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Fungal Isolation and the Production of its Biomass in a Palm Oil Medium

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ABSTRAK

Sejenis kulat yang telah dikenalpasti sebagai *Rhizopus arrhizus* telah dipilih sebagai mikroorganisma yang berpotensi dalam penghasilan biojisim menggunakan medium minyak kelapa sawit. Dengan menggunakan komposisi medium dan keadaan pengkulturan yang telah dioptimumkan (%,w/v): minyak kelapa sawit, 4.0; pepton, 2.0; $MgSO_4$. $7H_2O$, 0.07; NaCl, 0.5; $CaCl_2$. $2H_2O$, 0.01; Tween 20, 1.2, pH 7.0 pada suhu 37°C, kadar goncangan, 200 rpm dan saiz inokulum, 1×10^4 spora/ml, penghasilan biojisim dan penggunaan lemak yang maksimum masing-masing sebanyak 16.2 g/l dan 82% telah diperolehi. Berbanding dengan sebelum pengoptimuman, ini merupakan peningkatan sebanyak 62 dalam penghasilan biojisim dan 105% bagi penggunaan lemak.

Analisis anggaran ke atas biojisim menunjukkan kandungan protein dan asid nukleik masing-masing adalah 42.8 dan 2.2%. Profil asid amino didapati setanding dengan rujukan FAO.

ABSTRACT

A fungus which was identified to be *Rhizopus arrhizus* was selected as a potential microorganism for the production of biomass using a palm oil medium. Using the optimized medium composition and culture conditions (%, w/v) : palm oil, 4.0; peptone, 2.0; Mg SO_4. $7H_20$, 0.07; NaCl, 0.5; $CaCl_2.2H_20$, 0.01; Tween 20, 1.2, pH 7.0 at 37°C, agitation speed, 200 rpm and inoculum size, 1×10^4 spores/ml, a maximum biomass production and fat consumption of about 16.2 g/l and 82%, respectively, were obtained. Compared to the unoptimized conditions, this is an increase of 62% and 105% in biomass production and fat consumption, respectively.

Proximate analysis of the biomass revealed that the protein and nucleic acid content were 42.8 and 2.2%, respectively. Amino acid profiles were found to be comparable to those of the FAO reference.

Keywords: fungal isolation, biomass production, palm oil medium, *Rhizopus arrhizus*

INTRODUCTION

The interest in microbial biomass as a protein source was initiated during the First World War as a solution in overcoming food shortage. Today, several microorganisms have been used and produced commercially under the trade names of Pruteen (*Methylophilus methylotrophus*), Mycoprotein (*Fusarium graminearum*) and Waterloo (*Chaetomium cellulolyticum*). Various

raw materials such as methanol, glucose and cellulosic materials have been used as substrates. The choice of the raw materials for the growth of microorganisms depends on availability, cost and quality of the end product. Nevertheless, the eventual goal is to produce nutritive supplements for animals and human food.

Relatively few studies have been performed on the utilization of palm oil for biomass production (Nakahara *et al.* 1982; Koh *et al.* 1983). Prior reports on palm oil utilization other than for biomass production, were on the use of palm oil as substrate in the production of lipase (Ibrahim and Ng 1991; Ibrahim and Noor Izani 1991). Palm oil is also a renewable resource and its production is expanding annually. Microbial utilization of palm oil will be significant in supplying protein for animal and human consumption from safe and cheap raw material and also in resolving the overproduction of palm oil in the future.

Accordingly, we have attempted to produce fungal protein from palm oil medium. In this paper, we describe the isolation of an efficient fungal strain capable of assimilating crude palm oil for biomass production. Several governing parameters on the biomass production and the chemical analysis of the biomass were performed.

MATERIALS AND METHODS

Crude Palm Oil

Crude palm oil was the generous gift of Palmco Holdings (Co. Ltd), Seberang Jaya, Pulau Pinang.

Isolation of Microorganisms from Different Source Materials

Soil samples obtained around Pulau Pinang were used as the source of microorganisms. These materials were suspended in sterile distilled water and serial dilution was performed. Diluted samples were spread on potato dextrose agar and incubated at 37°C. Colonies that were formed were subcultured until pure cultures were obtained. These cultures were kept on potato dextrose agar slants at 4°C for further experiments. Pure cultures were subcultured routinely every 6 months.

