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Short communication

Relationship between saccharifying capacity and isolation sources for strains of the *Rhizopus arrhizus* complex

Ling-Dan Yao [a, b], Xiao Ju [b], Timothy Y. James [c], Jun-Zhi Qiu [a], Xiao-Yong Liu [b, *]

[a] Fujian Agriculture and Forestry University, College of Life Science, Fuzhou, Fujian 350002, China
[b] State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[c] Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA

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ABSTRACT

To discover the differences and modes of saccharifying ability among *Rhizopus arrhizus* strains isolated from various habitats, we grew strains in glutinous rice media to monitor the production of glucose from starch using spectrophotometry at regular time intervals. The saccharifying capacity of *R. arrhizus* var. *delemar* was found to be weaker than that of var. *arrhizus* and var. *tonkinensis*. These data provide additional phenotypic support for recognization of this genetically distinct lineage (var. *delemar*) as a separate species. Clinical isolates were generally low in saccharifying abilities, while all strains from southwest China had an excellent diastatic power.

Rhizopus arrhizus (synonymous with the more commonly used name *R. oryzae*) has three varieties, namely var. *arrhizus*, var. *delemar* and var. *tonkinensis* (Zheng, Chen, Huang, & Liu, 2007). Abe and others, however, considered *R. arrhizus* var. *delemar* to be the independent species *R. delemar* (Abe, Oda, Asano, Asano, & Sone, 2007, 2010). These three taxa are closely related in morphology and molecular phylogeny, and examination of many features is required for effectively distinguishing them (Liou et al., 2007; Liu, Huang, & Zheng, 2007, 2008). They also have a lot of similarities in ecological characteristics and industrial applications. The species complex is widely distributed in soil, dung, decomposing mushrooms, flowers, leaves, air, and so on and may occasionally arise as an opportunistic pathogen of humans. In daily cuisine, they are usually used to ferment foods such as grains, fruits, and legumes (Hu, Li, Li, & Gu, 2008; Igor, Ramón, & Igor, 2015); Among these, tempeh made from soybean in Indonesia and Malaysia may be the most well-known production (Dinesh Babu, Bhakyaraj, & Vildhyalakshmi, 2009). They can also produce fumaric acid (Peng et al., 2016; Wang, 2014; Ying, Jia, Yang, & Wan, 2015) and malic acid (He, Li, Fu, Xu, & Huang, 2008; Liu et al., 2015) on an industrial scale. Lipase (Vipin, Sebastian, Muraleedharan, & Santhiagu, 2016; Yi, Zhao, Gong, Wang, & Tan, 2013; Ying, Fu, Xu, & Tan, 2002) and other enzymes with applications for the food industry produced by the species complex have been studied.

However, the driving characteristic behind the dominant utilization of *R. arrhizus* is its production of amylase and glucoamylase that can decompose starch into glucose (He et al., 2014; Zhou, 2016) and produce flavoring substances such as lactic acid (Ge, Pan, Zhang, Cai, & Yu, 2013; Li, Zhang, Ren, Wang, & Zhang, 2015; Ming, Yu, Xiao, Yang, & Xiong, 2013) and higher alcohols (Yang & Tang, 2016; Zeng, Zhan, & Wu, 2012), which makes them very useful in the primary fermentation of rice wine (Cai, Yi, Cheng, & Wu, 2010; Hao et al., 2010; Zheng, Zhou, Tan, & Yao, 2015). However, the mixed microbial starter used in traditional Chinese liquor production may cause control issues and problems with reproducibility. For example, long production cycles and complicated ingredients often yield harmful substances such as fusel alcohols and fungal toxins. In recent years, pure fermentation has been widely considered, because it can better control the quality of production, ensure food security (Su, Jie, Xu, & Mu, 2013; Tang, Liu, & Xiao, 2016; Yang et al., 2015; Zhao, Yang, Ma, Ling, & Ling, 2014) and the production process can be automated. Saccharifying capacity is one of the most important factors for considering the value of a microorganism for pure fermentation. In this study, pure *R. arrhizus* from various parts of Asia was selected for investigation

* Corresponding author.
E-mail address: liuxiaoyong@im.ac.cn (X.-Y. Liu).

