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Effects of temperature and relative humidity on growth and enzyme production by *Actinomucor elegans* and *Rhizopus oligosporus* during sufu pehtze preparation

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Abstract

Sufu is a Chinese soybean cheese obtained after maturation of solid-state mould-fermented tofu. Ambient temperatures of 30–35 °C during the summer season prohibit the use of the usual starter *Actinomucor elegans*. We compared the properties of the latter with a potential alternative starter *Rhizopus oligosporus* that could be used at higher temperatures. The effects of temperature and relative humidity on growth rate of *Actinomucor elegans* and *Rhizopus oligosporus* were optimum at 25 °C at RH 95–97%, and 35 °C at RH 95–97%, respectively. Yields of protease (108 U/g pehtze), lipase (172 U/g) and glutaminase (176 U/g) by *A. elegans* were maximum after 48 h at 25 °C and RH 95–97%, and for α -amylase (279 U/g pehtze) and α -galactosidase (227 U/g) at 30 °C and RH 95–97% after 48 and 60 h of incubation. Highest protease (104 U/g pehtze), and lipase (187 U/g) activities of *R. oligosporus* were observed after 48 h at 35 °C and RH 95–97%, while maximum α -amylase (288 U/g pehtze) and glutaminase (187 U/g) activities were obtained after 36 h at 35 °C and RH 95–97%. Maximum α -galactosidase activity (226 U/g) by *R. oligosporus* was found after 36 h at 30 °C and RH 95–97%. It is concluded that *R. oligosporus* is a potential alternative to *A. elegans* as sufu pehtze starter during hot seasons.

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Keywords: Sufu pehtze; *Actinomucor elegans*; *Rhizopus oligosporus*; Enzyme activity

1. Introduction

Sufu, *Fu-ru* written in hieroglyphics, is a traditional Chinese fermented soybean curd and a highly flavoured, soft creamy cheese-type product, which can be used in the same way as cheese (Steinkraus, 1996; Su, 1986). This fermented product has been widely consumed by Chinese people as an appetizer for many centuries.

There are many different types of sufu, produced by different local processes in China (Wang & Du, 1998); mould-fermented sufu is the most popular type (Han, Rombouts, & Nout, 2001). Four stages are involved in preparing this type of sufu: (1) Preparing tofu (soybean curd), (2) Preparing pehtze (*pizi*) by fungal solid-state

fermentation of tofu, (3) Salting of pehtze, (4) Ripening in dressing brine (Wang & Hesseltine, 1970).

Pehtze, fresh soybean curd overgrown with fungal mycelium is produced by means of solid-substrate fermentation after inoculation (about 48 h) with pure culture moulds. In commercial practice, *Actinomucor* spp., *Mucor* spp and *Rhizopus* spp. are used for sufu preparation. Among them, *Actinomucor elegans* and *Actinomucor taiwanensis* seem to be the most frequently used for commercial sufu production in China. However, these two mould species only grow well at 25–30 °C, so it is impossible to produce sufu during the hot summer with indoor factory temperatures reaching 35 °C or even higher (Han et al., 2001). Since *Rhizopus oligosporus* grows well at higher temperatures (up to 40 °C; Han & Nout, 2000), it might be used as a starter to produce sufu during this season.

Protein is the main component in tofu. Among the fungal enzymes formed on tofu, proteases have received

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much attention. Using wheat bran, wheat, and soybean as substrates, Wang, Vespa, and Hesseltine (1974) investigated the incubation conditions for maximum production of acid protease by *Mucor dispersus*, *A. elegans* and *R. oligosporus*. Whereas Chou, Ho, and Tsai (1988) studied the enzymes produced by *A. taiwanensis* on tofu, there are few published data describing the enzymes produced by *A. elegans* and *R. oligosporus* during sufu pehtze preparation.

In the present study, we determined the effects of physical process parameters that can be monitored and controlled easily under factory conditions. As criteria for the comparison of the two fungal species, we selected relative humidity, incubation temperature and time, and studied their relation with the growth of *A. elegans* and *R. oligosporus* by measuring their biomass increment, and their enzyme production during the preparation of pehtze. Protease, lipase, α -amylase, glutaminase, and α -galactosidase, affecting the flavour and texture of sufu during the ripening period, were assessed.

