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Color mutants of *Monascus* sp. KB9 and their comparative glucoamylases on rice solid culture

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Abstract

Monascus sp. KB9 strain was used to convert carbohydrate base agricultural commodities to value added pigments. Mutagenesis of this strain using UV light and successive isolation was found to improve the yield of pigment product. As a result, two hyper pigment productive strains, red and yellow mutants were obtained. In addition, one white mutant was also obtained. These color mutants together with their parental strain were used to compare glucoamylase production in rice solid culture. Initial moisture content of rice at 35–43% is found to affect glucoamylase of these four strains. It is noteworthy that the white mutant which lost its ability to produce pigment and revealed morphology indifference to its parental strain could produce the highest glucoamylase enzyme at 4.07×10^5 U/gdw (units per gram-dry weight). This is about 3, 7, and 16 times higher than the wild type strain, red, and yellow mutants, respectively. The crude enzyme showed its thermophilic property at optimal temperature, 65°C, and its optimal pH, 4.7.

Keywords: Enzyme synthesis; Glucoamylase; *Monascus* spp. color mutants

1. Introduction

Rice is one of the major agricultural exporting products of Thai economic importance. This primary product could serve as the sustainable raw materials for secondary value-added products through fermentation of *Monascus* molds. Strain improvement, *Monascus* sp KB9 [1] strain was performed using various kinds of mutagenesis [2]. This resulted in three color mutants: red [2], yellow [3], and white [4]. Generally, *Monascus* converts carbohydrate to pigment as well as glucoamylase [5]. Our research revealed the comparative production of glucoamylase of the three color mutants and their parental strain on rice solid culture. This information is valuable for broadening the applications of the *Monascus* spp. for more value-added product besides its pigmentation.

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2. Experimental

2.1. Microorganisms and cultivation

Monascus sp. KB9, a potent cassava starch utilizing isolate for red pigment and glucoamylase production [1], was used as a parental strain for stepwise mutagenesis. The medium used for culture maintenance was MYS agar [3,6]. UV irradiation was used for mutagenesis. Solid culture media used for study on cultural characteristics were MYS [6], SS [6], GYP, and Potato Dextrose Agar (PDA). GYP medium is composed of 4% glucose, 1% yeast extract and 0.5% peptone.

2.2. Isolation of hyperpigment production mutants

Monascus sp. KB9 was used as a parental strain. It was cultured at 30–32°C for 10 days on medium C [7]. Conidia formed were collected and suspended in sterilized water. Ten milliliters of spore suspension were exposed to UV light at various time (0, 10, 15, 25, up to 60 min). The survival curve of conidia was shown in Fig. 1.

2.3. Solid-state fermentation of Monascus sp.

A local, non-sticky rice, Kao-Hom Mali cultivar was used throughout the experiments. All strains were incubated on moistured rice in Erlenmeyer flasks for 18 days at 30°C. The preparation of rice medium for solid culture was as follows: dehulled rice was soaked in tap water for 2 h. After the water was removed, the soaked rice was drained for 5–10 min and then a 500-ml flask containing 100 g rice was autoclaved for 15 min at 121.5°C and cooled to room temperature. Sterile distilled water of 0, 5, 10, and 20 ml were added into the flasks to adjust initial rice moisture content to 32%, 35%, 38% and 43%, respectively. Two milliliters of 10^6 spores/ml of *Monascus* molds were inoculated.

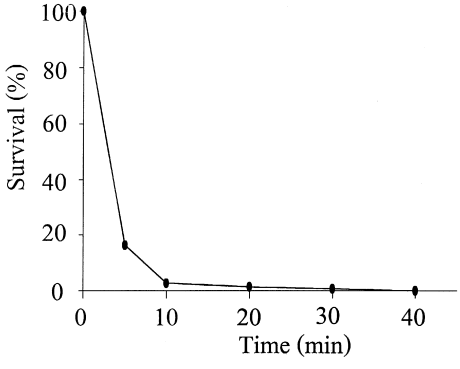

Fig. 1. Survival of conidia in *Monascus* sp. KB9. Treated with UV-irradiation.

