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Isolation and screening of molds from traditional look-pang starter cultures for rice syrup production

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Abstract

Rice syrup can be produced naturally by fermenting cooked rice with microorganisms derived from traditional starter cultures. In this study, we focused on identifying and assessing the sugar production of fungi present in Look-pang, a traditional starter. We employed both submerged fermentation in starch broth and solid-state fermentation techniques on cooked rice to isolate fungal strains from Look-pang. Of these, Aspergillus tamarii stood out for its superior sugar production. The reducing sugar and glucose concentrations achieved from solid-state fermentation using this culture were in line with findings from previous studies. Notably, A. tamarii is known to produce both beneficial health compounds and potential toxins under specific conditions. The significant role of A. tamarii in the fermentation dynamics of Look-pang underscores its potential in advancing organic rice syrup production, representing a step forward in sustainable food processing.

Keywords: Amylolytic fungi; Starter culture; Rice; Syrup

1. Introduction

Traditional starter cultures for rice fermentation are integral to many Asian countries, where they play a pivotal role in producing a diverse range of foods and alcoholic beverages. For instance, in India, the emao starter culture is used to make jou (a rice beer), while in Indonesia, ragi tape gives rise to brem (a rice wine). Similarly, Japan's koji is essential for its rice wine, and China's koji is vital for the red wine anchu. These fermentative processes also yield snacks and desserts, such as China's lao-chao and Indonesia's brem cake (Boro and Narzary, 2022; Mishra *et al*., 2022).

In Thailand, the Look-pang starter culture is particularly versatile, being used in the production of rice wine, rice vinegar, and khao mark, a fermented sticky rice dessert (Roongrojmongkhon *et al*., 2020). This culture harbors a rich diversity of microorganisms, including bacteria, yeasts, and molds. Among these, fungi, particularly molds, are more efficient than bacteria in degrading starch. This efficiency is attributed to their ability to thrive under the aerobic and low water activity conditions typical of solid-state fermentation. Compared to yeasts within the fungi group, molds exhibit superior amylolytic activity (Thancharoen and Malasri, 2023). While molds play a crucial role in hydrolyzing starch into sugars, yeasts primarily ferment sugars into ethanol and contribute to flavor development (Dejsungkranont, 2003). Therefore, in the context of producing rice syrup, which ideally has a low flavor profile, screening for molds is preferred. Notably, while previous studies have identified microorganisms with strong amylolytic activity within these cultures, few have delved into the amylolytic activity of Look-pang microorganisms during the solid-state fermentation of rice, a core step in khao mark production.

Rice syrup is a sweetener commonly used in various food industries due to its absence of allergens and the widespread availability of raw materials in many countries. The use of microorganism in Look-pang for rice syrup production is another sustainable and environmentally friendly practice that supports the preservation of local biodiversity and cultural heritage (Tamang *et al*., 2016). However, mixed culture in Look-pang may produce alcohol or other flavor compounds that affect rice syrup's sensory profile (Wongsa *et al*., 2018). Against this backdrop, our study aims to isolate, identify, and assess the amylolytic potential of microorganisms in traditional Look-pang starter cultures for rice syrup production. Our findings promise to spotlight new microbial strains with amylolytic capacities and propose an economical method for producing rice syrup from authentic, traditional sources.

2. Materials and Methods

2.1 Materials

Two Look-pang Khao Mak samples were sourced from Saraburi (sample A) and Suratthani (sample B) in Thailand. Organic sticky rice (RD6) was obtained from Ban Bua, Phayao. Potato dextrose agar was purchase from Himedia (India). Dinitrosalicylic acid (DNS) was from Aldrich (India). Acetonitrile (HPLC grade) was from RCI Labscan, Thailand.

2.2 Isolation of microorganisms

Local starter cultures were combined with 0.1% peptone, then streaked on potato dextrose agar (PDA). After a 72-h incubation, individual colonies were transferred to PDA slants.