2. Materials and methods

2.1. Microorganisms

Actinomucor elegans [Academia Sinica (AS) 3.227] and *Rhizopus oligosporus* (NRRL 5905) isolated originally from commercial sufu and tempe, respectively, were grown on malt extract agar (MEA, Oxoid CM 59) and maintained at 4 °C. After incubation at 30 °C for 5 days, spore suspensions ($\sim 10^5$ cfu/ml) were harvested by scraping the sporangia off the agar and suspending them into sterile distilled water with 0.85% salt, 0.1% peptone and 0.05% Tween 80. For each series a fresh spore suspension was prepared.

2.2. Determination of biomass formation rate $[d(x^{1/3})/dt]$ on membrane-covered tofu

The tofu used for biomass monitoring was provided by Beijing WangZhiHe sufu manufacture, and was cut into circular pieces (diam 8.5 cm $\times$ height 0.8 cm) and then immersed in 1% w/v citric acid solution for 5 min to inhibit growth of contaminating microbes. Proximate analysis of the tofu revealed that it contained approximately 76.8% moisture, 12.5% crude protein, 5.7% crude lipid, and 3.2% carbohydrate. The piece of tofu was fitted into a sterile glass Petri dish, and a membrane (Nylon Transfer Membrane, Schleicher & Schuell, no: 10416116, Pore hole 0.45 μ m) was placed onto the tofu (Nagel, Oostra, Tramper, & Rinzema, 1999). The membrane-covered tofu was inoculated at the centre of the membrane with a drop (0.02 ml) of spore suspension, and then incubated in a ventilated climate incubator at controlled temperature (20, 25, 30, 35 or 40 °C) and

controlled relative humidity (RH) (73–75, 84–86, or 95–97%).

At regular intervals duplicate Petri dishes were removed, and mycelium with membrane was taken off from a tofu. Subsequently the mycelium was dried for 2 days at 80 °C, and the mycelial dry weight was determined gravimetrically.

Growth curves based on the cubic root of mycelial dry weights were fitted using linear regression (Micro-soft Excel) to determine the biomass formation rate $[d(x^{1/3})/dt]$ (Han & Nout, 2000).

2.3. Pehtze preparation

Tofu, as mentioned earlier, was cut into pieces (3.2 $\times$ 3.2 $\times$ 1.6 cm) for pehtze preparation. The pieces were inoculated with *A. elegans* and *R. oligosporus*, respectively, by spraying spore suspension onto the surface of each. The inoculated tofu was placed evenly spaced in plastic trays, and incubated in a ventilated climate incubator, at controlled temperature (25, 30, or 35 °C) and controlled RH (73–75, 84–86, or 95–97%). Fresh pehtze (or tofu if not real pehtze) were obtained for analyses after being incubated for 12, 24, 36, 48, and 60 h.

2.4. Enzyme assays

2.4.1. Crude extracts

Duplicate pehtze samples (about 10 g) were mixed with 50 ml of 0.3 M NaCl and 0.2 M phosphate buffer (pH 6.8) and homogenized in a blender. They were kept at room temperature for 60 min with frequent stirring, and then centrifuged at 2100 $\times g$ for 5 min. The supernatant was used as crude enzyme extract. All samples were analysed in duplicate ($n=4$).

2.4.2. Protease activity

The method mentioned by Kruger (1973) was used. 2 ml of crude enzyme extract and 2 ml of 2.4% azocasein (Sigma A-2765) dissolved in 0.05 M McIlvaine citric acid–disodium phosphate buffer (pH 6.0) were mixed. The mixture was then shaken gently for 2 h in a shaking bath at 35 °C. The reaction was terminated by addition of 5 ml of 10% TCA, and the mixture was filtered. 5 ml of 0.5 M NaOH was added to 5 ml of the filtrate and after 20 min the absorbance of the solution was read at 440 nm. One unit of protease activity is defined as an absorbance (440 nm) change of 0.01 after 2 h at 35 °C. (UV–vis Spectrophotometer, UV mini 1240, SHIMADZU, Japan)

2.4.3. Lipase activity

Titrimetric determination of lipase (Lipase kit, Sigma Catalogue no. 800-B) was carried out. A mixture of 1.0 ml crude enzyme extract, 2.5 ml water, 3.0 ml Sigma

