

# Isolation and Identification of Fungi with Glucoamylase Activity from *Loog-pang-khao-mak* (A Thai Traditional Fermentation Starter)

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## Abstract

*Loog-pang-khao-mak* is a Thai traditional fermentation starter that has been used for production of Thai fermented foods for decades. This research aimed to isolate and identify the fungi that produce effective glucoamylase but low ethanol content from the starter. A total of 166 isolates were screened from twelve samples of *Loog-pang-khao-mak* accumulated from 12 provinces in Thailand using dichloran rose bengal chloramphenicol agar (DRBC Agar). Seventy-nine isolates that effectively hydrolyze starch were selected for glucoamylase activity and alcohol production assay. Three yeast isolates exhibited high glucoamylase activity ranging from 139.14 to 140.94 unit/ml and lowest alcohol yield of 0.41% (v/v) were *Saccharomycopsis fibuligera* using morphological and molecular identification. The five isolates of mold exhibited high glucoamylase activity (149.20 to 152.60 unit/ml) were identified as *Aspergillus niger*, *Aspergillus oryzae* and *Amylomyces rouxii*. These findings provide further knowledge on the fungi and their potential use as traditional inocula for fermentation of food products.

**Keywords:** *Loog-pang-khao-mak*, glucoamylase, alcohol assay, *Saccharomycopsis*, *Aspergillus*, *Amylomyces*

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## INTRODUCTION

For decades, *Loog-pang-khao-mak* has been used as a traditional fermentation starter of fermented Thai food and beverage products such as Kao-mak (Sweet fermented glutinous rice), Num Som Saichu (Rice vinegar) and Satoh (Rice wine). This kind of traditional starter has also been used in various other Asian countries with native names such as *Nuruk* in Korea, *Ragi* in Indonesia, *Murcha* in India, *Bubod* in the Philippines, *Fen-daqu* in China<sup>1</sup>, *Ragi-tapai* in Malaysia, *Koji* in Japan and *Banh-men* in Vietnam<sup>2</sup>. In the preparation of *Loog-pang-khao-mak*, rice or wheat is mashed and mixed with pepper and garlic. Water is added and thoroughly mixed, and then the solid mixture is shaped into small balls and putted on a weave tray<sup>3</sup>. Traditionally, the powder starter is then dusted over the balls. Then the balls are incubated at room temperature around 2 to 5 days. This kind of fermentation starter is prepared slightly differently in different geographical sources, can be stored for many months<sup>4</sup>. In the fermentation process, *Loog-pang-khao-mak* works in two steps: saccharification and alcoholic fermentation. It contains a mixture of microorganisms—molds, yeasts and bacteria therefore its fermentative capability is inconsistency. Our main goal was to isolate specific strains of fungi that provide high glucoamylase activity and low alcohol yield which is intended for a low alcohol fermented food product. *Rhizopus*, *Amylomyces*, *Mucor*, *Penicillium*, *Aspergillus*, *Monascus* and *Actinomucor* are the most common filamentous fungi from *Loog-pang-khao-mak*<sup>2,5</sup>. A group of yeasts, *Saccharomycopsis fibuligera*, *Wickerhamomyces anomalus*, *Rhodotorula philyla*, *Candida tropicalis*, *Candida rugosa*, *Issatchenkia orientalis* and *Saccharomyces cerevisiae* are commonly found in this type of starter<sup>5,6</sup>. Molds in *Loog-pang-khao-mak* be able to produce amylolytic enzymes such as glucoamylase and  $\alpha$ -amylase that can break down starch into glucose and oligosaccharide, while yeasts transform glucose into alcohol. The yeast *Saccharomycopsis fibuligera* is highly efficient at producing amylolytic enzymes<sup>5-8</sup>.

The purpose of this research was to isolate and identify the molds and yeasts from *Loog-pang-khao-mak* that provide a high amylolytic activity and a low alcohol yield. The most potential strains from this study can be used as the pure cultures

for development of low-alcohol fermentation products as well as static fermentation of Thai fermentation products.

## MATERIAL AND METHODS

### Sources of cultures collection of *Loog-pang-khao-mak* samples

All 12 samples of *Loog-pang-khao-mak* (a Thai traditional fermentation start) were purchased from local markets in twelve provinces in Thailand (Fig. 1). All samples were crush to flour and stored at 4°C.

### Isolation of molds and yeasts from *Loog-pang-Khao-mak* samples

Ten grams of each sample were resuspended into 90 ml of sterilized water and 10 - fold serial dilutions were performed ( $10^{-2}$  -  $10^{-6}$ ). One hundred microliters from each diluted sample were spread on dichloran rose bengal chloramphenicol agar (DRBC Agar) (Merck). After that the plates were incubated for 7 days at 25°C<sup>8</sup>. Colonies with different morphotypes were picked onto potato dextrose agar (PDA) then incubated at the same condition. The fungal culture was maintained in 15% glycerol at -80°C.

