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Isolation and Identification of Indigenous *Aspergillus oryzae* for Saccharification of Rice Starch

S.S. Sooriyamoorthy, K.F.S.T. Silva[1], M.H.W. Gunawardhane[2] and C.K. Illeperuma[3]

Postgraduate Institute of Agriculture
University of Peradeniya
Peradeniya, Sri Lanka

ABSTRACT. *A study was undertaken to isolate an indigenous Aspergillus oryzae strain for use in saccharification of high amylose rice starch. Bread, black gram, soya grains, 'kevum', and cooked rice samples assumed to be contaminated with Aspergillus oryzae were used in the isolation. Ten pure cultures obtained by culturing and sub-culturing on Potato Dextrose Agar (PDA) were maintained on PDA slant. A reference strain of Aspergillus oryzae obtained from Japan (Hiroshima Prefectural Food Technology Research Centre, Hiroshima), was used for comparison. All the isolates were inoculated on Aspergillus Flavus and Parasiticus Agar (AFPA) medium to differentiate them from Aspergillus flavus and Aspergillus parasiticus based on reverse colour. The isolate selected based on the reverse colour on AFPA was further identified using an identification scheme employed for common Aspergillus species and teleomorphs. This isolate and the reference strain were inoculated on Czapack Yeast Extract Agar (CYA) and the macroscopic characteristics between the two were compared. Microscopic characteristics of the two strains grown on slide cultures were also compared. Koji prepared using the two strains were tested for their ability to saccharify rice starch. Similar macroscopic and microscopic characteristics of the isolate and the reference strain and reverse colour on AFPA medium revealed that the former could most likely be Aspergillus oryzae. This Aspergillus strain isolated from bread could be used instead of the Japanese strain for saccharification of high amylose rice as reflected by the presence of 53 and 47 gl^{-1} of reducing sugar contents produced by the isolate and the reference strain, respectively, in the saccharified fermenting liquor.*

INTRODUCTION

Aspergillus flavus, *A. parasiticus*, *A. oryzae* and *A. sojae* belong to the *Aspergillus* section *Flavi*. *A. flavus* and *A. parasiticus* are known to produce the potent carcinogen aflatoxin. *A. oryzae* and *A. sojae* have been used for producing food grade amylase and fermentation of oriental foods for centuries (Lee *et al.*, 2004). The fungal mass of hyphae resulted by growing these two fungi on rice, soybean, bean, wheat, barley *etc*, is termed koji, which is used for producing sake, miso and tamari (Wood, 1977). Production of 'sake', a fermented traditional alcoholic beverage from rice, is a

[1] Department of Animal Science, Faculty of Agriculture, University of Peradeniya, Sri Lanka.

[2] Food Research Unit, Department of Agriculture, Peradeniya, Sri Lanka.

[3] Department of Food Science & Technology, Faculty of Agriculture, University of Peradeniya, Sri Lanka.

well established industry in Japan. Koji, *A. oryzae* grown on rice, is used as the major fermentation mass for sake production, which provides enzymes such as alpha amylase and glucoamylase to break down rice starch into fermentable sugars, thereby facilitating the subsequent fermentation process by yeasts.

Isolation and identification of *A. oryzae* has been reported to be problematic due to the intra-and interspecies variability of isolates and the morphological similarities with *A. flavus* (Lee *et al.*, 2004). Onions (1982) reported that *A. oryzae* could be differentiated from *A. flavus*, since the latter occasionally produces environmental survival structures like sclerotia. The present study was undertaken to isolate and identify *A. oryzae* from indigenous sources. Koji prepared using the isolated culture was compared with Japanese koji for their saccharifying capacity.

MATERIALS AND METHODS

Isolation

Samples, such as bread, cooked rice, black gram, soybean and 'kevum', assumed to be contaminated with *Aspergillus oryzae* were collected, and the fungus was isolated as given below. Samples of hyphae or spores, assumed to be *A. oryzae* based on the colour, were plated on PDA (Oxoid, U.K) as a point inoculum and incubated at 30° C until sporulation (Pitt and Hocking, 1985). Ten pure cultures of isolates were obtained by subculturing and maintained on PDA slants for further identification (Pitt and Hocking, 1985). Tane-koji (seed mold culture) obtained from Japan (Hiroshima Prefectural Food Technology Research Centre, Hiroshima) was plated on PDA and subsequently transferred to PDA slants and used as the reference strain (RJ).

Differentiation on AFPA selective medium

Ten isolates and RJ were plated in triplicate on AFPA selective medium, incubated at 30° C for 48 to 60 h and observed for reverse colour (Pitt and Hocking, 1991). All the plates were observed for further one week for changes in reverse colour during incubation at 30° C.

