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OPTIMIZATION AND PURIFICATION OF α-GLUCOSIDASE INHIBITOR FROM *BACILLUS SUBTILIS* YT20

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Abstract: α-glucosidase inhibitor has been drawing the researcher's interest world-wide because of its safe and convenient mechanism in the treatments of type 2 diabetes. However, as recent therapeutic molecules have side effects, alternative sources have been studied to limit the side effects and provide more options for the treatment of type 2 diabetes. In this study, after selecting microorganism strains capable of biosynthesis of α-glucosidase inhibitor from the collection of *Bacillus subtilis*, we have selected *B. subtilis* YT20 as the host to produce secondary metabolites. After optimization, the extracellular extract inhibited 91.5 % which is 1.34- fold higher than pre-optimization (68.1 %). After purification, the α-glucosidase inhibitory molecule of the *B. subtilis* YT20 has the molecular weight of 163 Da. The recovery efficiency at the final step reached 16.1 %. The combinational spectral data of MS showed that the compound isolated from the *B. subtilis* YT20 was 1-deoxynojirimycin.

Keywords: B. subtilis YT20, chromatography, 1-deoxynojirimycin, α-glucosidase inhibitor.

Classification numbers: 1.2.1, 1.3.1

1. INTRODUCTION

Diabetes is a recognized epidemic disease in Vietnam and over the world, due to the exponential increase of cases, the serious consequences of the disease on health and longevity, and the burdens of the treatment cost for patients and society. According to International Diabetes Federation, in 2015, 415 million people aged 20 to 79 years had diabetes in the world which is equivalent to 1 in 11 people diagnosed with this metabolic disorder. These numbers will go up to 642 million in 2040. In other words, there is 1 in 10 normal people who will have diabetes. In the treatment of type 2 diabetes, the therapy taking advantage of α-glucosidase inhibitor has been drawing the researcher's interests because of its safety and simplicity as a

neutral substance. For optimization and limitation of side effects, more studies are needed to find out alternative α-glucosidase inhibitor from various sources.

Domestic and international researches focus on selection, optimization and purification to discover new α-glucosidase inhibitors including plants [1, 2], and microorganism [3]. Alternatively, 1-deoxynomirycin (DNJ) is an iminiosugar that inhibits α-glucosidse enzyme to prevent the formation of sugar in the digestive tract [4]. The substance originated mostly from mulberry leaves in a small number. Thus, different sources have been exploited to produce DNJ via *Bacillus* sp. In *vivo* streptozotocin –induced diabetic white mice model, the crude extract of the *B. subtilis* S10 containing DNJ reduced the blood glucose level by 209.1 mg/dL (59.1%), lower than 510 mg/dL of the control group [5]. Alternatively, the α-glucosidase inhibitor was isolated from *B. lentimorbus* which is collected from the culture media containing 0.25% natri glutamate and 0.5% glucose; pH 8.0; at 30°C in 48 hrs [6]. Later, an α-glucosidase inhibitor was purified from the *B. subtilis* B2 whose structure was determined as 1-deoxynojirimycin of 163 Da by MS and NMR [7]. In 2013, Lee *et al* purified 1-deoxynojirimycin from the *B. subtilis* MORI which could enhance adiponectin level and its receptor in differentiated 3T3-L1 fat cells. The evidences indicated that this active substance proves effective in reducing blood glucose level which showed no sign of toxicity to differentiated 3T3-L1 cells at the concentration of 5 mM [8]. These are the basic foundations for the search of new compounds possessing α-glucosidase inhibitory activity used in the treatment of type 2 diabetes from the selected microorganisms in Vietnam. In this study we isolated and identified 1-deoxynojirimycin from the *B. subtilis* YT 20 in Vietnam oriented for the treatment of diabetes.