2.4. Extraction

The fermented rice was extracted at various times by the method described elsewhere [8,9]. Ten grams of samples were blended with 90 ml of distilled water in a Waring blender for 1 min. Forty milliliters of water was added to 10 ml of the blended mash, and extracted on a rotary shaker at 120 rpm for 30 min, followed by centrifugation at 10,000 rpm for 20 min. The supernatant was used for determination of glucoamylase activity, glucose, and ethanol concentrations.

2.5. Estimation of pigment concentration

The pigment concentration was measured by spectrophotometry at 370, 420 and 500 nm for yellow, orange and red pigments, respectively. Pigment in red rice samples corresponding to 1 g of initial rice substrate was extracted with 39 ml of 50% ethanol for 3 h on rotary shaker (300 rpm). The extract was then centrifuged for 15 min to remove suspended solids and the supernatant was analyzed by a spectrophotometer (UV-240, Shidmadzu, Kyoto) against a 50% ethanol blank. The moisture content of rice samples was determined by heating the fermented rice in a hot air oven overnight at 105°C and measured weight loss.

2.6. Glucoamylase activity

The glucoamylase activity was routinely assayed by the determination of reducing sugars liberated in the reaction mixture in 20 min at 55°C. The reaction mixture contained 0.5 ml of the enzyme solution and 0.5 ml of boiled 1% soluble starch in 0.05 M (final concentration) acetate buffer pH 4.7. The reducing sugar liberated was determined [10,11] using UV-240 spectrophotometer at 520 nm absorbance. Glucose was used as a standard. One unit of the glucoamylase was defined as the amount of enzyme to liberate reducing sugar equivalent to 1 μg of glucose/ml in 1 min at 55°C.

2.7. Protein determination

Protein content was determined [12] using bovine serum albumin (BSA) as a standard. The protein content in the column eluates was measured by the absorbancy at 280 nm.

2.8. Effect of temperature and pH on the enzyme activity and stability

Glucoamylase and activity was examined at different temperatures (30–70°C) and pH range [3–11] in the 0.05 M citrate phosphate buffer, pH 3.0–8.0, and 0.05 M phosphate buffer, pH 8.0–11.0. The stability of these enzymes was also investigated. The stability of pH was determined under the standard method after the treatment at desired pHs for 60 min at 30°C. The temperature stability was also performed under the standard method after the treatment at desired temperatures for 30 min.

2.9. Partial purification of glucoamylase of white mutant of Monascus sp. KB9

The crude enzyme was obtained from the filtrate of 9-day culture of white mutant on rice. This was obtained by extracting the mold rice solids with three volumes of tap water for 2 h at room temperature. The extract was then filtrated and centrifuged at 10,000 rpm for 20 min at 4°C. The crude enzyme could efficiently digest soluble starch and cassava starch.

Crude enzyme was then mixed with 0.05 M acetate buffer pH 4.7 at the ratio 1:9 and left for 30 min. Some part of protein together with pigments were precipitated and then centrifuged at 10,000 rpm for 15 min at 4°C. The cleared crude enzyme solution was concentrated by 20–40%, 40–60%, and 60–80% ammonium sulfate precipitation. The concentrated enzyme was dialysed in 0.05 M acetate buffer, pH 4.7 at 4°C for 12 h before being applied on a DEAE-cellulose column (2×9 cm) equilibrated with the same buffer. The enzyme was eluted from the column with stepwise buffer solutions containing 0.05 and 0.5 M KCl at the flow rate of 30 ml/h.