Morphological tests

The identification key for common *Aspergillus* species and teleomorphs (Pitt and Hockings, 1985) was used to identify the isolate that was selected based on the reverse colour on AFPA (B-1). B-1 isolate and RJ strains were inoculated in triplicate on Czapek yeast extract agar (CYA; Pitt and Hocking, 1985) and incubated at 25°C for 7 days. After the incubation period, all the plates were observed for macroscopic cultural and morphological characteristics, such as colony diameter, colony colour, conidial colour, mycelial colour, colony reverse, colony texture, nature of spore masses and resting structures.

B-1 isolate and RJ strain were used to prepare slide cultures according to the Riddle's method described by Uma (1997). The slide cultures were incubated at 31°C for 2-5 days and observed daily for microscopic characteristics, such as conidia, conidial heads, metulae (primary sterigmata) and phialides (secondary sterigmata).

Tane-koji and koji

B-1 isolate and RJ strain were used separately to prepare tane-koji according to the method described by Yoshizawa and Kishi (1985). In the preparation of koji, 1 g of tane-koji was sprinkled on 1 kg of rice (BG 358), which had been washed, soaked for 7 min, steamed for 1 h and cooled down to about 30-35°C followed by incubating at 35°C for three days. During incubation, the heap of rice grains was mixed after one day. After three days of incubation, the koji preparations, which had the growth of the mycelia on the grains, were packaged in airtight plastic boxes and stored at –18° C.

Saccharification of rice

Koji samples prepared using B-1 isolate (koji 1) and RJ strain (koji 2) as treatments and rice grains without the fungus as the negative control were used in a completely randomized design for saccharification experiment. Rice, BG 358, (100 g) was steamed for 1 h, cooled down to about 35° C and mixed with 25 g of koji 1, koji 2 or control rice grains in 500 mL conical flasks followed by addition of sterile distilled water (125 ml) in five replicates were used. The conical flasks containing the samples were loosely capped with cotton wool, mixed and held at room temperature (28±2° C) for 5 days with occasional stirring. Thereafter, the resulting suspension was filtered through two layers of sterile muslin cloth, and the filtrates were analyzed for pH, reducing sugar content and ethanol. The pH was measured using a pH meter (IM-40S TOA Electronics, Japan), ethanol percentage was determined by using an ebulliometer (Joslyn, 1970) and the reducing sugar content was determined by titration using Fehling's solution (Woodman, 1941). For determination of reducing sugar content, the filtrates were first diluted 10 times with distilled water.

RESULTS AND DISCUSSION

Isolation

Ten pure cultures assumed to be *Aspergillus oryzae* based on the yellow green colony colour on PDA (Onions, 1982) were obtained from contaminated bread, rice, black gram and soybean specimens. Though 'kevum' samples were contaminated with yellow green colour fungi, the pure cultures isolated from them did not show yellow green colony colour on PDA. This indicated that 'kevum', being a starch based oily food, was not a good source for isolation of *A. oryzae*. As other *Aspergillus* species also produce yellow-green conidial colour on PDA further confirmation tests were carried out. Pitt and Hocking (1991) reported that *A. flavus* and *A. parasiticus* produce bright orange-yellow reverse colour on AFPA medium as rapidly as 48 h during incubation at 30°C. Thus, confirmatory tests done on AFPA medium revealed that one (B-1) out of 10 pure culture isolates, which was isolated from contaminated wheat flour bread, and RJ strain did not produce any reverse colour, while the other nine pure culture isolates produced bright orange-yellow reverse colour within 48 h of incubation at 30°C. As reverse colour of B-1 isolate did not change during incubation at 30°C for further one week and was as same as RJ reference strain, the former was subjected to further morphological comparisons with the latter.

Morphological characteristics

The macroscopic characteristics such as colony diameter, colony colour, conidial colour, mycelial colour, colony reverse, colony texture, nature of spore masses and resting structures of B-1 and RJ grown on CYA medium are presented in Table 1.

Table 1. Macroscopic characteristics of B-1 isolate and RJ strain of *A. oryzae* observed after 7 days of incubation at 25° C on CYA medium.

Characteristics	CYA	
	B-1	RJ
Colony diameter (mm)	57	65
Colony colour	yellow	yellow
Conidial colour	yellow green	yellow green
Mycelial colour	whitish	whitish
Colony reverse	pale yellow	yellowish white
Colony texture	wet	wet
Nature of spore masses	powdery	powdery
Resting structures(sclerotia)	nil	nil

B-1: Isolated strain, RJ-reference strain.