2. MATERIALS AND METHODS

2.1 Microorganism strain and culture media

B. subtilis YT 20 was provided by the National key lab of gene technology at Institute of Biotechnology, Vietnam Academy of Science and Technology. The *B. subtilis* YT 20 was grown in culture media (pH 7.5), which comprised corn starch (10 g/L), soybean meal (5 g/L), and yeast extract (5 g/L), KH_2PO_4 (0.5 g/L), $(NH_4)_2SO_4$ (0.5 g/L).

2.2. Chemical reagent

Silica gel 60 with particle size 0.06–0.2 mm SiO_2, Sephadex G100 with particle size 40 - 120 µm Standard acarbose, 1-deoxynojirimycin (DNJ), p-nitrophenyl-α-D-glucopyranoside (*p*NPG), and intestinal rat enzyme α-glucosidase are purchased from Sigma-Aldrich (US). NaCl, Na_2HPO_4, NaH_2PO_4, $MgSO_4$, peptone, and glycerol are provided by Merck (Germany), active charcoal is from Japan. Maltose, glucose, ethanol, ethyl acetate, methanol, acid formic, and H_2SO_4 are from China; Corn powder, soybean powder, and monosodium glutamate are from Vietnam. All chemicals were of analytical grade otherwise stated.

2.3. Incubation and harvesting active ingredients

The *B. subtilis* YT20 was incubated for 5 days with shaking-speed of 200 rpm at 37 °C. The culture media consisted of: 1 % corn starch, 0.5 % soybean meal, 0.5 % yeast extract, 0.05 % KH_2PO_4; 0.05 % $(NH_4)_2SO_4$; pH value of 7.5. After incubation, the supernatant was centrifuged

at 12000 rpm for 15 mins, and 10 µL of supernatant was evaluated for the α-glucosidase inhibitory activity.

There are several factors which influence the optimal condition of the production of secondary metabolites. Therefore, we conducted optimal experiments to evaluate the effect of each factor by determining its α-glucosidase inhibitory activity following these categories. Effect of incubation times: 10 µL of extracellular extract was evaluated at 48; 72; 96; 120 and 144 hrs. The same volume of sample was used to analyze the effect of pH (4; 7; 7.5; 8 and 10), temperature (28°C; 30°C and 37°C), 1% media of different carbon sources (corn starch, glucose, maltose, galactose, sucrose and lactose), 0.5% media of different nitrogen sources (yeast extract, soybean powder, peptone, malt extract and beef extract).

2.4. Determination of α-glucosidase inhibitory activity

The α-glucosidase enzyme hydrolyzes substrate p-nitrophenyl-α-D-glucopyranoside to produce yellow p-nitrophenol that absorb 405 nm wavelength [9]. Each well of 96 wells plate was added 50 µL containing 40 µL of 0.1 M phosphate buffer pH 6.9 and 10 µL of sample, while 50 µL of 0.1 M phosphate buffer was used as control. Next, 100 µL of α-glucosidase 1U/ml in 0.1 M phosphate buffer pH 6.9 was added to previous mixture. The sample was incubated at 25 °C in 10 mins, shaking 300 rpm. Then, 50 µL 5 mM p-nitrophenyl-α-D-glucopyranoside in 0.1 M phosphate buffer pH 6.9 was added. The solution was mixed well and incubated at 25 °C in 5 mins. All the measurements are triplicated in 96 wells plate.

The α- glucosidase inhibitory activity was calculated by the following formula

$$\% \text{ Inhibition} = \frac{\Delta A_C - \Delta A_S}{\Delta A_C} \times 100$$

ΔA_C : The change in measurement of OD value before and after incubation of control. ΔA_S : The change in measurement of OD value before and after incubation of the sample.

2.5. Thin layer chromatography

TLC is an effective method to determine and analyze the number of different groups of substances presented in the collected fractions. The principal bases on the identical polarities and Rf of different compound. The crude extract was centrifuged at 4000 rpm for 10 mins, the upper phase was continuously centrifuged at 12500 rpm for 15 mins. The supernatant was collected and mixed with absolute alcohol by the ratio of 1 sample: 4 alcohol (v/v). After 30 mins, the fermented broth mixture was centrifuged at 12500 rpm in 15 mins, the supernatant was collected and evaluated by the TLC.