Screening for the Formation of Fungal Biomass

About 437 isolates were screened for the formation of fungal biomass. Screening was performed using the basal medium containing (%,w/v): yeast extract, 1.0; crude palm oil, 2.0; NaCl, 0.5, and $CaCl_2.2H_20$, 0.01 (Ibrahim *et al.* 1987) of pH 7.0 at 37°C. Thirty-five millilitres of the medium were added to a 100 ml Erlenmeyer flask. A loopful of spores from the slant culture was inoculated and incubated under shaking conditions of 200 rpm for 24 h. Biomass formation (fungal growth) was determined based on mycelium dry weight.

Cultivation Method for Fungal Biomass Production

The biomass production by the selected fungus was carried out using the basal medium composition as described previously. Based on the basal medium, optimization of the culture conditions and the effect of crude palm oil concentration, nitrogen sources, mineral salts and Tween 20 were investigated on biomass production. The biomass obtained from the optimized medium composition and cultural conditions was used in the determination of its chemical composition.

Identification of Selected Fungus

The isolated fungus was identified based on its morphological characteristics and microscopic observations as described by Ainsworth *et al.* (1973) and Fassatiova (1986).

Analysis

Growth was determined on the dry weight basis after fat removal using organic solvent, 1,4-dioxane and ethyl acetate of volume ratio, 3:2 (Koh *et al.* 1983).

Residual fat (as an index of oil consumption) was determined according to the method described by Nakahara *et al.* (1982). Ethyl acetate was used as the extraction solvent.

Protein was determined by the macro-kjeldahl method, lipid by chloroform-methanol method, and moisture, ash, fibre and carbohydrate were determined as described by Lovell (1981). Nucleic acid was determined according to the method described by Sambrook *et al.* (1989). Amino acid determination was carried out by hydrolyzing the samples in a 6N HCl containing 15 ml of 4.3 N $LiOH.H_2O$ for 16 h at 120°C. The hydrolyzates were dissolved in water before analysis using PICO-TAG$^{(R)}$ method. Tryptophan was determined after methanesulphonic acid hydrolysis. Cysteine was determined by performic acid oxidation.

RESULTS AND DISCUSSION

Isolation of Microorganisms from Different Source Materials

The soil samples used as source of microorganisms were obtained from the surroundings of palm oil refineries around Pulau Pinang. These materials were basically oily in nature with the pH ranging from 4 to 9 with most samples lying in the range of pH 5 to 7. The temperature of the sampling locations varied from 27 to 42°C.

A total of 437 isolates were obtained from the source materials after a visual observation of their morphological characteristics on the possible overlapping of similar microorganisms was made. These isolates were grouped into bacteria, yeast, actinomycetes and fungi, and were examined for their ability to consume palm oil for growth. Five fungal isolates

(K101, K314, K316, K317, K322) were selected for reconfirmation as potent consumer of palm oil. As shown in Table 1, isolate K101 was found to exhibit the highest biomass formation of about 5.4 g/l dry weight after 24 h cultivation. Therefore, isolate K101 was then selected to be used in subsequent experiments.

TABLE 1
Biomass dry weight of the selected isolates

Isolate	Biomass dry weight (g/l)
K101	5.43
K314	1.89
K316	2.72
K317	2.15
K322	1.81

Cultivation was performed for 24 h using the basal medium containing (%,w/v): yeast extract, 1.0; crude palm oil, 2.0; NaCl, 0.5; and $CaCl_2$. $2H_20$, 0.01; pH 7; 37°C.

Fig. 1 shows the profiles of biomass formation and fat consumption by isolate K101 in the medium containing 2.0% (w/v) of crude palm oil. As indicated in the figure, the biomass increased rapidly giving a maximum biomass formation of about 10 g/l after 42 h of cultivation. Fat consumption was observed to be parallel with the biomass formation. A maximum of about 40% of the fat supplied was consumed by the fungus after 42 to 48 h. The pH of the culture broth fluctuated from 7.0 to about 5.5.

