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Original Research Article

Screening for *Moniliella* sp. Glucosamine Biosysthesis

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ABSTRACT

Moniliella is a genus of black yeast with many prominent features such as the ability to ferment with high concentrations of sugar, biosynthesis of some kind of sugar alcohols... In this study, 25 strains of black yeast *Moniliella* were selected for glucosamine biosynthesis capacity screening in order to apply in functional food ingredients production technology. These strains were isolated from various sources of three regions in Vietnam and were grouped by PCR fingerprinting. Glucosamine biosynthesis capability were determined by HPLC method. Among the screened strains, 12 strains showed the glucosamine production superior to 0.5 g/L glucosamine). Almost tested *Moniliella megachiliensis* strains produced glucosamine, in which the *M.megachiliensis* TBY 3404.5 strain produced glucosamine yield at 5.66 g/L. *Moniliella* sp. TN18.2 strain produced the highest glucosamine yield at 6.06 g/L. Among 12 strains that produce glucosamine, is there significant different in glucosamine yield. Most of species *M. megachilliensis* have significantly higher glucosamine biosynthesis as TBY 3404.4, TBY 3404.5, TBY 3406.2 (reaching over 4.5 g/l). Some strains biosynthesize glucosamine in low concentrations such as *M. barkeri* sp. nov. or *M. byzovii* (less than 2 g/l) The results show potential applications in the production of glucosamine as a functional food ingredient from microorganisms.

INTRODUCTION

Glucosamine is an amino sugar synthesized from glucose and glutamine, a hydroxyl group of glucose is subtituted by an amine group. Glucosamine is an important component of the polysaccharides chitin and chitosan. It is also an important compound required for the formation of chondrocytes, which are one of the basic units of cartilage matrix and synovial fluid (Reginster JY *et al.*, 2001). Glucosamine has been widely used in the food, cosmetic, healthcare and pharmaceutical industries (Directorate General for Health & Consumers, 2003). Current biological production methods of glucosamine include: (1) microbial fermentation from glucose; (2) hydrolysis of chitin and chitosan of animal origin by acid or enzyme; (3) method using enzyme-catalyzed direct hydrolysis of chitin from fungi to glucosamine (Sitanggang et al. 2012).

For the purpose of conservation, genetic diversity of microorganism, Industrial Microorganisms Centre (Food Industries Research Institute, Vietnam) has isolated 200 species of black yeast of *Moniliella* strainss. To fully exploit the biological potential of these strains, and choose black yeast with high

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glucosamine biosynthesis capability for producing glucosamine at an industrial scale, screening glucosamine biosynthesis capability of 25 species of black yeast *Moniliella* kept at the center were carried out.

MATERIALS AND METHODS

Strains and media

Most of *Moniliella* species have only been isolated from man-made products or manufactured items. For example, *M. acetoabutens* has so far only been found in highly acidic fermented food samples such as fruit sauces, pickles, *M. carnis* from fermented meats and cuttings, *M. mellis* from honey, *M. nigrescens* from jam, spoiled marmalade, *M. spathulata* from buffalo milk… Species descriptions are mainly based on a small number of cultivars (*de Hoog et al., 2011*). The studied black yeast strains were isolated from different sources across the country, including *Lantana camara* flower, beach morning glory flower, convolvulus flower, Beach latex flower, sesame flower, meat slicing table, bottles of cooking oil, jar of soya sauce, soya sauce and some other sources like okra flowers, and Oleander flower, etc. The board samples, grease, soil, insects, garbage, spring rolls, soy sauce were collected and isolated within 2 hours from the time of sampling. After collecting the flower samples, they will be refrigerated and isolated for 24 hours.

Glucosamine fermentation method

Twenty five species of black yeast genus *Moniliella* was isolated from the Northern provinces (Hanoi, Ninh Binh, Lang Son), the Central (Nghe An), the Southern (Phu Yen). These strains were preserved in Malt - glucose 4°Brix by freeze-drying method at the Industrial Microbiology Center - Institute for Food Industry. The strains were activated in the Malt - glucose 2°Brix agar and cultivated medium contained 20% glucose, 1% yeast extract, 0,1% urea (GYU20) or 30% glucose, 1% yeast extract, 0,1% urea (GYU30).