The TLC was performed on the Merck 60 F254 thin silica gel plate, with a thickness of 0.25 nm and a solvent system 4 isopropanol : 1 acetic acid : 1 H_2O (v/v/v). The TLC plate was then dried and colorized by ninhydrin reagent.

2.6. Purification of α-glucosidase inhibitor

In order to purify α-glucosidase inhibitor from fermented broth of the *B. subtilits* YT20, the supernatant was centrifuged at 1200 rpm for 15 mins and precipitated with absolute alcohol at 4°C in 1 hr at the ratio of 1 sample: 4 alcohol (v/v) to eliminate protein and polysachharides. The upper phase was then dissolved in deionized H_2O and passed through the 0.45 µm filter

membrane. Next, the solution was filtrated with two membrane pore size 100 Da and 1000 Da, respectively. (Molecular weight cut off; Spectra/Por*, USA). The supernatant was loaded onto the activated charcoal column (Column size: 24 x 0.6 cm; flow speed 1.5 ml/min). Different concentrations of ethanol from 5 - 30 % were used to collect the fractions. These elutions later were examined by the TLC and α-glucosidase inhibitory activity. The potential segments were loaded onto sephadex G100 column G100 (24 x 0.6 cm), to collect putative compound. The fractions were eluted with potassium phosphate buffer 50 mM, pH 7.5. The flow speed was at 25 mL/h, the active substance was evaluated for the α-glucosidase inhibitiory activity and elucidated structure by mass spectroscopy.

2.7 Statistical analysis

All measurements were carried out in triplicate. The means were presented for averages of experiments.

3. RESULTS AND DISCUSSIONS

3.1 Alpha-glucosidase inhibitor activity of crude extract of *B. subtilis* YT20

The crude extract of *B. subtilis* YT20 showed a high α-glucosidase inhibitory activity at 68.1 %. While standard acarbose reached 75.2 % at the concentration of 1 mg/mL (Sigma). Equally, at the same concentration, the inhibitory activity of DNJ (Sigma) reached 70.1 % (Table 1).

Table 1. α- glucosidase inhibitory activity of the extracellular extract of *B. subtilis* YT20

The extracellular extract	α- glucosidase inhibitory activity (%)
B. subtilis YT 20	68.1 ± 2.1
Acarbose* (Merck)	75.2 ± 5.8
DNJ** (Sigma)	70.1 ± 3.4

***** Acarbose standard (Merck): 1 mg/mL; ** DNJ standard (Sigma): 1 mg/mL

The *Bacillus* sp. in particular, is a potential bacterial source which has been extensively investigated for production of α-glucosidase inhibitor, including the *B. subtilis* S10 [10], *B. subtilis* B2 [7], and *B. mycodes* TKU040 with 143 U/mL and IC_{50} value of the α-glucosidase inhibitors produced in the culture supernatant being 3 mg/mL [11]. Alternatively, the α-glucosidase inhibitory activity from the *B. subtilis* was recorded at as high as 8 5 % [12].

3.2. Optimization of production of α-glucosidase inhibitor

3.2.1. Effect of incubation time

The fermented conditions for production of the α-glucosidase inhibitors from the *B. subtilis* YT20 in a basic culture condition are 28 °C with shaking speed of 200 rpm, and pH 7.5. After 96 hrs of culture, the α-glucosidase inhibition activity of the extracellular extract from the *B.*