### Hydrolysis of starch by mold and yeast isolates

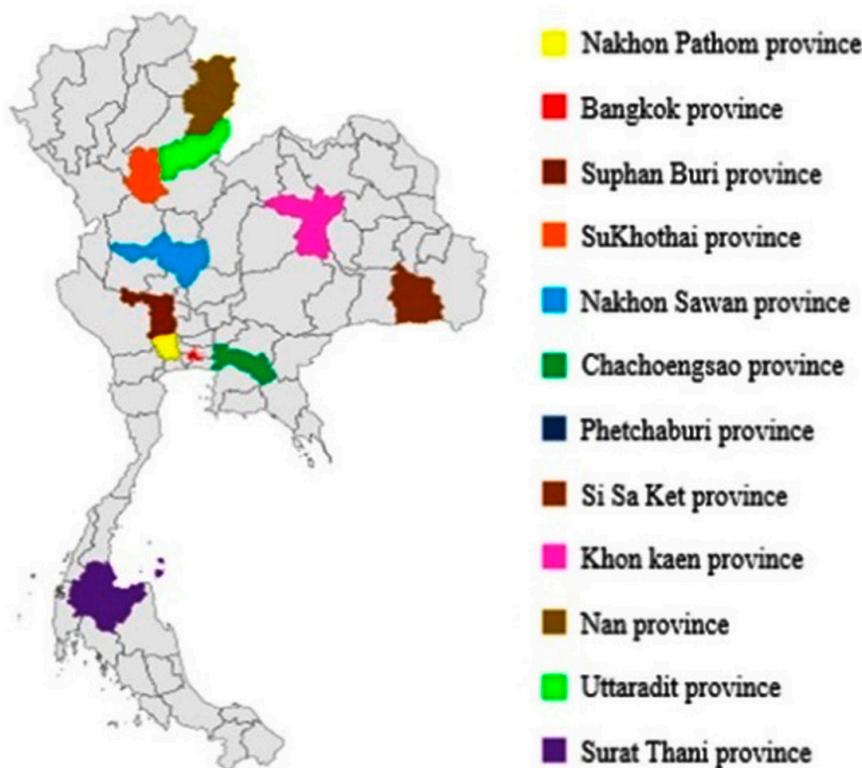
All isolates of yeast and mold were grown on a starch agar and the plates were incubated for 3 days at 30°C. After that, the iodine solution was flooded onto the plates and the diameters of the clear zone were recorded. The hydrolysis capacity of the isolate was calculated by the following equation reported by Limtong *et al.*<sup>6</sup>

### Alcohol assay of yeast isolates

Only yeasts with capability to hydrolyze starch were further screened for alcohol production. The selected isolates were grown in yeast peptone dextrose broth (YPD) and the cultured was agitated at 150 rpm and 30°C for 18 h. Then, cell growth was determined based on the optical density at 660 nm of the culture broth. Ten milliliters of an inoculum were transferred into 90 ml of YPD broth containing 10% glucose and incubated under a static condition at 30°C for 3 days<sup>9</sup>. Alcohol concentration was analyzed using a gas chromatography (Shimadzu GC-9A, Japan).

### Determination of Glucoamylase activity

Only yeasts and molds exhibiting the capability to hydrolyze starch were screened for glucoamylase activity. Yeasts were grown in



**Fig. 1.** Locations of *Look-pang-khao-mak* samples were collected in 12 provinces in Thailand.

YPD broth and shaken under the speed of 150 rpm at 30°C for 18 h. The optical density ( $OD_{660}$ ) of the culture was adjusted to 0.5 for inoculum. Ten milliliters of the active cell suspension were transferred to 90 ml of starch broth and shaken under the speed of 150 rpm at 30°C for 3 days. Molds were cultivated on PDA for 7 days at 30°C. A solution of 0.1% Tween 80 was used to disperse and suspend spores. Ten milliliters of spore suspension ( $1 \times 10^6$  spores/ml) was transferred into 90 ml of starch broth and agitated at 200 rpm and 30°C for 3 days. The yeast and mold cultures were centrifuged at 5,000 rpm and 4°C for 15 min and the supernatants were determined glucoamylase activity assay following a method previously reported by Ramadas<sup>10</sup>. One unit of glucoamylase activity was defined as the  $\mu\text{mol}$  of reducing sugar (in terms of glucose equivalents) produced per minute under the assay condition

#### Conventional identification of fungi

Three most potential isolates of yeasts were identified based on their morphological,

physiological, and biochemical characteristics according to the methods reported by Kurtzman and Smith.<sup>12</sup> as follows. They were cultivated on 5% malt extract agar (MEA) and incubated for 3 days at 25°C and morphological characteristics on MEA were recorded.

For the formation of hypha, the Dalmau plate culture technique was used: each isolate was cultivated on 5% MEA and incubated for 7 days at 25°C. The cells were microscopically examined and photographed. Studied on the growth at 37°C, each yeast isolate was cultivated on yeast malt extract agar (YMA) and then incubated for 3 days at 37°C.