Identification of Isolate K101

Table 2 shows the characteristics of isolate K101 grown on some selected solid media. Isolate K101 grew rapidly and showed good growth on the Sabouraud agar and potato dextrose agar. The mycelium was abundant at maturity (after 7 days incubation) and the colonies were overgrown with sporangiophores. *Plate 1(1)* shows the growth of isolate K101 on the Sabouraud agar. The fungus produced white and cottony aerial mycelium at young, which turned black at maturity. Microscopic observation of the colonies showed that the sporangiophores were colourless (hyaline) with a yellowish-brown coloration at the upper part, approaching the sporangium.

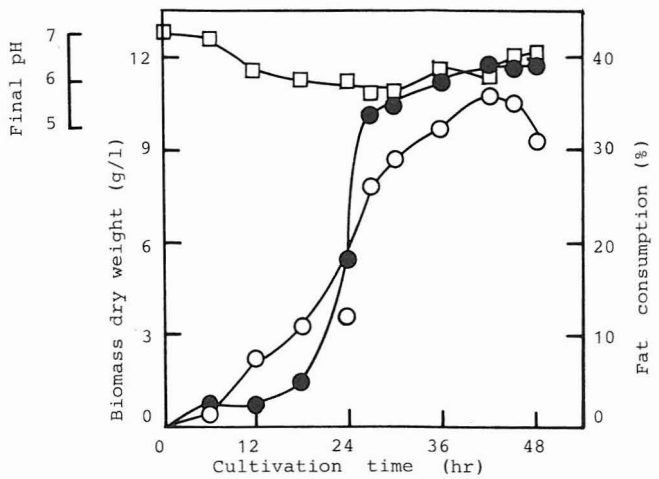

Fig. 1. Biomass production and fat consumption by isolate K101 using palm oil medium. Medium composition (%, w/v) and cultivation conditions: yeast extract, 1.0; crude palm oil, 2.0; NaCl, 0.5 and $CaCl_2.2H_2O$, 0.01; pH 7; 37°C; O biomass; ● fat consumption; □ final pH.

The sporangium is a distinct feature that arises from the mycelium and differentiates into a sporangiophore. Another characteristic of the isolate is the presence of rhizoid which functions as an anchor in the substrate [*Plate 1(2)*]. *Plate 1(2)* also shows the apophysis which supports the developing sporangiospores within the collumella. As illustrated in the figure, the collumella was ruptured, exposing the membrane that bound the spores in a globose shape. In *Plate 1(3)* the presence of chlamydospore was shown, which are formed from the concentration of cytoplasm.

Based on the growth characteristics on solid media (Table 2) and microscopic observation of the isolate (*Plate 1*), isolate K101 was identified as the genus *Rhizopus* (Ainsworth *et al.* 1973; Fassatiova 1986). An attempt was made to identify the species of *Rhizopus*. Based on the features such as mycelium, sporangium, rhizoid and apophysis, Ainsworth *et al.* (1973) and Fassatiova (1986) have identified the isolate to be either *R.arrhizus* or *R. cochnii*. The major difference between the two species is the spores. *R. arrhizus* possesses an oval-shape spore with bands on the surface of the spores, whereas *R. cochii* possesses a semi-globose to oval shape without the presence of bands. Based on the scanning electron microscopy performed (*Plate 2*), the spore of isolate K101 revealed distinct bands on the spore surface suggesting that the isolate is *Rhizopus arrhizus*.

TABLE 2
Some characteristics of isolate K101

Growth characteristics on solid medium	
Medium	Growth description
Rice	Rapid and good growth, grey to greyish black colonies, dense aerial mycelia of 10-15 mm high, abundant black sporangium.
Sabouraud Agar	Rapid and good growth, dense white to grey colonies, aerial mycelia of 10-15 mm high, black sporangium.
Cellulose Agar	Poor growth, scarce aerial mycelia of 1-2 mm high, white and fine mycelia. Few black sporangium.
Gelatine Agar	Good and dense growth, colonies white to yellow with aerial mycelia of 7 -12 mm high, few greyish sporangium present.
Czapex-Dox Agar	Poor growth, fine and scarce aerial mycelia of 1 mm high, few black sporangium.
Potato-Dextrose Agar	Good and abundant growth, dense colonies, greyish in colour with aerial mycelia of 8 -12 mm high, sporangium are black and abundant.