3. Results and discussion

3.1. Mutagenesis for various color mutants

Mutagenesis had the most effect on hyperpigmentation of *Monascus* spp. [2,7,13]. UV treatment was found to be the best method for improvement of *Monascus* spp. [2,7]. One of the primary mutants, strain KB10M16, provided two-fold increase in the concentration of red pigments. Whereas one of the secondary mutants, strain KB20M10.2, generated from KB10M16, showed yellow zone around its colony and provided six-fold increase in the concentration. The only primary white mutant, strain KB20M1, was obtained in 1992. The single white mutant was generated from the wild type, *Monascus* sp.KB9. All of these phenomena are shown in Fig. 2. Maximum absorption spectra of extracellular pigments are shown in Fig. 3. Pigments in the media of wild type cultivation showed double peaks of absorption spectra (A_{420} and A_{500}), whereas multipeaks ($A_{370,}$ $A_{420,}$ and A_{500}) were observed in the

pigment-producing medium of red mutant. It was noted that single peak of A_{370} was observed in the pigment producing medium of yellow mutant while no peaks appeared in the pigment producing medium of white mutant. These differences in pigment peaks among four strains obtained under the same optimal conditions: SS medium [3,6] 7 days cultivation at 28°C at agitation speed of 300 rpm.

3.2. Cultural and morphological characteristics

Various media was used to examine cultural characteristics. In addition to different pigment production, *Monascus* sp. KB9 and its white mutant posed similar cultural characteristics and able to grow abundantly on different media, e.g. MYS, SS, PDA, and GYP, with aerial lava mycelia of the same colonial sizes. Contrarily, red and yellow mutants showed smaller colonial size with short mycelia. Both mutants grew weakly on PDA. These two mutants, however, could excrete remarkable soluble pigment around their colonies (Table 1).

Under the microscopic observation, the wild type and its white mutant showed similarity in size of colonies and characteristics of mycelia, conidia, and cleistothecia, whereas the red and yellow mutants posed smaller size of mycelia, cleistothecia and less amount of conidia (Table 2).

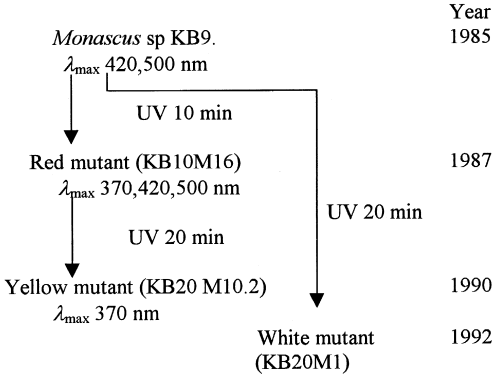

Fig. 2. Genealogy of cassava-utilizing *Monascus* mutants.

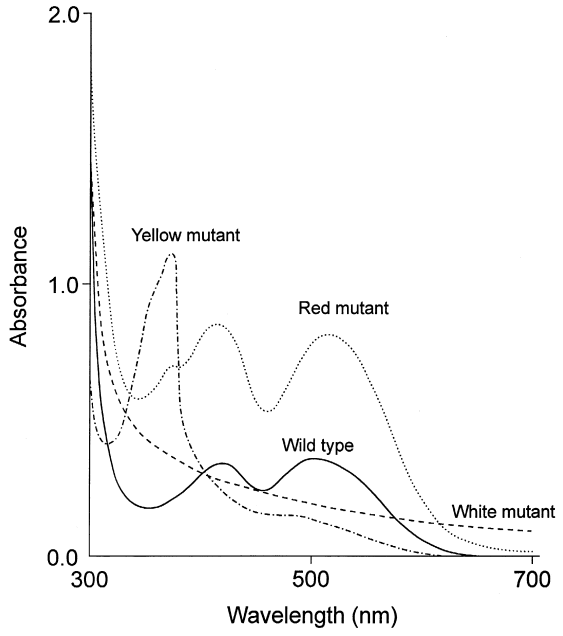

Fig. 3. Absorption spectra of pigments produced by wild type, red, yellow and white mutants. These spectra were measured in 50% ethanol.