For an examination of fermentation of carbohydrates, a basal fermentation medium containing 2% of glucose, sucrose, lactose, galactose, maltose, and trehalose, and 4% raffinose was used with a Durham tube. The colonies of yeast were suspended in sterilized water, and their turbidity values were estimated by comparing their apparent turbidity to a

**Table 1.** Biochemical tests of three yeast isolates from *Loog-pang-khao-mak* compared to those from a study by Kurtzman and Smith<sup>12</sup>. of *Saccharomyces fibuligera*

Isolate no.	Fermentation of carbohydrates										Growth on carbon compounds																
	D-Glucose	D-Galactose	Sucrose	Maltose	Lactose	Raffinose	Trehalose	Glucose	Glycerol	2-keto-D-gluconate	L-Arabinose	D-Xylose	Adonitol	Xylitol	D-Galactose	D-Sorbitol	Inositol	Methyl- $\alpha$ -D-Glucopyranoside	N-Acetyl-Glucosamine	D-Cellobiose	D-Lactose	D-Maltose	Sucrose	D-Trehalose	D-Melezitose	D-Raffinose	Reduction of nitrate
YSP04	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-
YSP14	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-
YSP16	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-
<i>S. fibuligera</i> (Kurtzman and Smith, 2011)	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-	-

Note: + = a positive result, - = a negative result, v = a variable result, nr = not reported

McFarland standard number 3<sup>13</sup>. Each test tube was inoculated with the cell suspension then incubated for 7 days at 25°C. If some gas was detected in the Durham tube in 7 days, the fermentation result was deemed positive. If gas was undetected then, the fermentation result was deemed negative. Assimilation of carbon compounds was determined with an API20C Aux kit (BioMérieux, France).

Five selected isolates of mold were identified based on their morphological characteristics according to the publication by Pitt and Hocking<sup>14</sup>. Mold isolates were grown on three different media: (CYA, MEA and PDA) and maintained for 5 days at 25°C. The fungal colonies were photographed and their fungal structures were stained with lactophenol cotton blue and microscopically photographed.

The analysis of aflatoxin production on the coconut agar medium (CAM) was studied<sup>15</sup>. Mold isolates were incubated for 5 days at 30°C. After that the colonies were observed under UV light (365 nm). Isolates producing aflatoxins on CAM would glow with green fluorescence under UV light.

#### **Molecular identification of the highest potential yeasts and molds**

Yeasts were cultured on YPD agar for 1 day at 30°C. Genomic DNA was extracted using the method followed by Ruiz-Gaitan *et al.*<sup>16</sup>. Briefly, a few colonies were resuspended into 20 µl of 20 mM NaOH solution and boiled at 100°C for 8 min. For molds, the genomic DNA was extracted using a method previously described by Zhang *et al.*<sup>17</sup>. The mycelium grown on PDA for 5 days was harvested and resuspended into 100 µl of sterile water. The content was centrifuged for 1 min at 10,000 rpm and supernatant was removed. After that, 100 µl of a lysis solution (50 mmol/L of sodium phosphate at pH 7.4, 1 mmol/L of EDTA and 5% of glycerol) was added and heated at 85°C. for 30 min. The extracted genomic DNA were stored at 4°C until use.

For PCR amplification, 1 µl of the genomic DNA was used as a DNA template. A pair of two fungal universal primers for internal transcribed spacer region (ITS) including ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC-3') were used for molecular identification<sup>18</sup>. The PCR cycling conditions were

as follows: initial denaturation for 5 min at 95°C; followed by 30 cycles of a denaturation for 30 sec at 95°C; an annealing for 30 sec at 55°C; an extension for 1 min at 72°C and then a final extension for 5 min at 72°C. The amplified products were purified with a FavorPrep™ GEL/PCR Purification Kit and examined in 1.2% agarose gel. The purified PCR products were sequenced using the same set of primers. The DNA sequences of the potential fungi were compared with reference sequences available in NCBI GenBank (<http://www.ncbi.nlm.nih.gov/blast>). A phylogenetic tree based on ITS regions was constructed using MEGA7 Software based on Neighbor Joining (NJ) method.

#### **Statistical analysis**

All data were determined using one-way ANOVA with the SPSS software, version 25. Significant differences between the means ( $p \leq 0.05$ ) were analyzed by Duncan's New Multiple Range Test (DMRT).

## **RESULTS AND DISCUSSION**

### **Screening of yeasts and molds by starch hydrolysis**

All 166 fungal isolates obtained from 12 *Loog-pang-khao-mak* samples were screened for their capability to hydrolyze starch. Forty-two isolates of yeasts produced a clear zone with the diameters in the range of 1.99 – 3.49 cm. Meanwhile, 37 isolates of molds showed a hydrolysis capacity in the range of 1.00 – 1.06 cm. According to previously studies indicated that the mold and yeast isolates were found in traditional rice wine starters collected from Vietnam, *Nuruk* (a traditional starter in Korea), and *Starter cakes* in India. All these fungi were able to produce the starch degrading enzyme<sup>20-22</sup>.

### **Glucoamylase activity**

Glucoamylase, which degrades starch and can be found in various microorganisms in particular molds and yeasts, is a very important amylolytic enzyme used in various processes of food industry<sup>22,23</sup>. In this research, 79 fungal isolates were screened for their glucoamylase activity by a dinitrosalicylic acid method, using 1% starch as the substrate. Three yeast isolates (YSP04, YSP14 and YSP16) showed a high extracellular glucoamylase activity ranging from 139.14 to 140.94 unit/ml (Fig. 2 A) with the YSP16 isolate had the highest glucoamylase activity. The 5 isolates of molds (ST02, SR02, UD01, UD02 and PB03) showed a

glucoamylase activity ranging from 149.20 to 152.60 unit/ml (Fig. 2 B) with the UD01 isolate produced the highest glucoamylase activity at 152.60 unit/ml. Previous studies have reported that amylolytic microorganisms were related to traditional starters used in traditional food fermentation. Some mold and yeast isolates from *Loog-pang-khao-mak* provided a high amylase activity<sup>2,5,6</sup>. In the same way, Carroll *et al.*<sup>8</sup> reported that most mold and yeast isolates found in *Nuruk* had a strong glucoamylase and  $\alpha$ -amylase activity similar to *Banh men* (an alcohol fermentation

starter in Vietnam)<sup>24</sup>. Also, mold isolates from *Stater cake* in India were capable of producing  $\alpha$ -amylase and glucoamylase<sup>22</sup>.