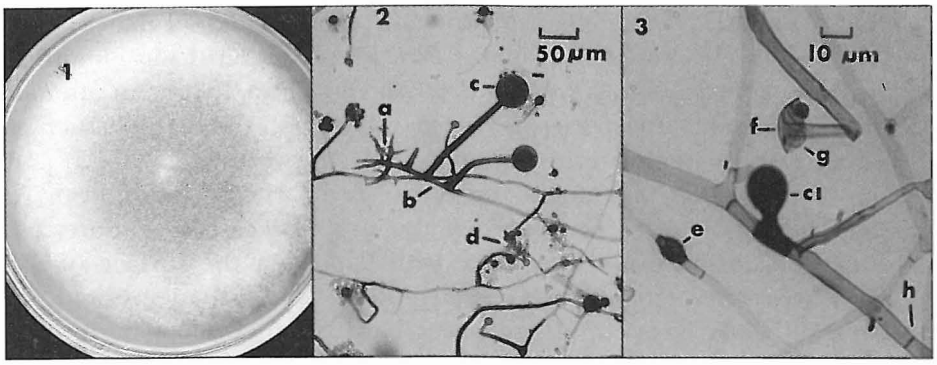

Plate 1. Morphological characteristics of isolate K101:1, Growth of isolate K101 on Sabouraud agar plate after 18 h at 37°C; 2 and 3, Some morphological features: a, rhizoid; b, stolon; c, sporangium; d, mature spores; e, chlamydospore; Cl; developing sporangium; f, apophysis; g, ruptured collumella and h, septum.

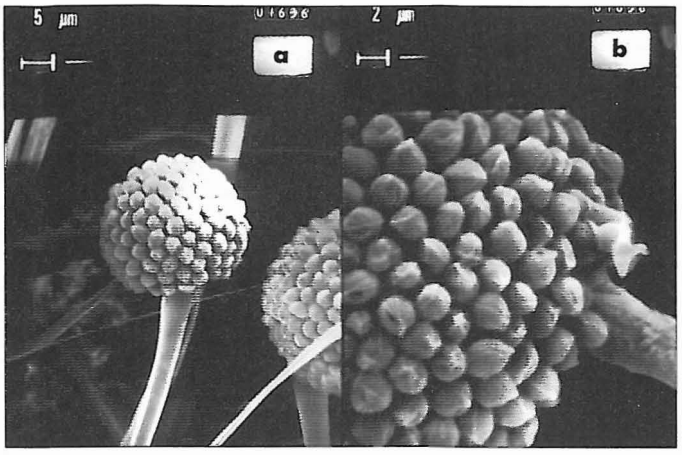

Plate 2. Scanning electron microscopy of sporangium with spores attached. a) Sporangium and sporangiophore, b) Cluster of oval-shape sporangiophores with distinct bands on the surface of the spore

Medium Optimization

Effect of Palm Oil Concentration

The effectiveness of crude palm oil as the carbon source was examined at different concentrations (*Fig. 2*). Without the addition of any oil, the fungus was able to produce about 2.8 g/l of dry weight biomass consuming basically nitrogen source after 24 h cultivation. As indicated in the figure, increasing the palm oil concentration enhanced biomass production with a maxium production of about 5.9 g/l at 4% (w/v) of crude palm oil. The fat consumption increased to about 71%. However, further increase in the oil concentration resulted in a decrease in biomass formation. High oil content may be related to the limitation of oxygen transfer within the culture system.

Effect of Mineral Salts

Various mineral salts [$(NH4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, KCl, $(NH_4)_2HPO_4$] at different concentrations were tested on the biomass formation by *R. arrhizus*. $MgSO_4.7H_2O$ at the concentration of 0.070% (w/v) was found to be the most effective, enhancing biomass production to about 8.3 g/l with the fat consumption of about 74.7% (Table 3). Other mineral salts resulted in varied quantities of biomass production from 2.5 to 6.5 g/l depending upon the concentration of the salt employed.