The activated strains was keep on agar plate for no more than 5 days before transferring to a liquid medium for starter culture preparation. The starter culture was inoculated in liquid media GYU20 at 28°C, 150 rpm for 48 hours, then the GYU 30 media was supplemented into the tank and the fermentation was carried out at 28°C, 150 rpm for 8 days. The cell biomass was removed by centrifugation at 10.000 rpm, 10°C for 10 min. The supernatant after centrifugation was used for analyzing glucosamine content by HPLC method. The strains with high glucosamine biosynthesis capacity was activated in medium of malt – glucose 2°Brix, then cultured in GYU20 media (incubated at 28°C, 150 rpm on rotary shaker). After 48 hours GYU30 was supplemented in the flask, continued incubate at 28°C, 150 rpm on rotary shaker. The content of polyols in fermented liquor was analyzed by chromatography method after each 48h for evaluating the effect of fermentation time to glucosamine yield.

Glucosamine and polyol content analysis method

The content of glucosamine and polyol was determined by ion-exchange chromatography method on HPLC Supercogel Carbohydrate column and UV-VIS detector (Supelcogel H^+, 30 cm × 7,8 mm (Supelco, USA)) with buffer solution of H_3PO_4 0,1%. The sample was diluted with H_3PO_4 0,1% and filtered through 0,2µm cellulose membrane. Twenty µl of sample was injected and column temperature was 80°C. Concentration of glucosamine was calculated basing on the pick area corresponding to retention time is 8.61 min. The way to calculate glucosamine concentration is based on the area of the corresponding pick in the chromatogram. Construct a calibration curve with known concentrations of glucosamine, each of which corresponds to a pick area. From there, a standard curve for concentration determination based on the area of the pick was constructed (the equation $y = ax + b$, where y is the corresponding pick area, x is the concentration of glucosamine).

RESULTS AND DISCUSSION

Screening results

Twenty-five strains were randomly selected and fermented, representative for 10 typical species of the genus *Moniliella*. including: *M. acetoabutans, M. barkeri sp. nov, M. byzovii, M. carnis, M. dehoogii, M. floricola, M. megachileiensis, M. Mellis, M. nemchuae sp. Nov, M. nigrescens, M. pollinis, M. sojae sp. Nov, M. spathulata, M. suaveolens, Moniliella sp1.* The results showed that some polyols such as glucose, erythritol and glycerol were detected in the fermented liquor. All studied strains were able to metabolize glucose into erythritol but only a few strains were able to accumulate glucosamine in the broth. In the chromatogram, the retention time of the glucosamine standard sample was 8.6 min. Based on the comparison of the retention time of the standard with the retention time of the test sample, it was that in the 25 test samples only a few strains were able to synthesize glucosamine.

The metabolism performance was different between species. *M. megachiliensis* and three new isolated species are the best species to metabolize glucose into glucsamine. Of the five *M. megachiliensis* species, two strains showed erythritol biosynthesis capacity superior to 5 g/L. Two newly isolated species, *Moniliella*.sp 18.2 and *Moniliella*.sp 10.3 also biosynthesis over 5g/L glucosamine. Three species with the lower glucosamine biosynthesis content (inferior to 2 g/L) are *M. barkeri sp. nov.*. *M. floricola* and *M. byzovii*.

Glucosamine biosynthesis ability from glucose is diverse between different species, and also between different strains belonging to the same species. *M.megachiliensis* TBY 3404.5 showed highest glucosamine yield at with 5.66 g/L, whereas for *M.megachiliensis* TBY 3406.3 and TBY 3403.1, the amount of glucosamine was only 3.04 g/L and 0 g/L, respectively. (Table1).

Recent studies about glucosamine biosynthesis in microorganism from different regions showed high glucosamine secreted. For *Monascus pilosus* the glucosamine level reached 0.26 g/L respectively (Yu et al, 2005), for *Aspergillus* sp the glucosamine content was 5.48 g/L (Chang et al., 2011). It can be seen that our *Moniliella megachiliensis* strains showed relatively high metabolizing ability from glucose into glucosamine, especially the *M. megachiliensis* TBY 3406.2, TBY 3404.4, and TBY 3404.5 strains.