3.3. Comparative glucoamylase activity among color mutants and their parental strain on rice solid culture

Initial moisture content in the solid substrate is one of the key factor affecting glucoamylase activity as well as pigmentation of the *Monascus*. We have adjusted the water concentration in the rice substrate at 32%, 35%, 38% and 43%, respectively. At the lowest initial moisture content of 32%, *Monascus* had low glucoamylase activity and slowly liberated glucose. This resulted in the lowest pigmentation for wild type strain, red and yellow mutants (Fig. 4). At higher initial rice moisture content, higher glucoamylase activity was produced, more and more glucose was liberated. Most glucose liberated from glucoamylase activity was found maximum at 43% of the initial rice moisture content in all strains. The amount of such glucose is about 10–18.5% at 6 to 9 days of incubation and later decreased to their minimum at 0.5–1.0% (Fig. 4). This provided

Table 1
Cultural characteristics of *Monascus* sp. KB9 and its mutants on various agar media after 10 days of incubation at 28°C

Culture	Cultural characteristics (colonial size, cm)			
	MYS	SS	PDA	GYP
Wild type	Thick colony deep red aerial, lava type mycelia (5.45)	Thick colony red aerial, lava type mycelia (5.8)	Thick colony pale red aerial, lava type mycelia (5.6)	Thick colony aerial, lava type mycelia (6.2)
Red mutant	Moderate dense colony short mycelia (5.13)	Moderate dense colony short mycelia (6.0)	Moderate dense colony short mycelia (2.3)	Moderate dense colony short mycelia (4.2)
Yellow mutant	Thin, moderate colony with pigment excretion in medium, short mycelia (5.3)	Thin, moderate colony with more excretion in medium, short mycelia (5.0)	Thin, small colony, short mycelia (2.3)	Moderate dense colony with dense excretion in medium, dense lava type mycelia (5.6)
White mutant	Thick white colony, aerial mycelia (6.2)	Thick white colony, aerial mycelia (4.9)	Thick white colony, aerial mycelia (4.2)	Thick white colony, highly aerial mycelia (5.9)

Table 2
Morphological characteristics of wild type of *Monascus* sp. KB9 and its mutants on cassava-soybean (SS) agar after 7 days of cultivation

Structure	Size (μm) and characteristics			
	Wild type	Red mutant	Yellow mutant	White mutant
Mycelium				
Size	3.8–5.5	3.8–5.6	5.0–7.7	3.0–5.0
Pigment	Hyaline, red	Red	Orange-yellow	Non-pigment
Shape	thin, long branched	thick, short branched	thick, short branched	thin, long branched
Conidium				
Size	5.1–10.0	3.8–8.0	6.4–10.3	7.0–11.5
Pigment	Hyaline	Hyaline	Hyaline	Hyaline
Number/chain	1–12	1–3	1–4	3–14
Shape	Obpyriform-globose truncate base, thin	Obpyriform-globose truncate base, thin	Obpyriform-globose truncate base, thin	Obpyriform-globose truncate base, thin
Cleistothecium				
Size	30–38.5	12.8–30.5	17.9–28.2	25.0–46.0
Pigment	Globose red	Globose red	Globose yellow	Globose, non-pigment

mycelial growth, pigments or some other metabolites. Red, as well as yellow, mutants could produce their maximum glucoamylase at 43% initial rice moisture culture while their pigmentation was at maximum at lower initial moisture content at 38%. Unlike these two color mutants, their wild type strain could produce the highest glucoamylase activity and pigmentation at the same initial single moisture content at 38%.