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of differences in saccharifying capacity, as well as possible genetic or environmental factors that may predict these differences. Ultimately, high quality strains for pure production practice may be chosen using the strategy we outline in the present study.

Thirty-four strains of *R. arrhizus* were utilized (Table 1). These cultures are from China, Japan, India and Indonesia, and their substrates include air, soil, mud, dung, flour, cake, candy wrapping, grass, stem, flowers, fruit, shells, rice fermentation starter, human skin and eyes. These strains were preserved in the State Key Laboratory of Mycology (R- or XY) and the China General Microbial Culture Collection, Institute of Microbiology, Chinese Academy of Sciences (CGMCC), or in the Biological Resource Center, National Institute of Technology and Evaluation, Japan (NBRC).

For preparation of glutinous rice media, polished glutinous rice (50 g) and 80 mL of distilled water were added into a 250 mL flask, covered with aluminum foil and heated in a water bath for 20 min on an induction cooker (C21-SDHC04, Zhejiang Supor Life Electric Co., Ltd.). After that, the flask was autoclaved under 1×10^5 Pa for 30 min (YXQ-LS-50A vertical high pressure steam sterilization pot, Shanghai Bo News).

Rhizopus arrhizus was cultivated on PDA (200 g potato, 20 g glucose, 20 g agar, 1 L distilled water) at 30 °C for 15 d. Hyphae, sporangiophores and sporangia were scraped from the surface of the culture with a surgical knife and transferred into a 50 mL centrifuge tube. Sterile distilled water (20 mL) was added and sporangiospores were released from sporangia by vigorously shaking. Then, the suspension was filtered through gauze to dislodge hyphae and sporangiophores. Finally, the number of sporangiospores was counted with a blood cell counting chamber, calibrated to 6.25×10^6 sporangiospores/mL, and used for cultivation as soon as possible.

Calibrated sporangiospore suspensions (20 mL) in sterile distilled water were inoculated into the glutinous rice media and incubated at 30 °C and 130 r/min. Zymotic fluid (1 mL) was sampled for all strains every 12 h until 168 h. After that, 18 strains were sampled once again at 372 h; 6 strains were sampled three more times at 180 h, 192 h and 372 h; and 10 strains were sampled four more times at 180 h, 192 h, 204 h and 372 h. This later period of sampling with longer intervals was used to investigate strains that showed latent growth patterns. Each strain was represented by three replicate cultures. Each biological replicate was tested three times as technical replicates, providing nine estimates for each strain for each time point.

A set of glucose (D-Glucose A.R., Beijing Chemical Works) standards in sterile distilled water was created at the following final concentrations: 0 mg/mL, 0.18 mg/mL, 0.36 mg/mL, 0.54 mg/mL, and 0.72 mg/mL. Then, 0.1 mL of DNS solution (6.3 g 3,5-Dinitrosalicylic acid, 185 g seignette salt, 5 g phenol, 5 g $NaSO_3$, 262 mL of 2 M NaOH, 738 mL distilled water) was added to 3.2 mL of each glucose standard and boiled for 5 min. The OD value was recorded at 520 nm and consequently a standard curve was created.

DNS solution (0.1 mL) was added into 0.2 mL of zymotic fluid and boiled for 5 min. When cooled to room temperature, 3 mL of distilled water was added to make a final volume of 3.3 mL. The OD

Table 1
Maximum glucose concentrations of zymotic fluid with *Rhizopus arrhizus*.