Lipase Substrate (Catalogue no. 800–1), and 1.0 ml TRIZMA Buffer (Sigma Catalogue no. 800–2) was shaken at 37 °C for 6 h. The reaction was stopped by adding 3.0 ml 95% ethanol, and 4 drops of Thymolphthalein Indicator Solution (Catalog No. 800–3) were added, followed by titration with 0.05 M NaOH solution until a slight blue colour was observed. Sigma-Tietz Units of lipase are equal to the volume (ml) of 0.05 M NaOH required to neutralize the fatty acids liberated during the incubation. Lipase activity in Sigma-Tietz Units/ml was converted to International Units multiplying by 280.

2.4.4. α -Amylase

The activity of α -amylase was determined by a colorimetric method as follows. The reaction mixture, which contained 10 ml of 1% soluble starch and 5 ml of 0.1 M phosphate buffer (pH 6.0), was equilibrated at 60 °C for 5 min. Then, 1.0 ml of the crude enzyme extract was added into the mixture and it was kept at 60 °C for 10 min. Next, 1 ml of the mixture was added to 5 ml of a solution containing 0.44 mg iodine and 0.2 g KI. The absorbance of the solution was measured at 660 nm, and α -amylase activity was calculated using a standard curve made with commercial α -amylase (Microbiological Media Product, Beijing). For practical reasons we defined one unit of α -amylase as the activity catalysing the hydrolysis of 1 ml of 1% starch solution in 1 h under the assay conditions, in comparison with α -amylase of known activity.

2.4.5. Glutaminase

A modification of the method of Moriguchi, Sakai, Tateyama, Furuta, and Wakayama (1994) was used, monitoring the formation of L-glutamate with L-glutamate dehydrogenase. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.5), 30 mM L-glutamine (Sigma G-3126), 5% NaCl and crude enzyme extract (0.1 ml) in a final volume of 0.5 ml. After being allowed to react for 10 min at 30 °C, the reaction was terminated by boiling for 3 min. 50 mM Tris–hydrazine buffer (pH 9.0), 1.5 mM NAD⁺ (Sigma N-7004), 0.5 mM ADP (Sigma A-2754) and 5 units/ml of glutamate dehydrogenase (Sigma G-2501) were added in a total volume of 1.0 ml to this supernatant after centrifugation (Centrifuge CT4D, HITACHI, Japan). The absorbance at 340 nm was measured after incubating the mixture for 1 h at 30 °C. One unit of glutaminase activity was defined as a change in the absorbance at 340 nm of 0.1 at 30 °C.

2.4.6. α -Galactosidase

The combined method described by Chou et al. (1988) and van den Broek, Ton, Verdoes, van Laere, Voragen, and Beldman (1999) was used. The reaction mixture, which contained 0.1 ml of 0.01 M *p*-nitrophenyl- α -D-galactopyranose (PNPG, Sigma N-0877), 0.3 ml of 0.2

M phosphate buffer (pH 6.4), and 0.1 ml of crude enzyme extract, was incubated at 37 °C for 20 min. The reaction was stopped by adding an equal volume of 0.5 M glycine/NaOH buffer (pH 9) containing 2 mM EDTA. The colour formation was measured at 400 nm. The α -galactosidase activity was then assayed by measuring the amount of *p*-nitrophenol liberated from PNPG at 37 °C in 20 min using a standard curve. One unit of α -galactosidase was defined as the activity liberating 1 μ g *p*-nitrophenol per min under the specified conditions.

3. Results and discussion

3.1. Effect of incubation temperature and RH on fungal growth rate on membrane-covered tofu

Previously, Chou et al. (1988) and Wang et al. (1974) monitored the growth by visual estimation, which only provides an indication of mycelial formation. In this study, a nylon membrane separated the mycelium from the tofu substrate. Previously, we found that fungal growth is not affected by the presence of a membrane, whereas this technique offers the possibility to separate the biomass and determine its weight. Similar experiments were reported by Mitchell, Doelle, and Greenfield (1989) and Nagel et al. (1999).