It showed that each strain preferred individual initial moisture content. Too much water reduced substrate porosity, deformed the structure of rice granule and caused stickiness of the substrate, leading to reduce O_2 transferred. Solid rice culture in sealed humid flasks where the air ventilation was poor, the CO_2 accumulated and promoted ethanol synthesis. Both CO_2 and ethanol could affect pigmentation. Han and Mudget [14] reported that the partial pressure of O_2:CO_2 at 0.50:0.02 was optimum for pigmentation in rice solid culture. Nevertheless, Lotong and Suwanarit [9] reported that at higher rice initial moisture content at 39.6%, *Monascus* sp. NP1, could liberate high glucose at 11.8% and produce ethanol at 7% that can inhibit pigmentation. Our experiments found that glucose accumulated high amount at 10% to 18.5% in the highest 43% initial rice moisture content. Ethanol accumulated in parallel with glucose accumulation. Noticeably, ethanol accumulated in tiny amounts not more than 0.6%. Both glucose and ethanol were decreased and consumed up by *Monascus*. Ethanol could not be detected in the initial rice moisture content at lower concentration (32% and 35%) of the three color strains. However, despite aeration in submerged cultivation, large amounts of ethanol were produced when 5% carbon source was used [15]. Alternatively, ethanol has been found as better substrate for pigmentation than maltose for *M. purpureus* [16]. However, Han [17] and Johns and Stuart [18] found that initial rice moisture content less than 40% gave less pigmentation, but that of 50–56% could give the highest pigmentation within 8 days. We would like to suggest that each *Monascus* strain has its own individual optimum initial rice moisture content for either pigmentation or glucoamylase activity or both. Prevention of undesirable air-tight condition of the solid rice culture in combination with optimization of another key factors such as temperature would improve any product of the *Monascus*. Our study also suggested that at the proper water concentration in the solid rice substrate between 35% and 43%