subtilis YT20 reached the highest peak of 80 %, followed by a slight decrease after 120 hrs, and almost remained unchanged at 75 % after 144 hrs of fermentation. After 96 hrs, the nutrition in the culture media slowly depleted, and led to some exotic stress on microorganism so other secondary metabolites was secreted to media and slightly reduced the inhibitory activity. Our results are in agreements with other studies in which the secondary metabolites showed the highest α-glucosidase inhibition activity after 96 hrs of incubation from the *B. subtilis* B2 [7] and the *B. mycodes* TKU040 [11]. However, the α-glucosidase inhibitory activity from the *B. subtilis* was over 85 % after incubation for 9 days [12]. The α-glucosidase inhibitory activity of the *B. subtilis* B2 fermentation was increased slightly after 6 days [7] and the *B. subtilis* S10 (24 hrs) [10]. Thus, the different strains have the ability to produce the α-glucosidase inhibitors whose activity is varied depend on the fermentation medium.

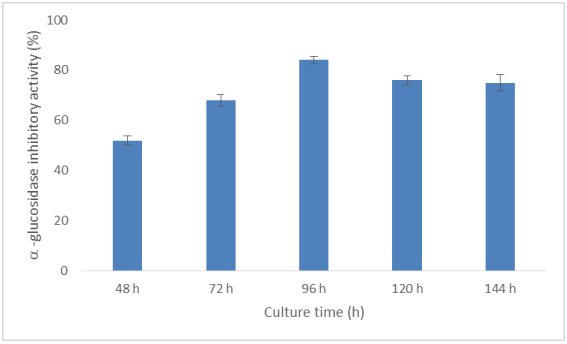

Figure 1. Effect of incubation on the production of α-glucosidase from *B. subtilis* YT20

3.2.2. Effect of pH

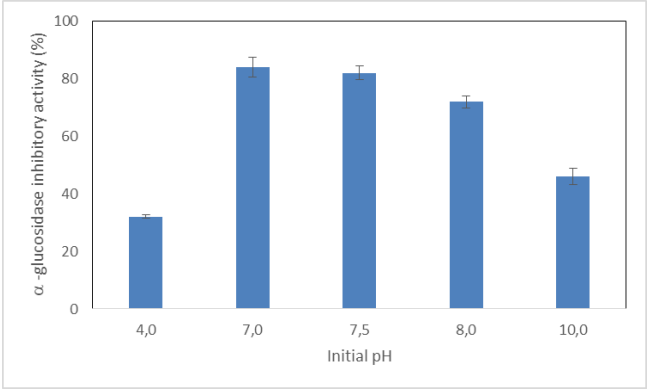

Figure 2. Effect of pH media on the α-glucosidase inhibitory activity from extracellular extract of *B. subtilis* YT20.

Five pH value at 4; 7; 7.5; 8 and 10 were evaluated for the biosynthesis of α-glucosidase inhibitors from the *B. subtilis* YT20. We found that the optimal pH is 7.0 and 7.5 possessing the highest activity of up to 82%. At lower or higher pH 7.0, it exerted negative effects on the production of the α-glucosidase inhibitors. This would suggest the active compounds were affected by pH of culture media which is agreeable with previous studies of the *Bacillus* sp. [13].

3.2.3. Effect of temperature

The *B. subtilis* YT20 strain was incubated in the basic fermentation media supplementing with corn starch, soybean powder, yeast extract and minerals at pH 7.0 with shaking-speed of 200 rpm, and incubated for 4 days. The results indicated the production of α-glucosidase inhibitory activity reached the highest point at 80 %.

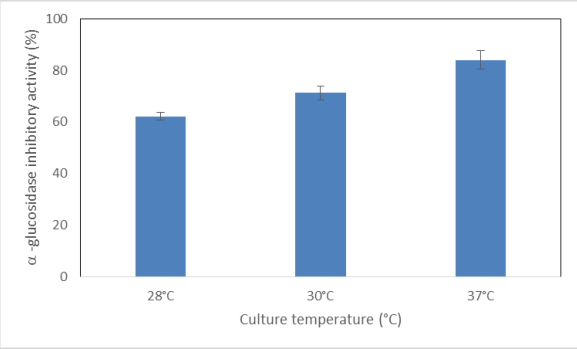

Figure 3. Effect of temperature on the α-glucosidase inhibitory activity from extracellular extract *B. subtilis* YT20

3.2.4. Effect of carbon source

Lactose producing media showed the highest activity of 86.5 %, followed by other media supplementing with galactose (81.8 %); corn starch (72.2 %). However, glucose and sucrose containing media showed lowest inhibition activity.