The microorganisms applied in industrial production are mostly recombinant strains and have been optimized for fermentation conditions for glucosamine biosynthesis (temperature, pH, oxygen, etc.). This study is only a preliminary study of glucosamine biosynthesis from completely natural and unoptimized strains. Therefore, with glucosamine production at 5 - 7 g/L, selected strains showed further potential application.

Differences in the levels of glucosamine production in fermented liquid depend on isolation source of yeast. Species with high metabolizing capability were primarily isolated from wilted convolvulus flower and five-color flower (in tropical regions). The strains isolated from meat processing tools showed relatively low glucosamine biosynthesis capability. Recently, studies related to

microbial fermentation to obtain glucosamine are attracting attention. The liquid fermentation method uses microorganisms such as bacteria and yeast to convert glucose into glucosamine. Compared with the two previous methods, the production method through microbial synthesis has many advantages, including short production time, high yield, efficiency and limited environmental impact. To date, strains used for glucosamine (GlcN) and GlcNAc production include the fungus *Aspergillus* sp. BCRC 31742 (Zhang J et al, 2012); Escherichia coli and recombinant Bacillus subtilis. After optimization, GlcNAc can be reached up to 17 g/L and 13.2 g/L by recombinant *E. coli* and *B.subtilis* when cultured in shakers (Chen X et al, 2012).

Table1. The content of glucosamine in the fermented liquor of species *Moniliella.sp*

Strains		Glucosamine (g/L)
M. acetoabutans	TBY 237.2	-
M. acetoabutans	TBY 38.4	-
M. barkeri sp. nov.	TBY 372	0.55
M. barkeri sp. nov.	TD 9	1.46
M. byzovii	TBY 1932	1.73
M. byzovii	TBY 2041.7	2.08
M. carnis	KFP 452	-
M. carnis	TBY 4372.3	-
M. dehoogii	TBY 4388.1	-
M. dehoogii	TBY 4549.1	-
M. floricola	TBY 5101.1	2.12
M. *floricola*	TBY 5105.1	1.02
M. megachileiensis	TBY 3403.1	-
M. megachileiensis	TBY 3404.4	5.03
M. megachileiensis	TBY 3404.5	5.66
M. megachiliensis	TBY 3406.2	4.58
M. megachiliensis	TBY 3406.3	3.04
M. mellis	TBY 4375.3	-
M. nemchuae sp. nov.	BY2	-
M. nemchuae sp. nov.	TBY 368.2	-
M.sp	TN18.2	6.06
M.sp	TN10.3	5.22
M.sp	TN12.3	1.47
M.sp	TN20.2	4.02
M.sp	TN23.2	3.3

Biological properties of some glucosamine biosynthetic strains

Cells of yeast strains *M.megachilliensi* TBY 3404.4, TBY 3404.5 and TBY 3406.2 are very diverse in morphology, size such as rods, ellipses, cells can be separated or arranged in chains and reproduction can be observed. cell shoots. Thereby, we found that there are many colonies with different shapes but with relatively similar cell shapes.

The experiment was conducted to ferment strains of TBY 3404.4, TBY 3404.5 and TBY 3406.2 in liquid medium at 28-30°C in a conical flask with 100ml of medium (glucose is substituted by different sugars). After 72 h, the fermentation broth was measured for OD at 600 nm.(Table 2).

From the results of evaluating the cell density of the fermenter by the OD method, that show are three strains are capable of fermenting glucose at the highest levels, simply because glucose is a simple sugar that is easily digestible. and is a common source of nutrients for many microorganisms. Besides, strain TBY 3404.4 can also use fructose and maltose.