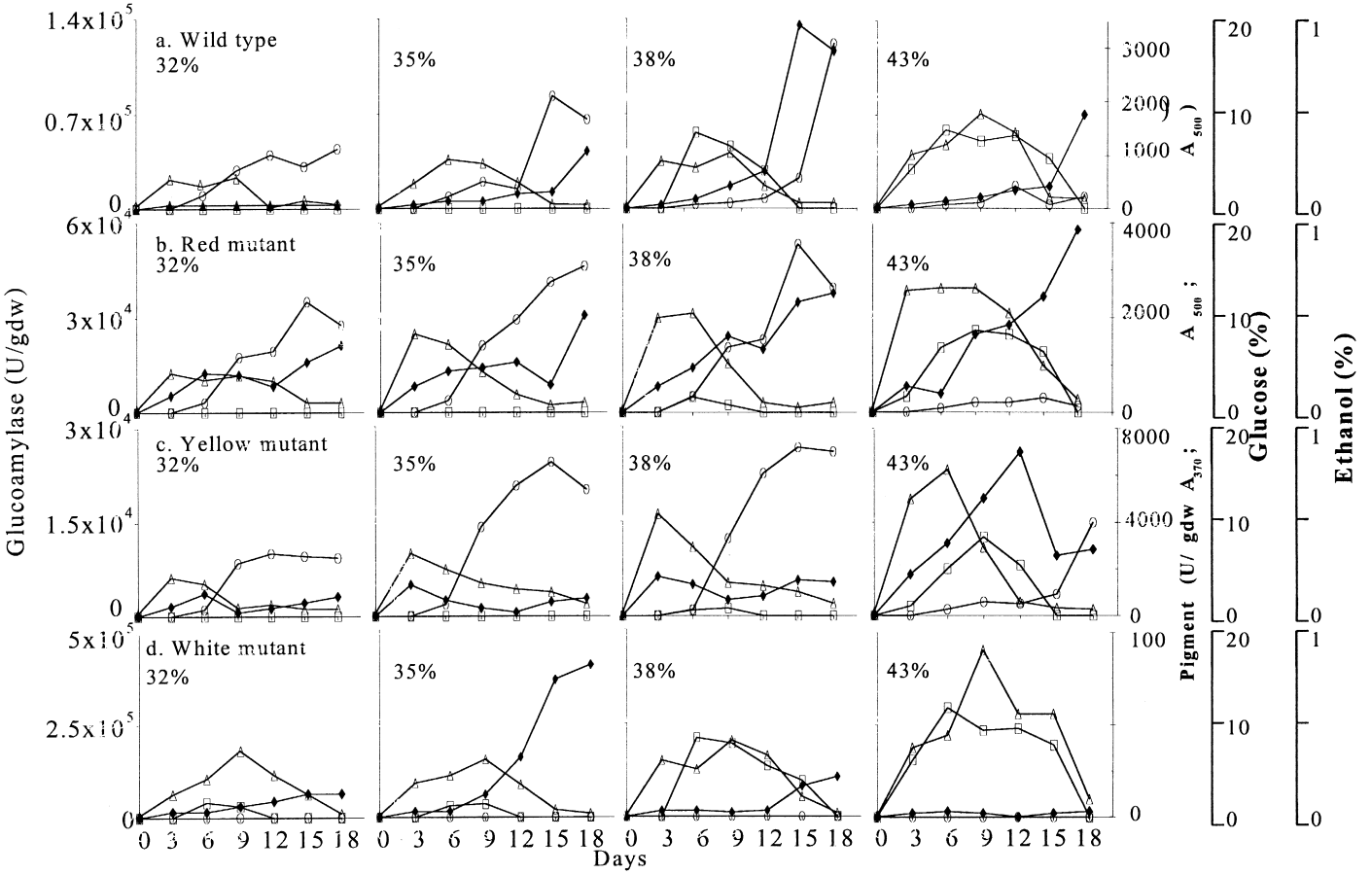

Fig. 4. Time-course of biochemical changes at various rice moisture contents of color mutants and parental strain at 32%, 35%, 38%, 43% initial rice moisture content. ♦, Glucoamylase; △, glucose; ○, pigment; □, ethanol.

was found to enhance the glucoamylase activity of these *Monascus* molds. Contrarily to the three color strains, the white mutant which could not produce any pigment but could produce glucoamylase at very high yield up to 4.07×10^5 U/gdw (units per gram-dry weight). This glucoamylase obtained from white mutant was 3, 7, and 16 times of those obtained from the wild type, red and yellow mutants, respectively. Glucose liberated at 32%, 35%, and 38% initial moisture by all color strains was found at similar content at less than 10% but it increased to more than 10% at 43% initial rice moisture content where their pigmentation was minimum. However, all glucose and ethanol liberation was further decreased and consumed up by the white (albino) mutant and was then converted to the other metabolites, e.g. malic acid [19], antibiotics [20], and fat [21], as previously reported instead of pigment production by the other color strains including wild type.

3.4. Effect of temperature and pH on the glucoamylase enzyme activity and stability

Fig. 5 showed the profile of temperature and pH on activity and stability of glucoamylase of white mutant. The crude enzyme exhibited the maximum activity at 65°C and pH at 4.7. Thermostability of enzymes was measured after incubating the enzyme glucoamylase at pH 4.7 at various temperature for 30 min. The remaining activities were examined under the standard assay condition. The results shown in Fig. 5a indicated that enzyme was stable at temperature below 50°C. However, the glucoamylase was fully stable up to 50°C, about 75% of activity remained at 60°C, and about 5% remained at 65°C and completely lost at 70°C.

pH stability of the enzyme showed that glucoamylase was stable over the pH range of 3–8 with its optimum at pH 4.7. Especially for glucoamylase, more than 75% of its activity remained at pH 2.2 or 9 and almost lost at pH 10.6.

Hydrolysis products of soluble starch was identified by thin-layer chromatography followed the method of Iizuka and Mineki [5]. It was found that glucose was only a liberated product and no other oligosaccharides could be found in all sample tests after 10, 20, and 30 min of hydrolysis.