2.3 Determination of reducing sugar production in starch broth

Spore suspension (10^7/mL) was prepared by mixing the single colonies with 0.1% Tween 80 and inoculated into sterile starch broth (Ramadas *et al.*, 1996). The sample was incubated at 30°C for 24 and 48 h. Total soluble solids were measured by hand refractometer (Atago, Japan) Reducing sugar content was determined by dinitrosalicylic acid (DNS) assay (Miller, 1959). Potential microorganisms which produce a high concentration of reducing sugar were selected for fermentation in cooked sticky rice.

2.4 Solid state fermentation of rice syrup

Organic sticky rice (RD6 variety) was mixed with water at a ratio of 100 g/300 mL and autoclaved at 120°C for 15 min. This process allowed both cooking and sterilization of raw material. Cooked rice was mixed with 10 mL of spore suspension (10^7/mL) and left to ferment at room temperature for 48 h. Afterward, the sample was passed through a double-layered cotton cloth and then centrifuged at 11,850 × g for 15 min. The collected supernatant was analyzed to measure rice syrup volume, total soluble solids, reducing sugar, glucose, and maltose concentration. All fermentation procedures were performed in triplicate.

2.5 Determination of glucose and maltose

Glucose and maltose concentrations were determined using high-performance liquid chromatography (HPLC, 4000 series, Jasco, Japan) with a Phenomenex Lunar Omega Sugar column (250 × 4.6 mm). Samples of 10 µL were eluted using a mix of acetonitrile and water (75 : 25) at a flow rate of 1.0 mL/min. The column temperature was maintained at 40°C, and sugars were detected using a refractive index detector.

2.6 Molecular identification

For the molecular characterization of fungal strains with significant potential for glucose and maltose production, we analyzed the internal transcribed spacer (ITS) region's base sequence. Genomic DNA was extracted from pure fungal cultures using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Inc., Lithuania). Amplification of the ITS region was performed using MyFi™ Mix DNA polymerase (Meridian Bioscience, USA), with the following primers: ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR conditions were as follows: an initial denaturation at 95°C for 1 min, followed by 35 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 15 seconds, with a final extension at 72°C for 1 min. The amplified products were stored at -20°C until further use. The ITS sequences obtained were compared with those available in the National Center for Biotechnology Information (NCBI) nucleotide database (GenBank) using a BLAST search. Fungal strains were identified based on the highest sequence similarity and query coverage.

2.7 Statistical analysis

Statistical analysis was performed by EZR version 1.61 (Kanda, 2013). Analysis of variance (ANOVA) and Tukey HSD tests were employed to compare means.

3. Results and Discussion

3.1 Isolation of microorganisms from local starter culture

From the starter cultures labeled as samples A and B, we successfully isolated 5 unique fungal isolates from sample A and 3 isolates from sample B. Each of these isolates was identified based on their distinct morphological characteristics on PDA agar. These fungi were evaluated for their reducing sugar production using starch broth. After a 24-h fermentation period, strains A2 and A3 produced notably high reducing sugar concentration at 3.95 ± 1.47 mg/mL and 4.81 ± 0.15 mg/mL, respectively (Fig 1). However, strain B2 demonstrated the highest reducing sugar content (24.01 ± 0.82 mg/mL) after 48 h of fermentation, followed by strain B3 (15.19 ± 1.11 mg/mL).