Fig. 1 shows the effect of incubation temperature and RH on the biomass formation rate expressed as $[d(x^{1/3})/dt]$. RH levels varied from 73–75% to 95–97% and temperatures from 20 °C to 40 °C. The optimum growth temperatures and RH for *A. elegans* and *R. oligosporus* were 25 °C at 95–97%, and 35 °C at 95–97%, respectively. At constant temperature, the formation rates were positively correlated with RH. The formation rate of *A. elegans* declined sharply at temperatures exceeding 30 °C. On the other hand, the formation rate of *R. oligosporus* was still quite high at 40 °C.

3.2. Effect of incubation temperature and RH on enzyme activities expressed in pehtze

Protease production by *A. elegans* and *R. oligosporus* is presented in Table 1. The protease activities produced by the tested mould strains on tofu were considerably affected by the incubation temperature and humidity. At higher RH, higher protease activities were obtained. The highest protease activity (108 U/g of pehtze d.m.) of *A. elegans* was found after 48 h incubation at 25 °C and RH 95–97%. For *R. oligosporus* the highest activity (104 U/g of pehtze d.m.) was obtained after 48 h incubation at 35 °C and RH 95–97%.

A. elegans and *R. oligosporus* produced relatively high lipase activities on tofu under optimum conditions (Table 2). The highest lipase activity (172 U/g pehtze

Fig. 1. Biomass formation rate $[d(x^{1/3})/dt]$ of *Actinomucor elegans* AS3.227 and *Rhizopus oligosporus* NRRL5905 as a function of temperature and relative humidity.

d.m.) produced by *A. elegans* was found after 48 h incubation at 25 °C and RH 95–97%. Although lipase activity produced by *R. oligosporus* was not strongly affected by incubation temperature and RH, the maximum activity (187 U/g pehtze d.m.) was observed after 48 h incubation at 35 °C and RH 95–97%.

Similarly to the production of protease and lipase, *A. elegans* produced more α -amylase activity at higher RH levels (Table 3). Although the optimum growth temperature for *A. elegans* was 25 °C, the highest yield of α -amylase (279 U/g pehtze d.m.) was observed after 48 h incubation at 30 °C and RH 95–97%. The enzyme activity declined after 60 h. *R. oligosporus* produced maximum α -amylase activity (288 U/g pehtze d.m.)

after 36 h of incubation at 35 °C and RH 95–97%; the enzyme activity decreased after 48 h.

Glutaminase is considered an important key enzyme for the palatable taste of fermented soybean foods (Lu, Yu, & Chou, 1996), and expectedly, the predominance of glutamate in sufu (Liu & Chou, 1994) results from glutaminase activity. Glutamate in combination with salt (NaCl) contributes to the flavour and hedonic characteristics of foods (Halpern, 2000). Table 4 shows that the highest glutaminase activity (176 U/g pehtze d.m.) of *A. elegans* was formed at 25 °C and RH 95–97% after 48 h of incubation. The highest yield of glutaminase (187 U/g pehtze d.m.) by *R. oligosporus* was observed at 35 °C and RH 95–97% after 36 h of cultivation. Large amounts

Table 1

Protease activity (U/g peptze dry matter) production by *Actinomucor elegans* AS3.227 and *Rhizopus oligosporus* NRRL5905 at various incubation temperatures and relative humidities (RH)