Strain	CGMCC	Variety	Substrate	Sources[b]	Country	Province	MGC[c] (g/mL)	Peak[c] Time (h)	Capacity[d]	Speed[e]	Type
R-027	3.9500	*delemar*	soil	W	China	Zhejiang	0.034	152	+	##	I
R-028	3.9501	*arrhizus*	flour	W	China	Hubei	0.034	160	+	##	I
R-029	3.9502	*delemar*	decaying flower	W	China	Beijing	0.034	96	+	##	I
R-030	3.9503	*delemar*	cake	W	China	Guangxi	0.034	144	+	##	I
R-141	3.9476	*delemar*	skin lesion	C	China	Shanghai	0.060	136	+	##	I
R-037	3.1136	*delemar*	air	W	China	Beijing	0.275	120	++	##	II
R-211	3.9493	*delemar*	skin lesion	C	China	Hebei	0.316	120	++	##	II
R-212	3.9531	*arrhizus*	eye socket	C	China	Beijing	0.416	120	++	##	II
R-720	3.15794	*delemar*	skinscab	C	China	Hebei	0.319	144	++	##	II
XY00406	3.4966	*arrhizus*	koji	D	Japan		0.234	128	++	##	II
XY00495	3.4995	*delemar*	distillery yeast	D	India		0.300	120	++	##	II
R-031	3.9504	*delemar*	mud	W	China	Guangxi	0.671	192	+++	##	III
R-032	3.15791	*arrhizus*	decaying fruit	W	China	Guangxi	0.683	192	+++	##	III
R-033	3.9505	*delemar*	decaying flower	W	China	Shanghai	0.909	136	+++	##	III
R-143	3.9477	*arrhizus*	soil	W	China	Gansu	0.685	156	+++	##	III
R-147	3.9478	*arrhizus*	decaying flower	W	China	Yunnan	0.925	144	+++	##	III
R-155	3.9516	*arrhizus*	decaying flower	W	China	Yunnan	1.084	180	+++	##	III
R-165	3.9518	*arrhizus*	decaying grass	W	China	Sichuan	1.075	136	+++	##	III
R-166	3.9483	*delemar*	candy wrapping	W	China	Guizhou	0.992	136	+++	##	III
R-171	3.9484	*delemar*	soil	W	China	Sichuan	0.746	132	+++	##	III
R-210	3.9492	*arrhizus*	decaying plant	W	China	Chongqing	1.114	168	+++	##	III
R-237	3.9495	*arrhizus*	Chinese yeast	D	China	Sichuan	1.026	160	+++	##	III
R-248	3.9510	*arrhizus*	distillery yeast	D	China	Guangdong	0.984	144	+++	##	III
R-324	3.9496	*arrhizus*	decaying stem	W	China	Zhejiang	0.687	192	+++	##	III
R-487	3.9533	*arrhizus*	decaying shell	W	China	Hebei	1.018	136	+++	##	III
XY00409	3.4967	*tonkinensis*	soil	W	Japan		0.681	192	+++	##	III
XY00410	5318[a]	*tonkinensis*	soil	W	Japan		0.896	204	+++	##	III
R-152	3.9479	*delemar*	soil	W	China	Yunnan	0.748	372	+++	#	IV
R-156	3.9480	*delemar*	candy wrapping	W	China	Yunnan	0.839	372	+++	#	IV
R-167	3.9519	*arrhizus*	soil	W	China	Guizhou	1.134	372	+++	#	IV
R-420	3.15792	*tonkinensis*	candy wrapping	W	China	Jilin	0.902	372	+++	#	IV
R-431	3.15793	*tonkinensis*	soil	W	China	Jilin	0.975	372	+++	#	IV
R-495	3.9534	*tonkinensis*	dung	W	China	Tibet	0.734	372	+++	#	IV
XY00438	3.4976	*tonkinensis*	Chinese yeast	D	China	Zhejiang	0.918	372	+++	#	IV

[a] This strain is received as NBRC 5318.
[b] C, clinical; D, domesticated; W, wild.
[c] MGC (maximum glucose concentration) and peak time from Fig. 1.
[d] +, ++, and +++ represent weak, medium, and strong saccharifying capacity, respectively.
[e] Strains reach MGC slowly (#) and fast (##).

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