Based on the control experiment (i.e. without the addition of any mineral salt; biomass production of 5.8 g/l), the conclusion that can be

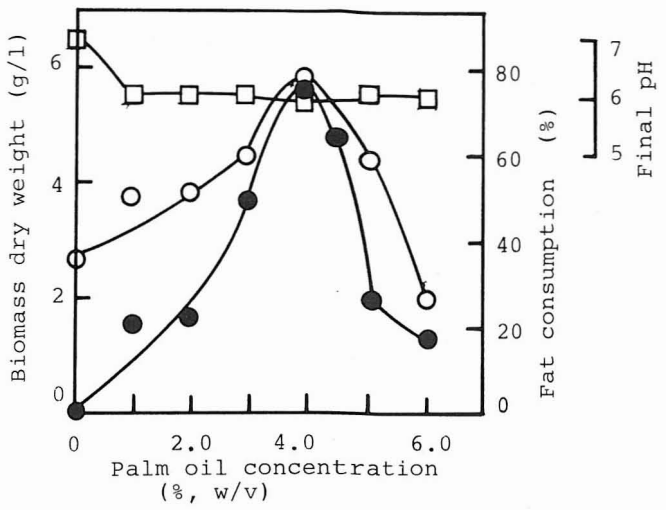

Fig. 2. Effect of palm oil concentration on biomass formation by Rhizopus arrhizus. *Medium composition (%, w/v) and cultural conditions: yeast extract, 1.0; NaCl, 0.5 and* $CaCl_2.2H_2O$, *0.01; pH 7; 37°C using different palm oil concentrations as indicated. Cultivation was performed for 24 h.* ○ *biomass;* ● *fat consumption;* □ *final pH.*

drawn was mineral salts may exhibit enhancing or inhibiting effects depending on the concentration. In most cases, higher concentration of mineral salts was inhibitory for biomass formation.

Effect of Nitrogen Sources

Table 4 shows the effect of nitrogen sources on biomass production. As indicated in the table, biomass was not produced in the absence of a nitrogen source. Organic nitrogen sources were found significant with peptone at 2% (w/v) concentration showing maximum biomass formation. On the other hand, inorganic nitrogen sources were not suitable for the growth of *Rhizopus arrhizus.*

Some Governing Parameters on the Biomass Production by R. arrhizus

The results of some governing parameters on the biomass production by *R. arrhizus* are depicted in *Fig. 3.*

Growth of *R. arrhizus* in palm oil medium occurred between pH 4.0 and 7.0, but not above 8.0. The optimum growth pH was 7.0 (*Fig. 3*a). *R. arrhizus* is a mesophilic fungus with a maximum temperature for biomass production of about 37°C (*Fig 3b*). At 42°C, a drop in biomass production was observed, suggesting that the fungus exhibited a narrow range of growth temperature.

TABLE 3
Effect of mineral salts on biomass formation by *Rhizopus arrhizus.*

Mineral salts	Concentration (%, w/v)	Dry weight (g/l)	Fat consumption (%)	Final pH
Control	-	5.87	72.07	5.93
$(NH_4)_2SO_4$	0.4	2.68	35.68	5.98
	0.5	2.78	43.64	5.92
	0.6	3.59	63.97	5.93
KH_2PO_4	0.2	3.71	64.04	6.24
	0.3	3.32	62.04	6.29
	0.4	2.70	41.84	6.25
$MgSO_4.7H_2O$	0.02	5.29	70.33	5.92
	0.05	6.55	71.57	5.97
	0.07	8.29	74.69	6.03
	0.09	6.71	71.95	5.92
	0.12	5.72	69.76	5.95
$FeSO_4.7H_2O$	0.001	5.01	68.03	5.97
	0.002	5.59	71.54	6.00
	0.003	4.53	65.41	6.01
KCI	0.7	4.72	62.94	5.72
	0.8	7.57	73.81	5.67
	0.9	5.11	68.83	5.65
$(NH_4)_2HPO_4$	4.0	-	4.69	7.19
	5.0	-	10.98	7.21
	6.0	-	6.23	7.23
0.8% KCl + $MgSO_4.7H_2O$	0.07	6.61	71.92	5.87
	0.09	4.45	65.46	5.87
	0.12	4.76	67.51	5.85

Medium composition (%, w/v) and cultural conditions: crude palm oil, 4.0; yeast extract, 1.0; NaCl, 0.5; $CaCl_2.2H_20$, 0.01 and the minerals added as indicated; pH 7.0; 37°C for 24 h

- insignificant quantity.