Table 2. Effect of carbon source on the production of α-glucosidase inhibitory activity from *B. subtilis* YT20.

Carbon source (1%)	α-glucosidase inhibitory activity (%)
Corn starch	72.2 ± 2.1
Glucose	27.1 ± 1.2
Maltose	67.4 ± 1.5
Galactose	81.8 ± 3.4
Sucrose	24.9 ± 0.8
Lactose	86.5 ± 4.5
Rice powder	47.3± 1.4
Corn powder	37.6± 0.7

3.2.5. Effect of nitrogen

Culture media supplementing with different nitrogen sources exhibited different α-glucosidase inhibitory activity (Table 3). Peptone and yeast extract adding media enhance the inhibition activity of the crude extract by 91.5 % and 76.5 %, respectively. In contrast, soybean powder and beef extract supplementing media exhibited a lower inhibition activity of 73.2 % and 73.5 %, respectively, and the lowest one is malt extract containing media.

Table 3. Effects of nitrogen source on the production of α-glucosidase inhibitory activity from *B. subtilis* YT20.

Nitrogen source (0.5%)	α-glucosidase inhibitory activity (%)
Soybean powder	73.2 ± 3.2
Peptone	91.5 ± 1.1
Yeast extract	76.5 ± 2.5
Malt extract	4.3 ± 0.41
Beef extract	73.5 ± 1.7
$(NH_4)_2SO_4$	17.15 ± 0.52

In conclusion, from the above data of optimizing the culture conditions and compositions, we found out the favorite media for enhancement of α-glucosidase inhibitor, which includes: lactose 1 %; peptone 0.5 %; pH 7.0; temperature 37 °C; shaking speed of 200 rpm, and 4 days of incubation. At the optimal conditions, the inhibition activity rose to the highest point of 91.5 % which is 1.34-fold higher than pre-optimization. Previously, the production of DNJ increased by 3.3 folds when fermentation media was supplemented with sodium citrate (0 h, 5 g/L), sorbose (0 h, 1 g/L), iodoacetic acid (20 h, 50 mg/L), and glucose (26h, 7 g/L) [14].

3.4. Purification of 1-deoxynojirimycin

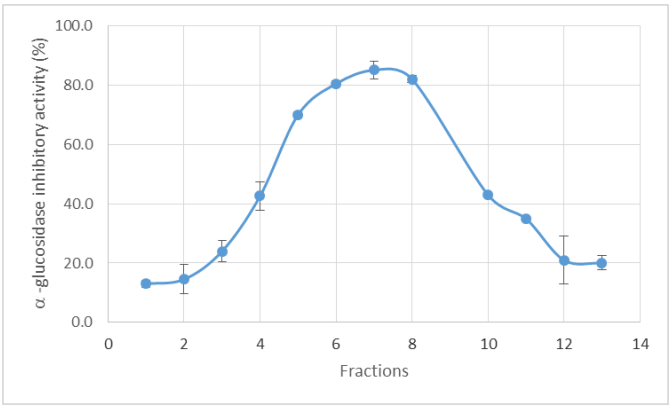

Figure 4. Fractional inhibitory activity of the samples was purified by sephadex G100 column.

At the final chromatography column with Sephadex G 100, fraction 6–8 showed the highest α-glucosidase inhibitiory activity of 85 % (fraction 7) and 80.4 % to 82 %, respectively. While other fractions exhibited a lower activity (Figure 4).