#### Alcohol fermentation of yeast isolates

Only yeast isolates were screened for alcohol fermentation. In general, all yeast isolates produced a low alcohol concentration which was less than 1% v/v and three isolates (YSP04, YSP14 and YSP16) produced the lowest alcohol concentration of 0.41% v/v (Fig. 3). Limtong *et al.*<sup>5</sup>, which previously reported on yeast isolates from *Loog-pang-khao-mak*, found that most of the

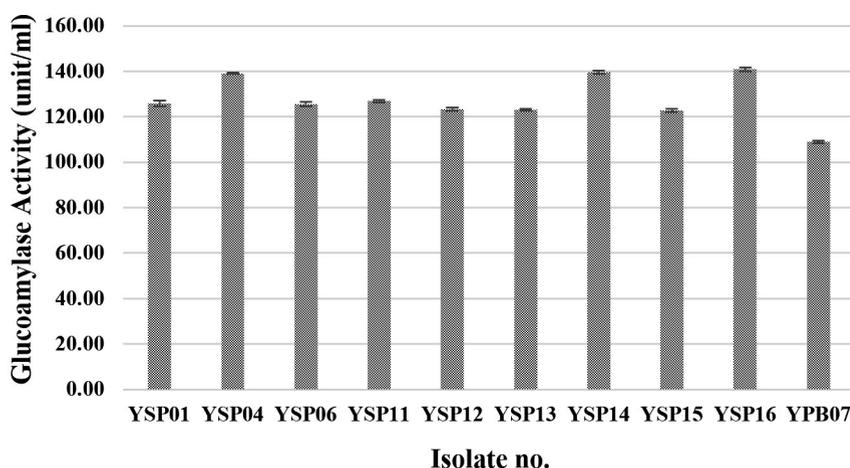


Fig.2.(A). Glucoamylase activities of fungi were isolated from *Loog-pang-khao-mak*; (A) Glucoamylase activities of ten yeast isolates

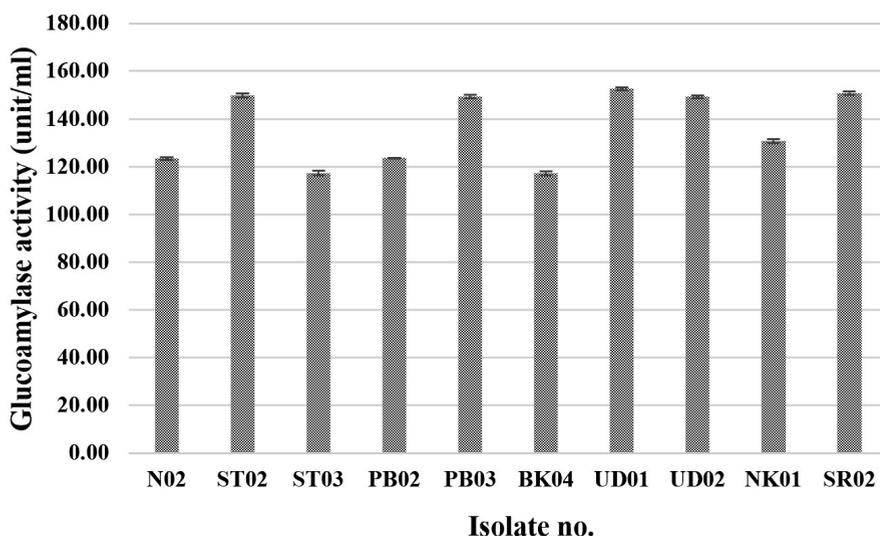


Fig.2.(B). Glucoamylase activities of fungi were isolated from *Loog-pang-khao-mak*; (B) Glucoamylase activities of ten mold isolates.

yeast isolates can ferment 18% glucose medium into ethanol, and some yeast isolates were able to produce a lower than 2% ethanol concentration. It is concluded that the yeast isolates yielding a high amylolytic activity could produce low ethanol concentration and vice versa.