Mould	RH (%)	Temperature (°C)														
		25					30					35				
		Incubation time (h)					Incubation time (h)					Incubation time (h)				
		12	24	36	48	60	12	24	36	48	60	12	24	36	48	60
<i>A. elegans</i>	73–75	4.4±0.8	36.4±8.8	58.3±10.0	61.2±4.2	60.3±5.6	6.4±1.3	43.4±7.4	52.3±8.4	61.2±5.4	60.1±3.4	2.3±0.1	8.1±2.0	13.3±3.0	14.5±1.3	14.3±1.1
	84–86	5.2±0.7	52.1±5.7	64.0±5.1	82.3±5.5	75.2±4.7	8.1±1.1	53.1±3.0	64.2±0.1	81.9±11.2	73.3±4.2	2.2±0.3	7.4±0.9	11.4±1.6	16.2±2.8	12.3±0.5
	95–97	5.2±0.8	58.3±4.2	80.4±5.9	108±13.8	90.3±8.6	12.1±0.5	56.3±1.2	85.4±4.6	96.3±5.9	81.3±7.9	2.1±0.2	10.5±1.1	12.3±1.1	15.4±1.3	16.4±1.6
<i>R. oligosporus</i>	73–75	5.2±1.8	38.4±4.0	46.2±5.6	61.9±1.9	72.2±7.9	6.3±1.6	46.2±5.9	69.5±4.5	77.3±5.8	58.7±6.7	11.8±1.6	57.9±4.9	76.2±5.2	86.8±6.2	77.2±7.4
	84–86	5.6±0.7	46.1±5.7	58.7±6.6	78.4±3.1	77.9±9.3	6.2±0.9	58.6±1.9	78.3±1.2	91.8±8.5	87.7±7.5	19.2±2.4	67.5±1.2	81.3±4.6	97.1±3.5	74.8±4.8
	95–97	7.4±1.0	39.3±4.2	63.9±1.1	72.3±8.3	72.7±4.7	7.2±1.3	56.1±1.9	82.5±6.2	97.9±4.3	91.4±5.6	20.4±2.5	64.5±1.5	94.4±6.7	104±8.8	103±9.8

Data represent averages±standard deviations of duplicate analyses of duplicate samples.

Table 2

Lipase activity (U/g peptze d.m.) production by *Actinomucor elegans* AS3.227 and *Rhizopus oligosporus* NRRL5905 at various incubation temperatures and relative humidities (RH)

Mould	RH (%)	Temperature (°C)														
		25					30					35				
		Incubation time (h)					Incubation time (h)					Incubation time (h)				
		12	24	36	48	60	12	24	36	48	60	12	24	36	48	60
<i>A. elegans</i>	73–75	15.6±2.3	56.2±7.4	113.8	123±9.9	105±7.0	14.2±1.7	62.3±6.1	96.4±7.3	131±5.7	105±6.1	10.2±0.9	35.2±2.6	44.6±7.1	56.4±3.1	55.3±5.1
	84–86	14.3±1.6	88.3±5.5	122±9.6	146±5.7	143±8.2	16.7±2.3	91.2±5.9	118±7.4	154±7.1	137±4.2	9.8±1.1	37.3±3.5	42.1±6.6	62.8±3.8	60.8±4.1
	95–97	17.4±2.3	93.6±6.7	135±10.0	172±11.3	168±9.4	17.3±1.1	102±8.5	120±5.8	147±4.2	135±10.0	11.7±1.5	31.2±2.8	45.9±3.6	59.2±4.1	61.6±7.8
<i>R. oligosporus</i>	73–75	12.3±1.2	78.4±4.8	90.6±7.7	119±5.7	123±9.3	13.3	84.3±6.1	93.3±7.7	128±8.4	119±6.1	12.5±1.0	87.2±4.0	99.1±9.7	129±7.8	115±5.1
	84–86	16.8±1.4	83.9±1.8	127±3.8	154±5.7	158±6.9	18.4±1.2	96.8±4.0	125±7.5	169±11.3	172±8.4	19.7±2.3	96.4±5.1	143±12.4	156±8.5	155±9.7
	95–97	15.8±1.7	92.2±5.1	133±10.2	179±9.9	170±6.4	18.9±1.6	106±5.7	144±7.2	177±10.1	183±8.1	20.1±2.6	95.1±5.0	148±11.6	187±5.7	178±9.9

Data represent averages±standard deviations of duplicate analyses of duplicate samples.

Table 3

α -Amylase activity (U/g pehtze d.m.) production by *Actinomucor elegans* AS3.227 and *Rhizopus oligosporus* NRRL5905 at various incubation temperatures and relative humidities (RH)