The purified efficacy of 1-deoxynojirimycin was listed in the table below

Table 5. Purification efficacy of 1-deoxynojirimycin

Purification steps	Volume	Active DNJ	Yield of step

	(ml)	(mg)	(%)
The broth extract after condensation	1000	1.73	100
Condensate column (membrane 1000 MWCO)	134	0.52	71.2
Activated column	75	0.41	56.1
Sephadex G100	4.5	0.28	16.1

The result from table 4 demonstrated the purified efficacy reached 16 %. These fractions were determined on TLC (Figure 5 A). There is an appearance of one band constrained at Rf 0.34 which corresponds to standard DNJ. In 2008, DNJ was isolated from the *B. subtilis* S10 yielding 0.75 g/L. In the next 2 years, Zhu and his team isolated DNJ from the *B. subtilis* B2 by loading the fermented broth on active charcoal column and chromatography CM-sepharose column [7].

The MS spectrum data showed that the purified α -glucosidase inhibitor was 1-deoxynojirimycin with a molecular weight of 163 Da (Figure 5 B). From the collected evidence, we can confirm that the putative compound was DNJ- alpha glucosidase inhibitor purified from the *B. subtilis* YT20 strain with molecular weight of 163 Da which is similar to the previous report [7].

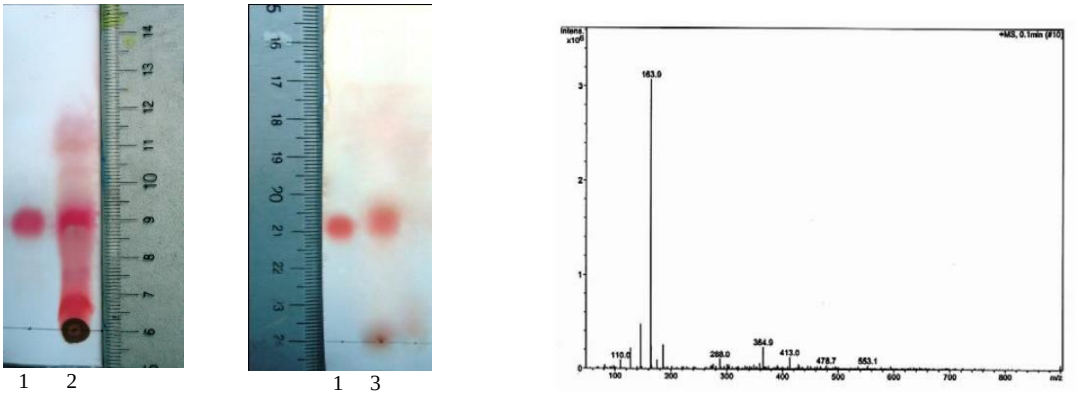

Figure 5. The appearance of standard 1-deoxynojirimycin (1), extracts sample (2), and purified 1-deoxynojirimycin (3) on TLC chromatography; (B) MS spectrum of purified 1-deoxynojirimycin.

4. CONCLUSION

We have determined the optimal culture media for fermentation of the α-glucosidase inhibitior from the *B. subtilis* YT20 composed of (g/L): Lactose 1 %; Peptone 0.5 %; KH_2PO_4 0.05 %; $(NH_4)_2SO_4$ 0.05 %; pH 7.0. The optimal incubated conditions for the biosynthesis of 1-deoxynojirimycin was 96 hrs, 37°C and shaking speed of 200 rpm. Combination of all the optimal conditions, the α-glucosidase inhibition activity exhibited the highest point at 91 %. The putative 1-deoxynojirimycin was purified by continuous steps including ethanol sedimentation,

condensation, activated charcoal column chromatography, sephadex G100 chromatography and TLC. 1-deoxynojirimycin was purified from the *B. subtilis* YT20 with the yield of 16% (w/w) and confined at Rf 0.34 corresponding to standard 1-deoxynojirimycin (Sigma). This proves that the 1-deoxynojirimycin can be mass produced in Vietnam by the native *B. subtilis* YT20.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