#### Identification of yeasts from *Loog-pang-khao-mak*

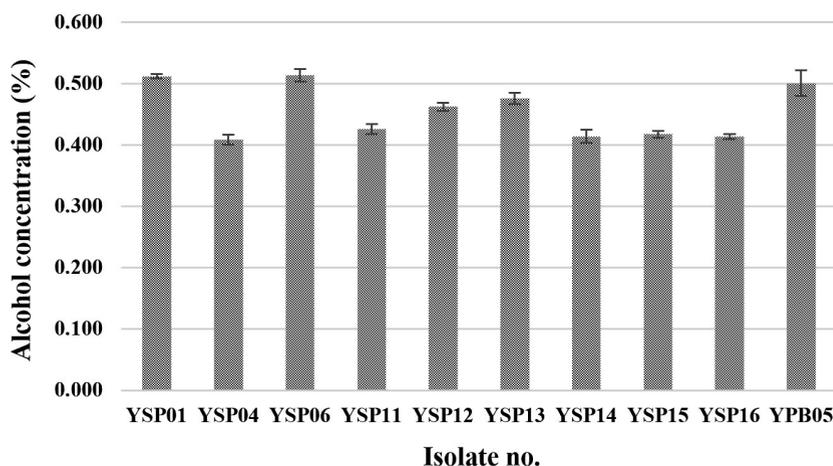
Three yeast isolates (YSP04, YSP14 and YSP16) were identified based on morphological and molecular characteristics. On MEA, the three isolates grew into circular, umbonate colonies with off-white to cream-colored mycelia (Fig. 4 A). Budding cells were multilateral, and the formed cells were ovoid to elongate. After 7 days at 25°C on MEA, the cells grew into a large number of blastoconidia with true hyphae. All yeast isolates were able to grow at 37°C. Biochemical tests are shown in Table 1. All three yeast isolates were able to ferment D-glucose, D-maltose and D-maltose. These isolates utilized different carbon sources: glucose, glycerol, D-cellobiose, D-maltose, and sucrose, but none of them was able to assimilate potassium nitrate. Their results were compared to those reported by Kurtzman and Smith<sup>12</sup>, indicating that the three isolates could be *Saccharomyopsis fibuligera*.

The identity of three yeast isolates was further confirmed by the molecular analysis based on ITS region. They formed a closely relationship with three strains of *Saccharomyopsis fibuligera* with a high statistical support (100% NJBS), while

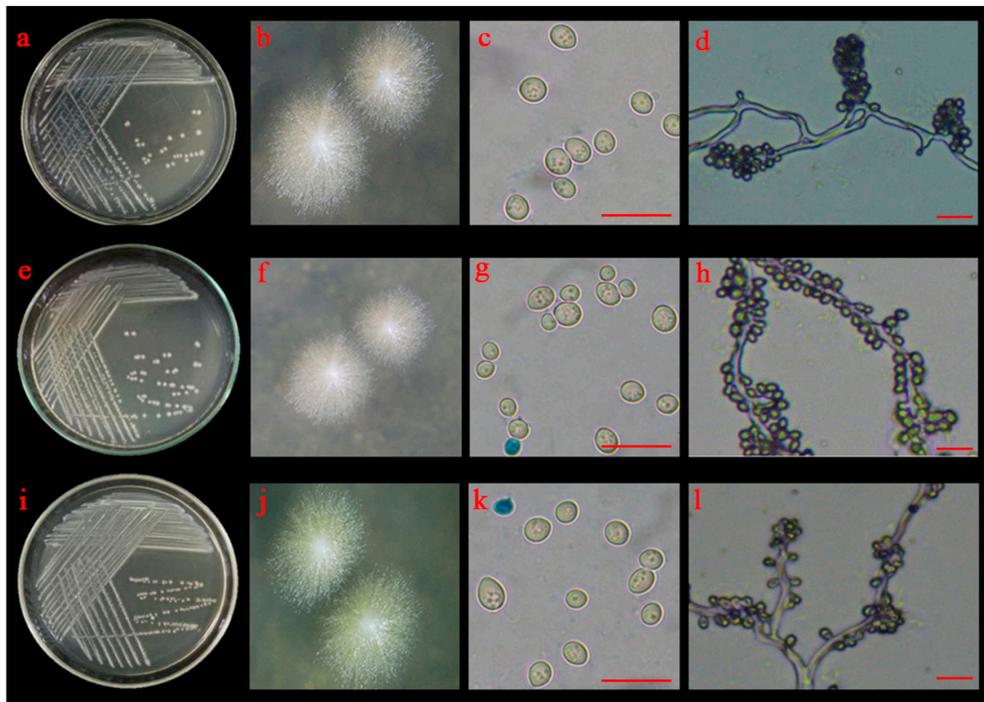
other species of *Saccharomyopsis* were placed in the lower clades (Fig. 4 B), therefore our three isolates (YSP04, YSP14 and YSP16) were identified as *S. fibuligera*. In this study found that *S. fibuligera* strain YSP04, YSP14 and YSP16 exhibited a high glucoamylase activity with *S. fibuligera* strain YSP16 providing the highest activity. *S. fibuligera* is a dimorphic yeast that is also called *Endomyopsis fibuligera*<sup>12</sup>. It is widely used in the traditional fermentation starters of various Asian countries such as *Loog-pang-khao-mak* in Thailand<sup>5,6</sup>, *Banh men* in Vietnam<sup>24</sup>, *Fen daqu* in China<sup>1</sup>, *Yao qu* in China<sup>25</sup> and *Nuruk* in Korea<sup>8</sup>. Daroonpant *et al.*<sup>5</sup> reported that *S. fibuligera* isolated from *Loog-pang-khao-mak* produced the highest glucoamylase activity among various yeast strains similar to *Nuruk*<sup>8</sup>. Moreover, *S. fibuligera* strain YSP04, YSP14 and YSP16 were able to produce alcohol less than 1% v/v. In contrast, Chi *et al.*<sup>26</sup> reported that *S. fibuligera* was unable to ferment ethanol from glucose. Conversely, research by Limtong *et al.*<sup>6</sup> presented that *S. fibuligera* produced alcohol content of less than 2 % v/v from 18% glucose medium at 48 h and they remarked that it may have other roles in producing a pleasurable flavor.