3.5. Partial purification of glucoamylase of white mutant (KB20M1)

We reported here the partial purification of the glucoamylase of the white mutant. The dialyzed solution was applied to a DEAE column (2×9 cm). The column was eluted with the same buffer (0.05 M acetate buffer, pH 4.7) at a flow rate of 30 ml/h and 3-ml fractions were

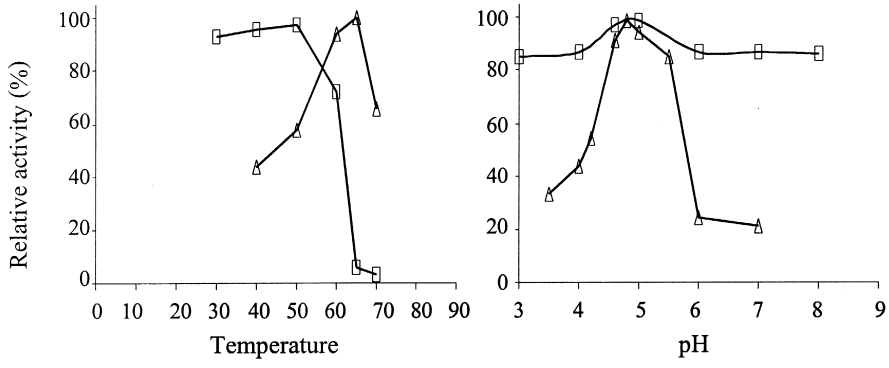

Fig. 5. Effects of temperatures and pH on the activity and stability for glucoamylase from white mutant. To determine the effects of pH, different buffer solutions were used as explained in the text. Symbols: △ activity; □, stability.

collected. There were two peaks of proteins found at 3–7 and 13–20 fractions but only single active component of glucoamylase (fraction 13–20) was separated by this process. These partial purified glucoamylase showed its activity toward both soluble and raw starch as same as crude enzyme.

4. Conclusions

Intitial rice moisture content at 35–43% (w/w) was found to be one of the major factors affected glucoamylase activity as well as pigmentation of four *Monascus* comprising one parental strain and its three closely color mutants. Even though they posed distinguished characteristics on morphology and pigmentation, they favoured individual specific initial rice moisture content for glucoamylase or pigmentaion. Red and yellow mutants favoured the highest initial rice moisture content at 43% for glucoamylase activity and accumulated high glucose with some ethanol that could inhibit their pigmentation to some extent, while wild type strain favoured initial rice moisture content at 38% for its glucoamylase as well as pigmentation. Unlike these three strains, white mutant cannot synthesize any pigment, however, it synthesizes glucoamylase while it liberates higher amount of glucose and ethanol at all levels of initial rice moisture contents (32%, 35%, 38%, 43%), while other color strains could liberate ethanol only at 38–43%. Surprisingly, this white mutant gave the highest glucoamylase activity of 4.07×10^5 U/gdw at 3, 6, and 17 times of those of wild strain, red and yellow mutants, respectively. Its crude enzyme as well as partially purified glucoamylase showed the highest activity at pH 4.7 and 65°C. These enzymes could digest soluble starch as well as raw starch. Even though intensive purification of glucoamylase is needed, the study on raw starch digestion is in progress. Remarkable glucoamylase activity of white mutant using low moisture content solid culture indicates possibility for low process costs. This white mutant may offer economically favourable alternative for glucoamylase applicable to other carbohydrate substrates available for the country.

Based on the morphological study of parental strain on various culture media, *Monascus* sp. KB9 seems to be similar to *M. purpureus*. However, the fungus was considered as *M. kaoliang* using biochemical test on growth resistance to 30% ethanol and 6% NaCl [22]. The taxonomy study of this fungus using RAPD assay and electrophoretic karyotyping technique [23] were used to confirm that *Monascus* sp. KB9 is *M. purpureus*. Further study on transformation of *Monascus* using chromosome-specific fragments carrying putative color gene(s) is under investigation.

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