REFERENCES

1. Benalla W., S. Bellahcen, and M. Bnouham - Antidiabetic medicinal plants as a resource of α-glucosidase inhibitors. Curr. Diabetes Rev. **6** (4) (2010) 247-254.
2. Yin S., ZhangW., Zhang Y., Kang W. - α-glucosidase inhibitors isolated from medicinal plants. Food Science and Human Wellness **3** (3-4) (2014) 136-174.
3. Nguyen V. S. and S. L. Wang - New novel α-glucosidase inhibitors produced by microbial conversion. Process. Biochem **65** (2018) 228-232.
4. Onose S., Ikeda R., Nakagawa K., et al. - Production of the α- glycosidase inhibitor 1-deoxynojirimycin from *Bacillus* species. Food Chem. **138** (1) (2013) 516-23.
5. Cho Y. S, Park Y. S., Lee J. Y., Kang K. D., Hwang K. Y., Seong S. I. - Hypoglycemic effect of culture broth of *Bacillus subtilis* S10 producing 1-deoxynojirimycin. J. Korean Soc. Food Sci. Nutr. **37** (11) (2008) 1401-1407.
6. Kyoung-Ja K., Y. J. Yang, and J. Kim - Production of α-glucosidase inhibitor by β-glucosidase inhibitor-producing *Bacillus lentimorbu*s B-6. J. Microbiol. Biotechnol. **12** (6) (2002) 895-900.
7. Zhu Y. P., Yamaki K., Yoshihashi T., Kameyama M. O., Li X. T., Cheng Y. Q., Mori Y., Li L.T. - Purification and identification of 1-deoxynojirimycin (DNJ) in okara fermented by *Bacillus subtilis* B2 from Chinese traditional food (Meitaoza), J. Agric. Food Chem. **58** (7) (2010) 4097-103.
8. Lee S. M., Do H. J., Shin M. J., Seong S., Hwang K. Y., Lee J. Y., Kwon O., Jin T., Chung J. H. - 1-deoxynojirimycin isolated from a *Bacillus subtilis* stimulates adiponectin and GLUT4 expressions in 3T3-L1 adipocytes, J. Microbiol. Biotechnol. **23** (5) (2013) 637-43.
9. Yamaki, K. and Y. Mori - Evaluation of α-glucosidase inhibitory activity in colored foods: a trial using slope factors of regression curves. Nippon Shokuhin Kagaku Kogaku Kaishi **53** (4) (2006) 229-231.
10. Zhu, Y .P., Yin, L.J., Cheng, Y.Q., Yamaki, K., Mori, Y., Su, Y.C., Li, L.T. - Effects of sources of carbon and nitrogen on production of α-glucosidase inhibitor by a newly isolated strain of *Bacillus subtilis* B2. Food Chemistry **109** (4) (2008) 737-742.
11. Hsu, C.H., Nguyen, V.B., Nguyen, A.D., Wang, S.L. - Conversion of shrimp heads to α-glucosidase inhibitors via co-culture of *Bacillus mycoides* TKU040 and *Rhizobium* sp. TKU041. Research on Chemical Intermediates **44** (2018) 4597-4607.

12. Kim, Y.S., Ju, W.T., Kim, H.B., Sung, G.B. - Optimization of culture conditions of *Bacillus subtilis* with α-glucosidase inhibitory activity. Int. J. Indust. Entomol. **33** (1) (2016) 24-30.
13. Bamola, N. and R.K. Gupta - Effect of pH and temperature on secondary metabolite isolated from soil bacteria. Int. J. Life. Sci. Scienti. Res. **4**(2) (2018) 1729-1735.
14. Wu, H., Guo, Y., Chen, L., Chen, G., Liang, Z. - A novel strategy to regulate 1-deoxynojirimycin production based on its biosynthetic pathway in *Streptomyces lavendulae* Front. Microbiol. **10** (2019) 1968.