#### Identification of molds isolated from *Loog-pang-khao-mak*

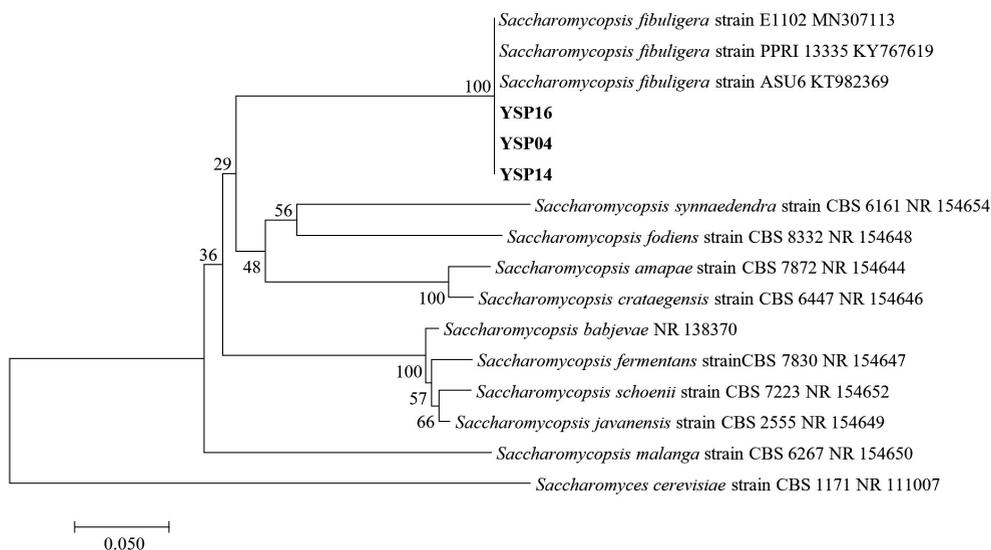
Five isolates (ST02, UD01, UD02, PB03 and SR02) were identified based on their morphological and molecular characteristics. They were cultured on three different media (PDA, MEA and CYA) for



**Fig. 3.** Alcohol contents of ten yeast isolates fermented in 10% glucose medium under a static condition for 3 days incubation.



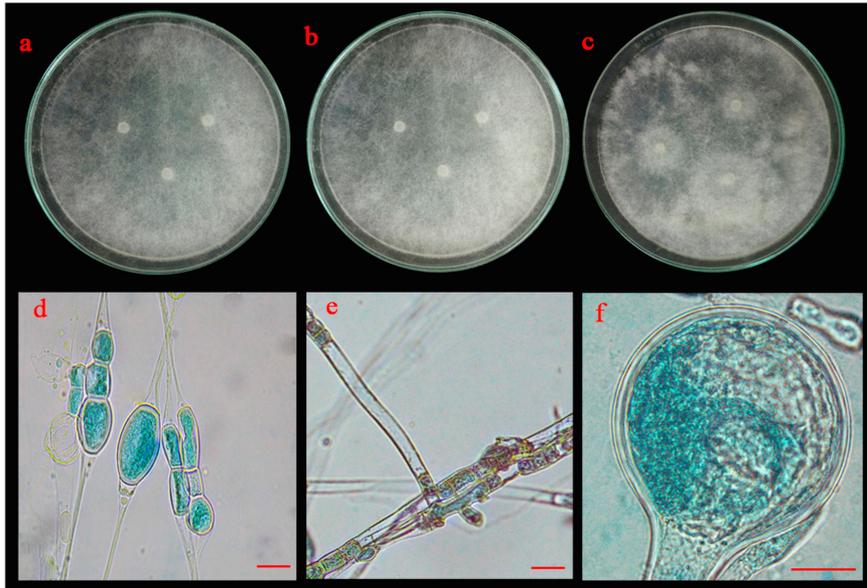
**Fig. 4.(A).** Morphological characteristics of YSP04; YSP14 and YSP16 isolates: colonies of YSP04 (a and b), YSP14 (e and f) and YSP16 (i and j) on 5% malt extract agar; budding cells of YSP04 (c), YSP14 (g) and YSP16 (k) isolates and blastoconidia with true hyphae of YSP04 (d), YSP14 (h) and YSP16 (l) isolates; the scale bar = 10  $\mu$ m.



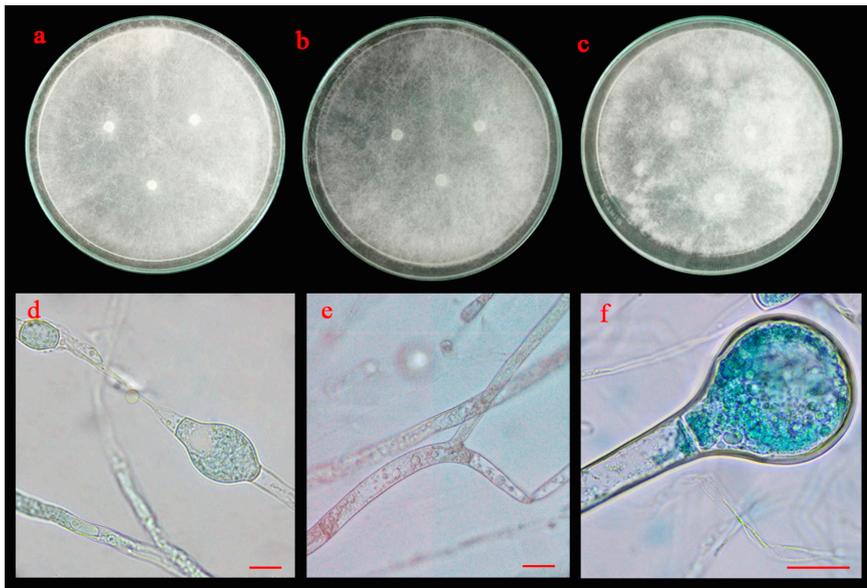
**Fig. 4.(B).** A phylogenetic tree of 3 isolates of *Saccharomycopsis fibuligera* (YSP04, YSP14 and YSP16) constructed with the dataset based on ITS gene sequences. The tree was generated from Neighbor Joining Analysis (NJ), and NJ Bootstrap (NJBS) values were calculated and shown on the tree.