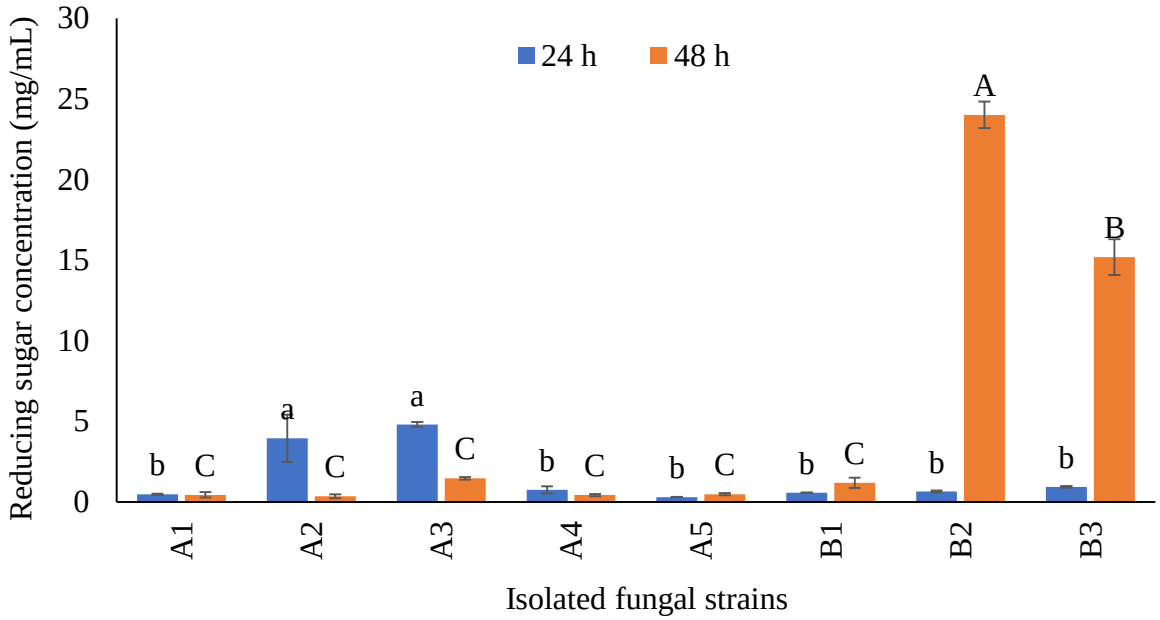

Fig 1 Reducing sugar content of starch broth after fermentation using isolated fungal strains for 24 and 48 h

Note: Bar graph and error bar represent mean ± SD (n = 3). Lower and upper script letter indicate a statistically significant difference ($P<0.05$) for fermentation at 24 and 48 h, respectively.

The main ingredients in Look-pang production include rice flour, sodium bicarbonate, and various herbs like galanga, liquorice, and garlic. In the production process, the flour is combined with water, chosen herbs, and original Look-pang, subsequently shaped into spheres. Within 24 h, mold hyphae start to grow, followed by drying the starter culture, fostering the growth of specific microorganisms. Look-pang contains a mix of molds and yeasts. Molds like *Mucor* spp., *Amylomyces rouxii*, and *Rhizopus oryzae* generate amylolytic enzymes, transforming starch into sugars. In contrast, yeasts such as *Saccharomyces* sp., *Hansenula anomala*, and *Endomycopsis* are instrumental in converting sugars to alcohol. The fungi composition in Look-pang can differ based on the particular formulation. For instance, Look-pang for khaomark, a sweet fermented rice dessert, typically yields lower alcohol content than the Look-pang used for rice whisky (Kaweewong, 2014).

Another investigation found 79 isolates capable of starch hydrolysis from 12 Look-pang samples out of 166 fungal isolates. Notably, strains like *Aspergillus niger*, *Aspergillus oryzae*, and *Amylolyces rouxii* showed significant glucoamylase activity in starch agar, ranging from 149.20 to 152.60 units/mL (Roongrojmongkhon *et al.*, 2020).

3.2 Solid state fermentation of rice syrup

Isolates B2 and B3, due to their pronounced ability to produce reducing sugars in starch broth, were evaluated for sugar production during the solid-state fermentation of cooked rice. For comparative analysis, isolate B1, which showed minimal sugar production in starch broth, was also assessed. After a fermentation period of 48 h, isolate B2 achieved the highest concentrations of glucose (8.38 ± 0.30%) and reducing sugars (10.56 ± 0.15%) and yielded the largest syrup volume (88.0 ± 9.0 mL). In contrast, isolate B3 had the highest maltose concentration, registering at 2.07 ± 0.08% (Fig 2).