The identification key for common *Aspergillus* species and teleomorphs (Pitt and Hockings, 1985) was followed when making comparisons of macroscopic colonial and morphological characteristics between B-1 and RJ to further identify the former. As the colony diameters were above 35 mm at 25°C, colonies were yellow in colour and conidia being yellow green in colour without developing cleistothecia, B-1 could be *A. flavus*, *A. oryzae* or *A. parasiticus*. However, all the macroscopic characteristics of B-1 were similar to that of RJ, indicating that the former was most likely a strain of *A. oryzae*. Furthermore, reverse colour observed on CYA medium for both B-1 isolate and RJ strain (Table 1) was in agreement with the observations made by Jernejc and Cimerman (2003) for *A. oryzae*. Moreover, smooth conidial surface and heads consisting predominantly of metulae and phialides revealed that B-1 was either *A. oryzae* or *A. flavus* (Pitt and Hocking, 1985). As these microscopic characteristics of B-1were similar to that of RJ (Plate 1) and bright orange-yellow reverse colour was not produced within 48 h when inoculated on AFPA it could be concluded that B-1 isolate was a strain of *A. oryzae* isolated locally from bread. The current methods employed for identification of the economically valuable members of *Aspergillus* section *Flavi* are still dependent primarily on macroscopic (cultural and morphological) and microscopic characteristics. In order to confirm the identity of B-1 isolate, genomic level analysis using amplified fragment length polymorphism (AFLP) could be easily carried out as it has been done recently for the same purpose (Lee *et al.*, 2004).

Preparation of koji and saccharification

Tane-koji preparation resulted in abundant yellow green colour spore formation for both B-1 and RJ strains. As koji containing significant number of spores cannot be used in the rice fermentation process due to off-flavour development (Wood, 1977), it was important to ensure that method of koji preparation would not encourage

sporulation. Preparation of koji from tane-koji, during which mixing after one day was a must to ensure uniformity of growth, maintain proper temperature and moisture content (Wood, 1977), resulted in a finished product of *A. oryzae* which, to some extent looked like rice with white frosting. This indicated that there was no spore formation during the preparation of koji.

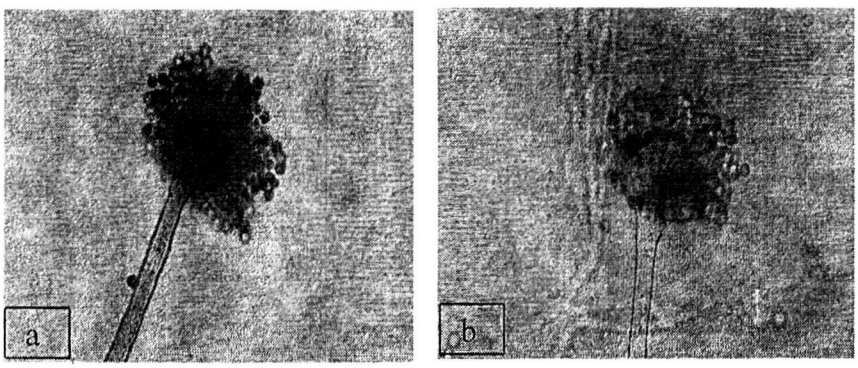

Plate 1. Asexual fruiting heads of (a) B-1 isolate and (b) RJ strain.

Conversion of rice starch into fermentable sugars by using koji is a key step in rice fermentation. Low amylose rice known as Japonica rice and koji are used in Japan to obtain fermentable sugars for subsequent sake production (Japan International Corporation, 2002; Gauntner, 1999). The ability of the *A. oryzae* strain isolated in this study to convert starch into fermentable sugars in high amylose rice, which is predominantly grown in Sri Lanka, was tested and compared with that of RJ strain. Saccharification, break down of starch by enzymes such as alpha amylase and glucoamylase present in koji, produces fermentable sugars. As the natural flora present in the environment can convert these sugars into either acids or ethanol, reducing sugar content as well as ethanol content and pH of the saccharifying solutions were analyzed to find out the saccharification ability of the koji prepared with the isolated *A. oryzae* strain. Amount of reducing sugar produced by koji-1 was significantly higher than koji-2 and the ethanol content were not significantly different (Table 2). This indicated that the strain isolated in this study could be used instead of the Japanese strain for saccharification of high amylose rice. This strain can be made available for industrial use in the form of tane-koji.

Table 2. Reducing sugar, ethanol contents and pH values resulted after saccharification of rice with *Aspergillus* strains.

Parameter	Control	Koji-1	Koji-2
pH	6.0 [a]	4.6 [b]	4.3 [c]
Ethanol (%)	2.8 [a]	4.3 [b]	4.3 [b]
Reducing sugar (g/l)	14 [a]	53 [b]	47 [c]

Each value represents mean of five replicates. Means in each row followed by the same letter are not significantly different ($p<0.05$). Koji-1 = Koji samples prepared using B-1 isolate; Koji-2 = Koji samples prepared using RJ strain.

CONCLUSIONS

Aspergillus oryzae strain isolated from bread and identified based on macroscopic and microscopic characteristics could be used for saccharification of high amylose rice.

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