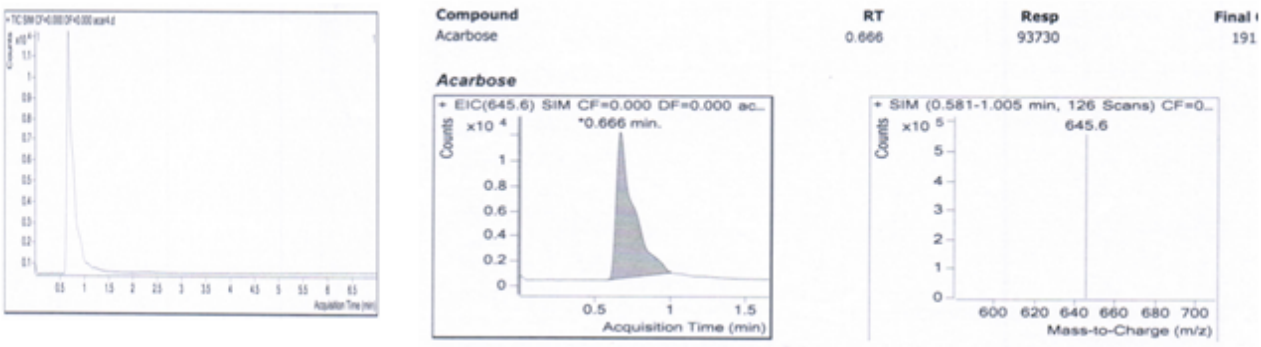

Figure 5. HPLC mass spectrometry chromatograph of purified acarbose substance from fermentation solution *Actinoplanes* sp. KCTC 9161-L14 mutant

3.3. Purification, concentration of acarbose powder product

Fragments, after TLC check having only one acarbose band correlate standard band, were cleaned by 100% ethanol, then air vacuum until gone out of solution and water, acarbose was collected under white pounder type.

From the obtained result, we propose process for fermentation and production of acarbose from *Actinoplanes* sp. KCTC 9161-L14 mutant: *Strain activation→ Culture for reproduction→ Fermentation cultivation→ Fermentation solution→ Active carbon column chromatography 1 (absorption range by ethanol from 5-20%)→ Active carbon column chromatography 2 (absorption range by ethanol from 5-15%)→ Purification acarbose solution (collect*

fragments TLC, obtained only one band correlate with standard) → Crystallize for product → Acarbose product.

4. Discussion

4.1. The adsorbent of acarbose on active carbon

In this study, we did experiment active charcoal for purification, based on structural characteristics, surfactants, absorption and binding affinity between maltose, glucose, acarbose (acarbose is a pseudo-oligosaccharide) and active charcoal can purify some sugars like fructooligosaccharides (Kuhn and Filho, 2010) and monosaccharides, disaccharides. The adsorption behavior of each sugar to active charcoal depends on the micropores diameter distribution of the pore channel. The strength of the interaction between the sugar and active charcoal increases with increasing molecular weight and size of the adsorption molecules. The bigger molecular will be difficult to pass through the channel, and be kept inside the matrix, while small molecular pass through. Therefore, it is clearly that oligosaccharides adsorb more than other small saccharides (e.g. glucose or maltose). In addition, the major portion of the carbon surface of the charcoal is nonpolar (or hydrophobic). Therefore, oligosaccharides that have much – CH group more adsorbed to the active charcoal than other small saccharides. It is the reason for the retaining of acarbose on charcoal column after washing step by step, and total eluted with high concentration of ethanol (20%).

4.2. The effect of glucose and maltose on acarbose production

The results showed that maltose and glucose had significant and positive effects on acarbose production by *Actinoplanes* sp. KCTC 9161-L14 mutant strain. Due to maltose directly incorporated into acarbose (Lee et al., 1997), the level of maltose in culture broth probably plays an important role in acarbose production. Choi and Shin (2003) investigated the effect of maltose on acarbose production in *Actinoplanes* sp. CKD485-16, and the results revealed that maltose concentrations should be maintained at high levels during cultivation to obtain high acarbose yields.
Corn power is an excellent source of nitrogen for most microorganisms (Shah and Cheryan, 1995; Silveira et al., 2001). Furthermore, corn power has been widely and successfully used as a low-cost medium for a variety of fermentations, e.g., the production of solvents, antibiotics, and enzymes (De Azeredo et al., 2006). Our results revealed that corn steep liquor was another significant and negative factor for acarbose production. Therefore, corn steep liquor can be used as the inexpensive nitrogen source but limited for production of acarbose by *Actinoplanes* sp. KCTC 9161-L14mutant.

5. Conclusions

Maltose and glucose have positive effect on the production of acarbose by *Actinoplanes* sp. KCTC 9161-L14 mutant strain. Continue research to find the concentration of these in the fermentation medium to have highest acarbose amount is necessary.

Acarbose substance purification by chromatography through active carbon column, acarbose amount in purification solution was 191.5 g/L. Successfully crystallization and production of purified acarbose product in order to attend trial target.