5 days at 25°C. There were three morphotypes of fungal colonies, so their identifications were based on the morphotypes. Although the three isolates (UD02, PB03 and SR02) were cultured on three different media, they produced a similar type

of colony (Fig. 5 A, B and C). Colonies were fast-growing and spreading, dense cottony colonies with white mycelia and chlamydo spores were present within 5 days. These chlamydo spores were produced in both the substrate and aerial hyphae.



**Fig. 5.(A).** Morphological characteristics of UD02 isolate: colonies of UD02 isolate on three different media (a; PDA, b; MEA and c; CYA medium); chlamydo spores (d), sporangiophore with lake of rhizoid (e) and sporangia and sporangiospore (f); the scale bar = 10 μm.



**Fig. 5.(B).** Morphological characteristics of PB03 isolate: colonies of PB03 isolate on three different media (a; PDA, b; MEA and c; CYA medium); chlamydo spores (d), sporangiophore with lake of rhizoid (e) and sporangia and sporangiospore (f); the scale bar = 10 μm.



genus *Amylomyces*. Although *A. rouxii* is closely related to *Rhizopus oryzae*<sup>27</sup>, these two genera can be differentiated by their morphological properties—*A. rouxii* produces a large number of chlamydo-spores but does not form stolon, rhizoids or black sporangia, while *Rhizopus oryzae* does not produce only a few chlamydo-spores but forms stolon and rhizoids<sup>28</sup>. The results from our molecular analysis also emphasize the difference between these two genera.

Morphological characteristics of isolate UD01 is shown in Fig. 6 A. On PDA, it produced circular, flat colonies with white mycelia that later developed into greenish-yellow. It grew well on MEA but with different colony type because it produced a flat colony without white margin. It also produced very distinct type of colonies on CYA with velvety colony and olive-green conidia. The conidiophores of the UD01 isolate developed from foot cells and produced globose vesicles with metulae and phialides around the vesicles with yellowish-green conidia on the phialides

Colonies of isolate ST02 are shown in Fig 6 B. Its colonies on PDA were circular, flat colony with white to bright yellow mycelia that later developed into dark brown conidia. On MEA, it grew into a circular, flat colony with white mycelia that developed into black and dusty. Isolate ST02 grew slowly on CYA with cottony and circular

colonies, raised colony with white to pale yellow mycelium that developed into pale brown conidia. The conidiophores developed from basal cells (foot cells). It produced globose vesicles with metulae and phialides around the vesicles and pigmented conidia on the phialides.

Isolates UD01 and ST02 had closest relationships with *Aspergillus oryzae* and *Aspergillus niger* with statistical supports 67% and 87% respectively as shown in Fig. 6 C, therefore the two isolates were identified as *A. oryzae* UD01 and *A. niger* ST02. The sequences of rRNA genes of the UD01 strain were closely related to those of *A. oryzae* and *A. flavus*, but they can be differentiated by their aflatoxin production—*A. flavus* produces aflatoxin, while *A. oryzae* does not produce aflatoxin<sup>29,30</sup>. Aflatoxin production of *A. oryzae* UD01 was undetected when examined with coconut agar medium (CAM) and confirmed its identity as *A. oryzae*.

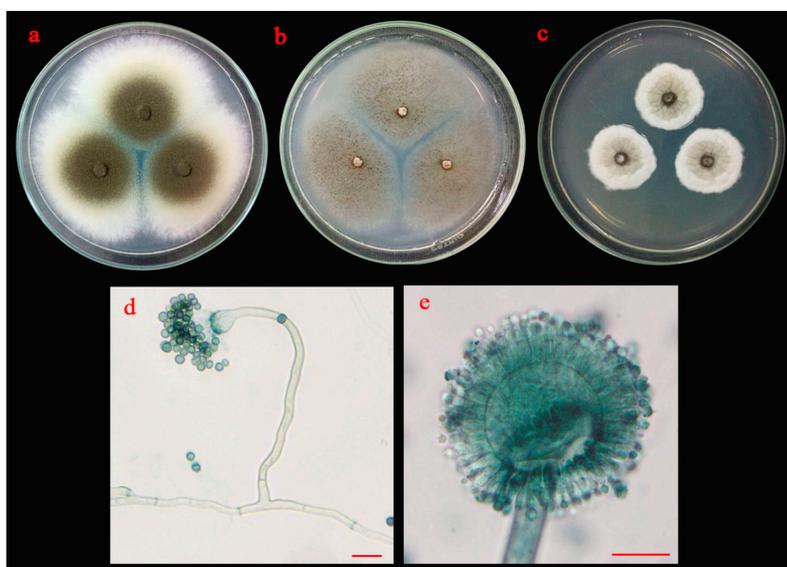
*A. rouxii* has been used for centuries as a culture starter for production of traditional fermented food and alcoholic beverage in East Asia countries. The use of *A. rouxii* which were reported from *Loog-pang-khao-mak* in Thailand<sup>2,5</sup> and *Banh men* in Vietnam<sup>24</sup> has been well known to be a producer of amylolytic enzyme<sup>31</sup>. Meanwhile, *Aspergillus oryzae* and *A. niger* have also been isolated from traditional fermentation starters in