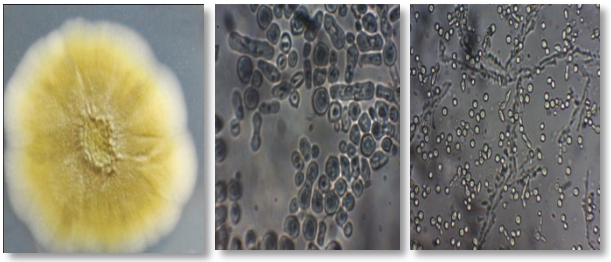

Figure 1. Colonies and cells strain *M.m* TBY 3406.2

Biosynthesis of some extracellular enzymes

To test the ability to degrade organic compounds of 3 strains TBY 3404.4, TBY 3404.5 and TBY 3406.2, we cultured these 3 strains on agar containing starch, casein and tween 80 substrates. Tween 80 serves as the sole carbon source. If any strain has lipase activity, it will degrade Tween 80 and release oleic acid, which will precipitate salts with Ca2+ in the medium and form an opaque white resolution ring around the colony. Bacterial strains were scored on agar medium containing 1% tween 80, after 5 days of culture, the diameter of the lipase-degrading ring was checked. Evaluation of protease activity by agar well method. The fermentation solution after centrifugation to remove the biomass was poured into the agar well, kept in the refrigerator for 2 hours for the enzyme to diffuse evenly. Continue incubation at 28-30°C for 5h, then observe the decomposition ring around the agar well. (Table 3)

The ability of different yeast strains to biosynthesize different enzymes is different. The strain TBY 3404.4 was able to produce the most amylase among the three strains, and the strain TBY 3406.2 produced the least amylase but the highest biosynthesis of protease and lipase compared to the other two strain.

In order to have an overview on glucosamine biosyntheis potential capacity of *Moniliella megachiliensis* TBY 3404.5, a survey on effect of fermentation time to polyol content was conducted for this strain. The strains *M. megachiliensis* TBY 3404.5 was activated in medium of malt-2°Bx glucose, then cultured in media GYU20 (incubated at 28°C, 150 rpm on rotary shaker). After 48 hours GYU30 was supplemented in the flask, continued incubate at 28°C, 150 rpm on rotary shaker. The content of polyols in fermented liquor was analyzed by chromatography method after each 48h.

Table 2. The OD in fermenting sugars

Strains	Glucose	Fructose	Lactose	Maltose	Arabinose	Galactose
M.m TBY3406.2	5,200	2,660	3,02	1,537	0,783	0,615
M.m TBY3404.4	6,030	1,020	0,680	2,002	0,666	0.352
M.m TBY3404.5	5,521	0,645	0,064	1,070	0,768	0,126

Table 3. Biodegradation activity of organic compounds

STT	Strains	Resolution ring diameter (D-d, mm)		
		Amylase	Protease	Lipase
1	TBY 3406.2	10	20	24
2	TBY 3404.4	16.5	18	21.5
3	TBY 3404.5	14	19.5	14

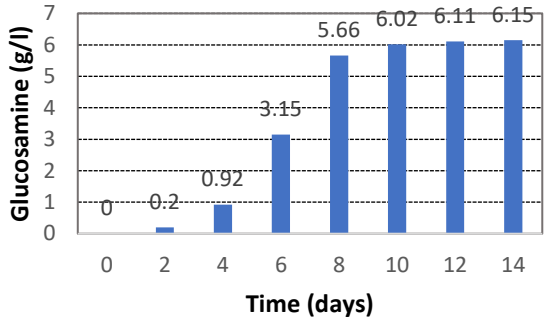

Figure 2. Content of glucosamine in fermented liquor by the time

The result showed that the longer the fermentation time, the higher-level production of glucosamine obtained. The amount of glucosamine ascended in the first 10 days, the glucosamine concentration in fermented liqour was at 6.02 g/L.

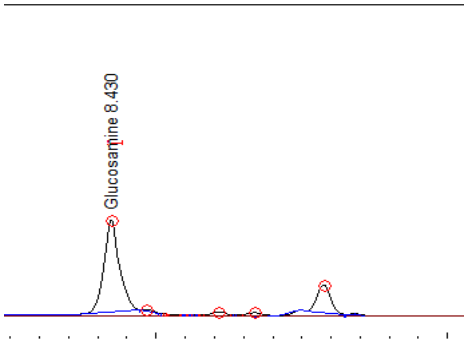

Figure 3. Chromatogram of glucosamine fermentation after 10 days

However, in order to increase the metabolic efficiency, further studies on the factors effecting on the process of biosynthesis of glucosamine and in strains selection as well as alternatives to appropriate fermentation method should be performed.

CONCLUSIONS

In this study, the capacity of glucosamine metabolism from glucose of 25 black yeast strains has been initially evaluated. *M. megachiliensis* was found as the highest glucosamine producing species with the glucosamine content reaching 6 g/L. Three newly isolated strains including M.sp TN18.02 and TN 10.03 showed high ability to synthesize glucosamine. However, biological data on these strains are limited. The results showed potential application in functional sugar production for industrial purpose.

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