Mould	RH (%)	Temperature (°C)														
		25					30					35				
		Incubation time (h)					Incubation time (h)					Incubation time (h)				
		12	24	36	48	60	12	24	36	48	60	12	24	36	48	60
<i>A. elegans</i>	73–75	56.4±3.8	157±4.2	198±9.9	231±4.2	167±17.0	67.4±3.5	162±5.7	206±5.7	248±18.4	187±9.9	12.5±3.9	34.3±4.2	58.1±4.4	79.2±11.8	75.8±4.2
	84–86	64.5±4.0	167±5.7	219±4.2	251±4.2	213±9.9	76.2±9.9	173±7.1	227±4.2	267±4.4	235±14.1	13.4±3.7	38.6±1.1	64.2±5.7	86.7±6.4	76.1±6.1
	95–97	76.2±8.8	178±5.7	236±14.1	269±5.7	223±11.3	88.4±4.5	187±8.5	243±4.2	279±12.7	226±5.7	11.4±1.7	33.9±2.6	65.2±4.1	57.2±2.6	67.9±10.6
<i>R. oligosporus</i>	73–75	34.6±3.5	126±5.7	176±9.9	204±8.5	187±7.1	56.3±3.4	187±2.8	238±8.5	212±11.3	209±4.2	49.5±5.5	197±7.1	249±4.2	225±11.3	217±4.3
	84–86	46.5±5.9	134±5.7	198±9.9	226±5.6	223±4.2	57.7±5.2	198±9.9	265±9.9	243±21.2	224±18.3	68.7±3.3	228±8.5	282±17.0	264±25.5	245±14.1
	95–97	58.4±4.5	149±5.7	213±9.9	238±11.3	225±5.7	67.5±3.8	176±8.5	287±9.9	257±5.7	253±8.5	76.1±5.8	249±7.1	288±17.0	274±8.5	259±12.7

Data represent averages±standard deviations of duplicate analyses of duplicate samples.

Table 4

Glutaminase activity (U/g pehtze d.m.) production by *Actinomucor elegans* AS3.227 and *Rhizopus oligosporus* NRRL5905 at various incubation temperatures and relative humidities (RH)

Mould	RH (%)	Temperature (°C)														
		25					30					35				
		Incubation time (h)					Incubation time (h)					Incubation time (h)				
		12	24	36	48	60	12	24	36	48	60	12	24	36	48	60
<i>A. elegans</i>	73–75	25.3±1.8	57.1±4.2	91.2±5.5	105±7.7	110±5.4	24.5±2.7	68.1±3.7	95.7±8.9	111±4.8	121±10.4	8.35±1.1	23.8±1.6	36.4±3.1	31.5±1.6	32.6±3.3
	84–86	34.2±1.5	76.4±7.9	128±9.4	147±9.6	136±9.3	35.4±1.9	77.6±7.1	128±5.6	167±13.2	155±9.8	8.26±2.0	24.6±3.3	28.4±2.0	27.8±2.4	24.5±2.8
	95–97	49.2±3.4	95.8±7.7	158±11.1	176±12.0	167±7.1	36.5±3.6	72.3±4.0	139±10.2	168±11.8	159±4.7	8.41±0.8	31.2±2.1	33.4±1.9	36.1±3.7	35.6±4.2
<i>R. oligosporus</i>	73–75	16.2±1.2	48.7±6.4	98.3±4.3	106±9.4	103±5.0	19.3±1.7	55.8±2.5	110±6.1	118±6.9	116±9.3	34.2±4.5	67.5±3.9	127±9.1	145±8.4	138±11.2
	84–86	27.1±1.8	61.4±3.8	119±2.8	128±6.7	118±10.4	43.6±2.8	71.5±3.8	146±11.3	154±10.9	136±13.2	46.3±5.3	97.5±7.9	167±12.0	178±14.7	164±8.6
	95–97	27.9±2.6	79.4±6.9	164±10.3	169±9.4	171±12.3	58.4±4.4	83.2±7.7	179±8.9	170±11.0	176±12.1	58.4±3.2	106±6.5	187±11.8	176±12.6	177±11.5

Data represent averages±standard deviations of duplicate analyses of duplicate samples.

of glutaminase are a favourable prerequisite for production of pehtze with highly appreciated palatability.

Soybean contains low-molecular-weight saccharides such as stachyose and raffinose, which may be involved in flatulence resulting from ingestion of soybean products. Hydrolysis of stachyose and raffinose by α -galactosidase has been suggested as a way to resolve the flatulence problem (Hayakawa, Mizutani, Wada, Masai, Yoshihara, & Mitsuoka, 1990; Sugimoto & van Buren, 1970). Therefore, it is of interest to investigate α -galactosidase production by *A. elegans* and *R. oligosporus*.