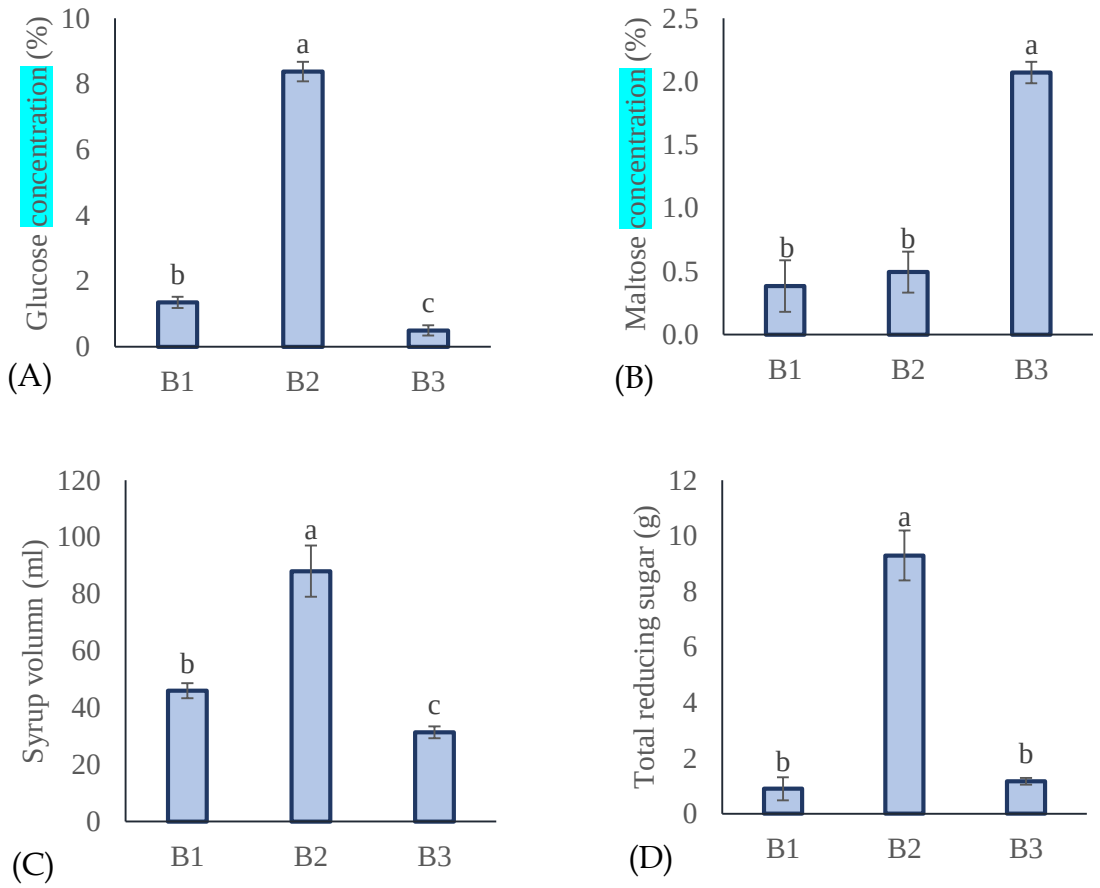

Fig 2 Glucose, maltose, syrup volume and total reducing sugar obtained from 48-h solid-state fermentation of cooked rice by 3 isolates.
Note: Bar graph and error bar represent mean ± SD (n = 3). Different letters on the bar chart indicate statistically significant difference ($P<0.05$).