TABLE 4
Effect of nitrogen sources on the biomass formation by *Rhizopus arrhizus*

Nitrogen source	Concentration (%, W/V)	Dry Weight (g/l)	Fat Consumption (%)	Final pH
Control*	0.0	0.03	7.1	6.90
Yeast extract	1.0	7.17	73.05	5.96
	2.0	5.94	70.64	6.04
Malt extract	1.0	2.23	35.76	5.86
	2.0	2.95	46.31	6.61
Peptone	1.0	5.13	69.37	5.74
	2.0	8.24	74.72	5.96
NH_4NO_3	1.0	0.02	6.44	6.12
	2.0	0.01	9.14	6.26
NH_4Cl	1.0	-	-	6.40
	2.0	-	-	6.49
$(NH_4)_2CO$	1.0	-	-	8.52
	2.0	-	-	8.69
$NaNO_3$	1.0	-	-	4.79
	2.0	-	-	5.33
$NaNO_2$	1.0	-	-	6.37
	2.0	-	-	6.59

Medium compostion (%, W/V) and cultivation condition: crude palm oil 4, NaCl 0.5; $MgSO_4.7H_2O$, 0.07; $CaCl_2$. $2H_2O$ 0.01, pH 7, 37°C and nitrogen sources as indicated.

* without any addition of nitrogen source

\- insignificant quantity

One of the limitations for good growth in a biphasic medium system is poor mixing of the substrate. The ultimate consequence that can be observed is its hindrance on the substrate accessibility by the fungus for growth (Ibrahim and Noor Izani 1991). The effect of agitation speed on the growth of *R. arrhizus* was investigated (*Fig. 3c*). The maximum biomass production was observed with the agitation speed of 200 rpm. Higher agitation speed resulted in cell lysis at the early stage of fermentation which subsequently prevented further cell growth.