Within the conditions investigated (Table 5), *A. elegans* produced the highest α -galactosidase activity (227 U/g pehtze d.m.) at 30 °C and RH 95–97% after 60 h of incubation. *R. oligosporus* yielded the highest α -galactosidase activity (226 U/g pehtze d.m.) at 30 °C and RH 95–97% after 36 h of incubation. The optimum temperature for production of α -galactosidase by both moulds was 30 °C, which did not exactly follow the trends of optimum growth temperatures. We observed that *A. elegans* grows well at 25–30 °C and RH 84–97%, and produces considerable enzyme activities after 48 h, whereas it poorly tolerates higher temperatures (35 °C). On the other hand, *R. oligosporus* could grow very well at 35–40 °C, and yield a very similar pattern of enzyme activity when compared with *A. elegans*. We conclude that, from the point of view of growth and extracellular enzyme production, *R. oligosporus* is able to produce similar levels of biomass and enzyme activities as *A. elegans* at temperatures that are about 10 °C higher than those tolerated by the latter. Therefore, *R. oligosporus* could be an alternative for *A. elegans* as the starter of sufu production during hot seasons. It remains to be established whether or not sufu produced with *R. oligosporus* has a flavour and taste similar to sufu from *A. elegans*.

The flavour and texture of sufu that develop during the aging are determined by the enzymes produced by the mould in pehtze. There is so far no indication of metabolites also contributing to the flavour. The data obtained in this study demonstrate that growth and enzyme production by *A. elegans* and *R. oligosporus* on tofu were influenced by incubation temperature, humidity, and time. From these results it is obvious that sufu manufacturers should pay attention to the control of these conditions. The fluctuation of incubation conditions could result in sub-optimum flavour and texture, or even in putrefaction. Control of incubation conditions will also contribute to reduce the variability in quality from batch to batch.

In conclusion *R. oligosporus* has been shown to have similar growth and enzyme production abilities as *A. elegans*. Consequently it will now be of interest to evaluate the feasibility and acceptability of the use of *Rhizopus* strains in sufu production.

Table 5
 α -Galactosidase activity (U/g pehtze d.m.) production by *A. elegans* AS3.227 and *R. oligosporus* NRRL5905 at various incubation temperatures and relative humidities (RH)

Mould	RH (%)	Temperature (°C)	25						30						35					
			Incubation time (h)						Incubation time (h)						Incubation time (h)					
			12	24	36	48	60	12	24	36	48	60	12	24	36	48	60			
<i>A. elegans</i>	73–75	23.2±2.1	58.8±6.3	98.3±6.4	129±9.4	124±9.5	41.6±3.6	87.2±6.7	119±9.1	167±10.7	174±7.3	12.4±2.2	26.1±2.1	41.3±2.9	32.7±3.0	34.6±2.5				
	84–86	31.2±3.0	84.5±7.5	137±7.3	181±13.5	184±10.8	48.1±2.9	91.2±7.9	164±9.9	210±15.5	216±15.5	7.2±1.6	24.1±1.5	35.4±1.8	31.3±2.4	32.4±2.4				
	95–97	42.1±1.9	95.4±4.7	158±11.0	197±12.1	184±7.6	56.2±4.8	110±5.4	189±16.3	225±12.4	227±14.3	7.2±1.0	21.5±1.1	38.4±1.7	24.1±1.3	26.4±2.8				
<i>R. oligosporus</i>	73–75	35.7±4.2	65.2±6.1	158±14.3	179±13.4	142±10.8	34.6±4.4	120±7.8	189±12.8	187±10.6	178±11.8	58.4±2.6	91.2±4.9	178±11.2	192±11.7	176±15.4				
	84–86	48.3±2.3	87.2±5.8	149±10.6	197±9.4	168±13.0	56.2±3.3	91.4±6.9	167±10.3	215±11.9	208±12.9	69.3±4.7	98.1±5.6	188±9.9	216±16.5	213±12.6				
	95–97	57.4±3.5	130±9.0	189±11.2	178±12.6	168±11.3	42.6±2.9	140±12.0	226±16.6	208±10.8	201±9.1	57.2±3.5	133±10.4	210±13.5	198±13.8	188±11.9				

Data represent averages ± standard deviations of duplicate analyses of duplicate samples.