The values obtained from this study were higher than those found in rice beverages made with red koji, which had a reducing sugar concentration of 4.42% (Kim *et al*., 2008). In their methodology, cooked monascal rice was mixed with a water extract of barley sprout before fermentation. However, the absence of a specified rice-to-water ratio in their study limits a direct comparison of sugar yields based on the rice content. Conversely, Japanese sweet rice beverages fermented with yellow and white koji exhibited reducing sugar contents ranging from 13.7 to 16.9%, which notably exceed the values obtained in our study (Saigusa and Ohba, 2007). Additionally, our study's glucose concentration was slightly lower than the 13.5% reported in another Japanese sweet rice beverage, where rice koji was saccharified in hot water (Oguro *et al*., 2019). A limitation in comparing our findings with these latter two studies is the lack of detailed information on the total volume of the products, which is essential for calculating the sugar yield based on rice content. These comparisons underline the variability in sugar concentrations across different fermentation methods and starter cultures. They also emphasize the potential for further research in optimizing the solid-state fermentation process using the isolated strain. Specifically, there is a need to focus on enhancing the sugar concentration in the culture, potentially by modifying fermentation conditions, substrate composition, or by exploring synergistic effects with other microbial strains. Such research could significantly contribute to the development of more efficient and higher-yielding rice syrup production methods.

3.3 Molecular identification of amylolytic isolates

The isolates B1, B2, and B3 were identified using ITS sequence analysis and compared with the NCBI database via a BLAST search. The results revealed that isolate B1 was identified as *Flavodon ambrosius*, with a 91.05% similarity, and has been assigned the GenBank accession number [OR995568]. Isolates B2 and B3 were both identified as Aspergillus tamarii, with a 100% similarity to sequences in the database, and have been designated the GenBank accession numbers [OR996353] and [OR995569] respectively. Despite their genetic alignment, B2 and B3 were phenotypically distinct, displaying different spore colors (Fig 3).

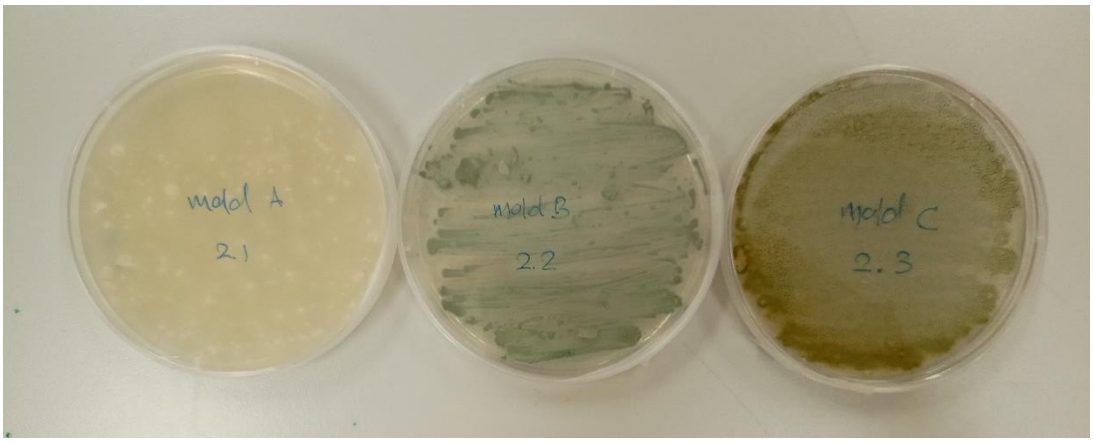

Fig 3 Characteristics of B1, B2 and B3 isolates on PDA agar.

Previous research indicated that *Saccharomycopsis fibuligera* was predominant in 22 out of 38 Look-pang khaomak samples, exhibiting amylolytic activities with minimal ethanol production (Limtong *et al*., 2002). Another study from Northeastern Thailand revealed that starter cultures consisted of 68 yeast and 32 mold isolates, with five mold isolates from the *Rhizopus* and *Penicillium* genera displaying amylolytic activity (Thancharoen and Malasri, 2023).

Traditional starter cultures are widely utilized across various regions of Asia. For instance, in Assam, India, the 'Emao' starter culture for ethnic beer production comprises 11 isolates, with *Wickerhamomyces anomalus, Candida glabrata* and *Hyphopichia burtonii* as the dominant fungi. Among these, only five isolates, including *Hyphopichia burtonii*, *Saccharomycopsis fibuligera, Mucor indicus, Mucor circinelloides* and *Penicillium citrinum*, show amylolytic properties (Boro and Narzary, 2022). In Japan, the 'Koji' starter culture used for sake and soy sauce production primarily contains *Aspergillus oryzae* (Yamashita, 2021). Meanwhile, in Korea, 'Nuruk', which is used for rice wine production, consists of fungi from the *Mucor* and *Aspergillus* genera (Yang*et al*., 2011).