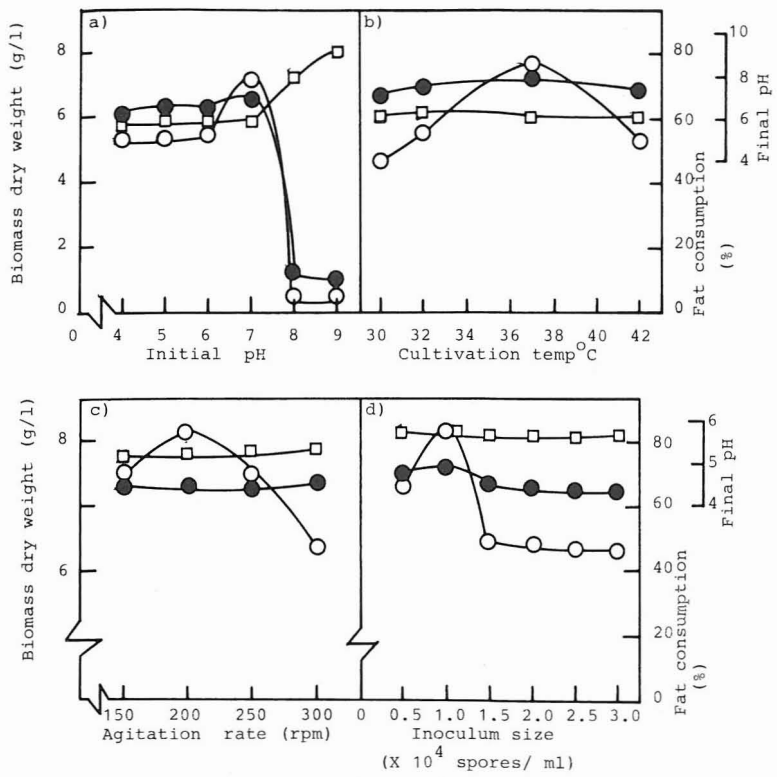

Fig. 3. Some governing parameters on biomass formation by Rhizopus arrhizus. *Medium composition (%, w/v) and cultivation conditions: crude palm oil, 4; peptone, 2; NaCl, 0.5; and* $CaCl_2.2H_2O$, *0.01; a) Effect of initial medium pH, b)effect of cultivation temperature, c) effect of agitation speed and d) effect of inoculum size. Symbols:* ○ *biomass;* ● *fat consumption;* □ *final pH.*

Hofsten and Hofsten (1974) and Hofsten and Ryden (1975) have shown that inoculum size for most fungi determines its morphological appearance which subsequently affects biomass propagation. The effect of inoculum size on biomass formation by *R. arrhizus* indicates that the inoculum size of 1×10^4 spores/ml gives the highest production (*Fig. 3d*). The morphology of the biomass was clumpy and coalescent. Byrne and Ward (1989) have demonstrated that the shift from the inoculum size of 4×10^3 spores/ml to 2×10^5 spores/ml results in morphological changes of clumpy and coalescent growth to disperse growth. These morphological changes reflect a drop in biomass formation. Higher inoculum size also results in rapid growth during the early stages. This condition may result in increased oxygen demand in the culture system, giving rise to an oxygen-limited condition. Such a condition would obviously retard rapid growth thereafter.

Growth Profiles of R. arrhizus *after Optimization*

The growth profiles of *R. arrhizus* were determined after the optimization of medium composition and cultural conditions (*Fig. 4*). The results before optimization are incorporated in the figure as a comparison. As shown in the figure, the optimized culture conditions resulted in improved biomass production of almost 14.0 g/l with about 81% of fat consumption by the fungus. Compared to before optimization, this was about 47% and 100% increment in the biomass production and fat consumption, respectively. Similarly, the generation time was reduced from 7.9 h to 6.6 h and the specific growth rate was improved from 0.088 to 0.104 h^{-1}.

A slight variation in the pH was observed within the range between 6 and 7.

Effect of Tween 20 Addition on the Growth of R. arrhizus

In a previous paper, it was shown that the production of lipase by *Corynebacterium* sp. grown in palm oil medium was improved in the presence of Tween 20 in the medium (Ibrahim and Ng 1991). Tween 20 (Polyethylene sorbitan monolaurate) helped to disperse the oily phase of

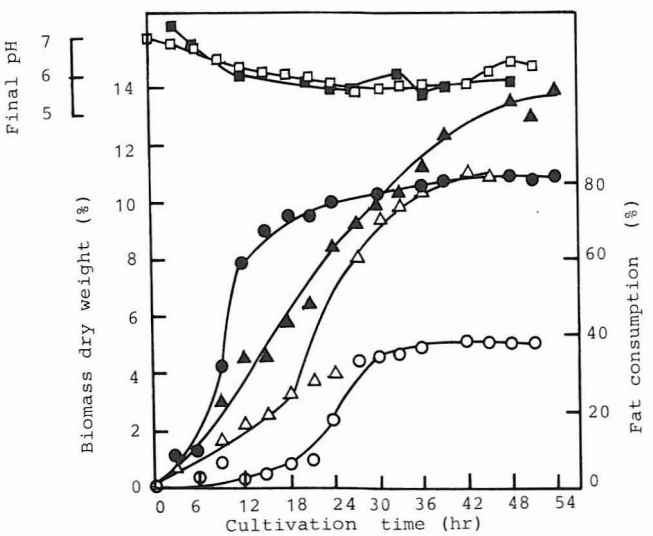

Fig. 7. Growth profiles of Rhizopus arrhizus *using the optimized cultivation conditions. Medium composition (%, w/v) and cultivation conditions: Before optimization (open symbols): yeast extract, 1.0; crude palm oil, 2.0; NaCl, 0.5 and* $CaCl_2.2H_2O$*, 0.01; pH 7; 37°C. After optimization (closed symbols): peptone, 2.0; crude palm oil, 4.0;* $MgSO_4.7H_2O$*, 0.07; NaCl, 0.5 and* $CaCl_2.2H_2O$*, 0.01; pH 7; 37°C. Symbols: △ ▲ biomass; ○ ● fat consumption; □ ■ final pH.*

the medium allowing good accessibility for consumption by the microorganism. The time course of biomass production in the presence of Tween 20 in the optimized medium composition was plotted (*Fig. 5*). As indicated in the figure, the maximum biomass production of about 16.2 g/l with about 85% of fat consumption, was obtained.