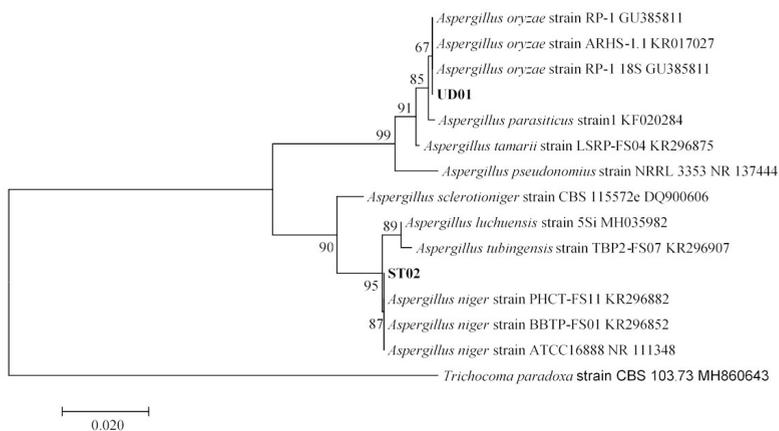
**Fig. 6.(A).** Morphological characteristics of UD01 isolate: colonies of UD01 isolate on three different media (a; PDA, b; MEA and c; CYA medium); sporangiophore with stolon (d) and conidia (e); the scale bar = 10 μm.

various Asian countries such as *Loog-pang-khao-mak* in Thailand<sup>2,5</sup>, *Hong qu* and *Yao qu* in China<sup>25</sup>, *Koji* in Japan<sup>32</sup> and *Nuruk* in Korea<sup>8,33</sup>. The two species *A. niger* and *A. oryzae* play an important role for many bio-based industries; for example, they are used for food fermentation, enzyme production, and organic acid production<sup>34</sup>. *A. oryzae* and *A. niger* are able to produce effective amylase (such as  $\alpha$ -amylase and glucoamylase) for starch digestion<sup>32,35</sup>. In addition, Jasani *et al.*<sup>36</sup> found that *A. niger* have able to produce cellulase enzyme.

Our results indicate that *A. rouxii* PB03, SR02 and UD02, *A. oryzae* UD01 and *A. niger* ST02 exhibited a high glucoamylase activity. In previous studies of amylolytic fungi associated with starter traditional fermentation, Limtong *et al.*<sup>2</sup> and Daroonpant *et al.*<sup>5</sup> presented that *A. rouxii* and *Aspergillus* spp. were commonly isolated from *Loog-pang-khao-mak*, and they also noted that *A. rouxii* provided a high amylolytic activity. Carroll *et al.*<sup>8</sup> which studied on enzyme activity of fungi obtained from *Nuruk* found that *A. oryzae* and *A. niger* were able to produce various kinds



**Fig. 6.(B).** Morphological characteristics of ST02 isolate: colonies of ST02 isolate on three different media (a; PDA, b; MEA and c; CYA medium); sporangiophore with stolon (d) and conidia (e); the scale bar = 10  $\mu$ m.



**Fig. 6.(C).** A phylogenetic tree of *Aspergillus oryzae* (UD01) and *Aspergillus niger* (ST02) constructed with the dataset based on ITS gene sequences. The tree was generated from Neighbor Joining Analysis (NJ), and NJ Bootstrap (NJBS) values were calculated and shown on the tree.

of enzymes including glucoamylase,  $\alpha$ -amylase and acid protease. In particular, *A. oryzae* strain CN1102-08 produced more  $\alpha$ -amylase and glucoamylase activity than *A. niger* strain LNBS02-03. Similarly, the result of this study presents that *A. oryzae* strain UD02 produce glucoamylase activity than *A. niger* strain ST02.

## CONCLUSION

Fungi isolated from *Loog-pang-khao-mak* (a traditional fermentation starter in Thailand) were screened for their glucoamylase activity and ethanol production. The highest potential isolates were selected and identified based on their morphological and molecular characteristics. Three isolates of yeast, *Saccharomycopsis fibuligera* YSP04, YSP14 and YSP16, exhibited the highest extracellular glucoamylase activity but with the lowest alcohol yield. For molds, *Amylomyces rouxii* PB03, UD02 and SR02, *Aspergillus oryzae* UD01 and *Aspergillus niger* ST02, gave the highest glucoamylase activity. These findings expand the key knowledge for the inoculum preparation. Using an inoculum from a mixture of pure cultures rather than an inoculum produced by mixture on unknown species which is sold in the market may improve the productivity of traditional fermentation.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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None.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

This article does not contain any studies

with human participants or animals performed by any of the authors

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