Aspergillus tamarii, previously isolated from soil, has been observed to produce α-amylase and glucoamylase when utilizing starch or maltose as carbon sources. Notably, its amylase production is viable within a pH range of 4 to 10 and a temperature range of 25 to 42°C (Moreira *et al*., 1999). Beyond amylases, *A. tamarii* also boasts the capacity to produce tannase in submerged fermentations involving tannic or gallic acids. Additionally, it can generate xylanase from mediums rich in corn cob powder and protease from those containing wheat bran or soybean meal (Boer and Peralta, 2000; Costa *et al*., 2008; Kadowaki *et al*., 1997). Furthermore, during the solid-state fermentation of wheat bran, this fungus produces both protease and xylanase (Anandan *et al*., 2007).

The specific enzyme production by *A. tamarii* largely hinges on the substrate. For instance, wheat bran fermentation induces high proteolytic activity, yet this activity diminishes when using corn cob or sugar cane bagasse (Ferreira *et al*., 1999). There are instances of isolated *A. tamarii* strains employed in the production of pectin methylesterase through the solid-state fermentation of coffee pulp, as well as α-amylase from both solid-state and submerged fermentations of wheat bran (Nava *et al*., 2011; Premalatha *et al*., 2023). Moreover, a fusant cell fusion of *A. flavus* and *A. tamarii* has been utilized to produce ascorbic acid via brewery waste fermentation (Banjo *et al*., 2019).

A. tamarii isolated from root of *Ficus carica* produced malformin E, a cyclic peptide with antimicrobial activity against certain foodborne pathogens (Ma *et al*., 2016). However, various strains of this fungus are known to produce mycotoxins. A survey of 45 strains of *A. Tamarii* found that a majority produced the mycotoxins cyclopiazonic acid and kojic acid, while certain strains also produced aflatoxin B_1 and B_2. Specific characteristic of aflatoxin-producing strain of *A tamarii* was yellow-green colonies, which notably remain unchanged in color throughout their growth phase (Goto *et al*., 1997; Klich *et al*., 2000).

In addition to Look-pang, *A. tamarii* is present in other traditional fermented products. Its first isolation was from a soybean sauce known as "tamari," which inspired its name (Goto *et al*., 1997). Additionally, it has been identified in fermented tea, where it coexists with other *Aspergillus* species, contributing to a unique flavor profile and potential health benefits (Ma *et al*., 2016).

The literature suggests that *A. tamarii*, sourced from various origins, can produce both health-promoting compounds and toxins. These outcomes may depend on several factors, such as the strain, substrate, growth conditions, and the presence of other microorganisms. Further research is necessary to examine the interactions between microorganisms in traditional starter cultures, particularly in relation to the production of desired and undesired compounds. Additionally, there's a need for food safety evaluations of traditional products, especially concerning potential toxic compounds produced during fermentation.

4. Conclusion

This investigation into the fungal constituents of Look-pang revealed that *A. tamarii* produced the highest contents of glucose and reducing sugar in both submerged fermentation in starch broth and solid-state fermentation on cooked rice. While the results surpassed some existing benchmarks, particularly in rice beverages made with red koji, they did not match the sugar levels observed in certain Japanese rice beverages. This underscores the need for future work to optimize fermentation processes using the isolated *A. tamarii*. Moreover, given its potential to produce undesired compounds, there's an evident need for rigorous food safety evaluations. Future research should prioritize exploring microbial interactions in traditional starter cultures and their subsequent impact on product safety and quality. Such insights will be invaluable for refining fermentation processes, ensuring food safety, and preserving the rich heritage of traditional fermentation practices.

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