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7. References

[1] Baron, A.D., Eckel, R.H., Schmeiser, L., and Kolterman, O.G. (1987). The effect of short-term a-glucosidase inhibition on carbohydrate and lipid metabolism in type II (noninsulin-de- pendent) diabetics. Metabolism 36, 409-415.

[2] Choi, B.T., Shin, C.S. (2003). Reduced formation of byproduct component C in acarbose fermentation by *Actinoplanes* sp. CKD485-16. Biotechnol Prog 19(6), 1677-1682.

[3] De Azeredo, L.A.I., De, L.M.B.D., Coelho, R.R.R., and Freire, D.M.G. (2006). A Low- Cost Fermentation Medium for Thermophilic Protease Production by *Streptomyces* sp 594 Using Feather Meal and Corn Steep Liquor. Curr Microbiol 53 335-339.

[4] Derosa, G., Maffioli, P., Ferrari, I., Fogari, E., D'Angelo, A., Palumbo, I., Randazzo, S., Bianchi, L., and Cicero, A.F.G. (2011). Acarbose actions on insulin resistance and inflammatory Parameters during an oral fat load. European Journal of Pharmacology 651, 240-250.

[5] Hemker, M., Stratmann, A., Goeke, K., Schroder, W., Lenz, J., Piepersberg, W., and Pape, H. (2001). Identification, cloning, expression, and characterization of the extracellular acarbosemodifying glycosyltransferase, AcbD, from *Actinoplanes* sp. strain SE50. J.Bacteriol 183, 4484-4492.

[6] Inoue, I., Takahashi, K., Noji, S., Awata, T., Negishi, K., and Katayama, S. (1997). Acarbose controls postprandial hyperproinsulinemia in non-insulin dependent diabetes mellitus Diabetes Research and Clinical Practice 36, 143-151.

[7] Klein, A., Selber, K., Wehlmann, H., Rosen, W., Puhler, A., Schwientek, P., Kalinowski, J., and Wehmeier, U.F. (2013). "New Actinomycete intergrative and conjugative element from *Actinoplanes* sp. SE50/110 as plasmid for genetic transformation of related Actinobacteria". International Publication Number, WO 2013/083566 A083561.

[8] Kuhn, R.C., and Filho, F.M. (2010). Purification of fruc-tooligosaccharides in an activated charcoal fixed bed column. New Biotechnology 27(8), 862- 869.

[9] Lebovitz, H.E. (1998). A-glucosidase inhibitors as agents in the treatment of diabetes. Diabetes Rev 6, 132-145.

[10] Lee, S., Saueribrei, B., Niggemann, J., and Egelkraut, E. (1997). "Biosynthesic studies on the alfa-glucosidase inhibitor acarbose in *Actinoplanes* sp. source of the maltose unit". J Antibiot (Tokyo) 50, 767-778.

[11] Rockser, Y., and Wehmeier, U.F. (2009). The gac-gene cluster for the production of acarbose from *Streptomyces glaucescens* GLA.O: identification, isolation and characterization. J Biotechnol 140(1-2), 114-123.

[12] Schmidt, D.D., Frommer, W., Junge, B., Muller, L., Wingender, W., Truscheit, E., and Schafer, D. (1977). Alpha-glucosidase inhibitors. New complex oligosaccharides of microbial origin. Naturwissenschaften 64(10), 535-536.

[13] Schwientek, P. (2012). Genomics and Transcriptomics of the inductrial Acarbose producer *Actinoplanes* sp. SE50/110. Bielefeld University, Germany, 155.

[14] Schwientek, P., Szczepanowski, R., Ruckert, C., Kalinowski, J., Klein, A., Selber, K., Wehmeier, U.F., Stoye, J., and Puhler, A. (2012). The complete genome sequence of acarbose producer Actinoplanes sp. SE50/110. BMC Genomic 13, 18 pages.

[15] Shah, M.M., and Cheryan, M. (1995). Acetate production by Clostridium thermoaceticum in corn steep liquor media. J. Industrial Microbiol 15, 424-428.

[16] Silveira, M.M., Wisbeck, E., Hoch, I., and Jonas, R. (2001). Production of glucose-fructose oxidoreductase and ethanol by *Zymomonas mobilis* ATCC 29191 in medium containing corn steep liquor as a source of vitamins. Appl Microbiol Biotechnol 55 442-445.

[17] Wang, Y.J., Liu, L.L., Feng, Z.H., Liu, Z.Q., and Zheng, Y.G. (2011). Optimization of media composition and culture conditions for acarbose production by *Actinoplanes utahensis* ZJB-08196. Microbiol Biotechnol 27, 2759-2766.

[18] Wei, S.J., Cheng, X., Huang, L., and Li, K.T. (2010). Medium optimization for acarbose production by *Actinoplanes* sp. A56 using the response surface methodology. African Journal of Biotechnology 9(13), 1949-1954.