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References

- Chou, C.-C., Ho, F.-M., & Tsai, C.-S. (1988). Effects of temperature and relative humidity on the growth of and enzyme production by *Actinomucor taiwanensis* during sufu pehtze preparation. *Applied and Environment Microbiology*, 54(3), 688–692.
- Halpern, B. P. (2000). The use and utility of glutamates as flavoring agents in food—glutamate and the flavor of food. *Journal of Nutrition*, 130, 910S–914S.
- Han, B.-Z., & Nout, M. J. R. (2000). Effects of temperature, water activity and gas atmosphere on mycelial growth of tempe fungi *Rhizopus microsporus* var. *microsporus* and *R. microsporus* var. *oligosporus*. *World Journal of Microbiology & Biotechnology*, 16, 853–858.
- Han, B.-Z., Rombouts, F. M., & Nout, M. J. R. (2001). A Chinese fermented soybean food. *International Journal of Food Microbiology*, 65(1–2), 1–10.
- Hayakawa, K., Mizutani, J., Wada, K., Masai, T., Yoshihara, I., & Mitsuoka, T. (1990). Effects of soybean oligosaccharides on human faecal flora. *Microbial Ecology in Health & Disease*, 3, 293–303.
- Kruger, J. E. (1973). Change in the levels of proteolytic enzymes from hard red spring wheat during growth and maturation. *Cereal Chemistry*, 5, 122–131.
- Liu, Y.-H., & Chou, C.-C. (1994). Contents of various types of proteins and water soluble peptides in sufu during aging and the amino acid composition of tasty oligopeptides. *Journal of the Chinese Agricultural Chemical Society*, 32(3), 276–283 (in Chinese, with English abstract).
- Lu, J.-M., Yu, R.-C., & Chou, C.-C. (1996). Purification and some properties of glutaminase from *Actinomucor taiwanensis*, starter of sufu. *Journal of the Science of Food and Agriculture*, 70, 509–514.
- Mitchell, D. A., Doelle, H. W., & Greenfield, P. F. (1989). Suppression of penetrative hyphae of *Rhizopus oligosporus* by membrane filters in a model solid-state fermentation system. *Biotechnology Techniques*, 3(1), 45–50.
- Moriguchi, M., Sakai, K., Tateyama, R., Furuta, Y., & Wakayama, M. (1994). Isolation and characterization of salt-tolerant glutaminases from marine *Micrococcus luteus* K-3. *Journal of Fermentation & Bioengineering*, 77(6), 621–625.
- Nagel, F. J. I., Oostra, J., Tramper, J., & Rinzema, A. (1999). Improved model system for solid-substrate fermentation. *Process Biochemistry*, 35, 69–75.
- Steinkraus, K. H. (1996). Chinese sufu. In K. H. Steinkraus (Ed.), *Handbook of indigenous fermented foods* (pp. 633–641). New York, Basel, Hong Kong: Marcel Dekker.
- Su, Y. C. (1986). Sufu. In N. R. Reddy, M. D. Pierson, & D. K. Salunkhe (Eds.), *Legume-based fermented foods* (pp. 69–83). Boca Raton, Florida, USA: CRC Press.
- Sugimoto, H., & van Buren, J. P. (1970). Removal of oligosaccharides from soy milk by an enzyme from *Aspergillus saitoi*. *Journal of Food Science*, 35, 655–660.
- Van den Broek, L. A. M., Ton, J., Verdoes, J. C., van Laere, K. M. J., Voragen, A. G. J., & Beldman, G. (1999). Synthesis of α -galactooligosaccharides by a cloned α -galactosidase from *Bifidobacterium adolescentis*. *Biotechnology Letters*, 21, 441–445.
- Wang, H. L., & Hesseltine, C. W. (1970). Sufu and Lao-Chao. *Journal of Agricultural and Food Chemistry*, 18(4), 572–575.
- Wang, H. L., Vespa, J. B., & Hesseltine, C. W. (1974). Acid protease production by fungi used in soybean food fermentation. *Applied Microbiology*, 27, 906–911.
- Wang, R.-Z., & Du, X.-X. (1998). *The production of Sufu in China* (in Chinese). Beijing, China: China Light Industry Press.