Chemical Composition and Amino Acid Analysis of the Biomass of R. arrhizus
Elementary analysis of the biomass is shown in Table 5. Protein, lipid and carbohydrate content were 43.0, 20.6 and 17.7%, respectively. The protein content of most moulds and higher fungi used in single cell protein production vary considerably from 26-55 % (Litchfield 1979).

One significant result was the very low content of nucleic acid of about 2.2%. The low content is a positive indication for use as a protein source in animal feeds. While most microorganisms are reported to contain nucleic acid between 6-15% (Goldberg 1985), the low content in *R.*

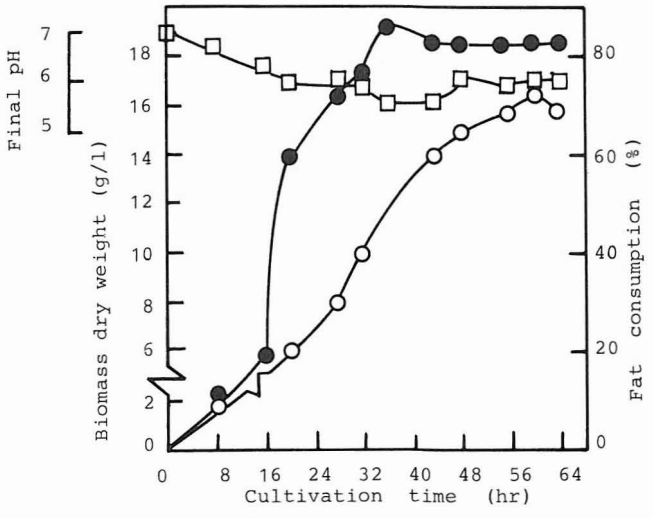

Fig. 5. Effect of Tween 20 addition on the formation of biomass by Rhizopus arrhizus. *Optimized medium composition (%,w/v) and cultivation conditions: peptone, 2.0; crude palm oil, 4.0; $MgSO_4.7H_2O$, 0.07; NaCl, 0.5 and $CaCl_2.2H_2O$, 0.01; pH 7; 37°C; 200 rpm; 1 × 10^4 spores/ml of inoculum size. Tween 20 was added to the final concentration of 1.2% (w/v) in the cultivation medium. Control experiments (without addition of Tween 20) are similar to those shown in Fig 4. Symbols: ○ biomass; ● fat consumption; □ final pH.*

TABLE 5
Proximate analysis of *Rhizopus arrhizus* biomass

Components	Quantity (%)
Protein (% N × 6.25)	42.81
Lipid	20.60
Carbohydrate	17.72
Moisture	7.15
Ash	6.57
Crude fibre	5.17
Nucleic acid	2.21

TABLE 6
Amino acid analysis of *Rhizopus arrhizus* biomass

Amino acid	*R. arrhizus* biomass		FAO/WHO reference
Phenylalanine	4.58	}	
		} 7.47	6.0 (Tyr + Phe)
Tyrosine	2.89	}	
Methionine	1.39	}	
		} 2.65	3.5 (Met + Cys)
Cysteine	1.26	}	
Valine	4.26		5.0
Threonine	3.23		-
Tryptophan	6.60		-
Isoleucine	5.39		4.0
Histidine	2.81		-
Arginine	4.83		-
Leucine	7.50		7.0
Lysine	5.33		5.5
Aspartic acid	8.43		-
Glutamic acid	20.89		-
Serine	5.25		-
Glycine	3.97		-
Alanine	7.17		-
Proline	2.89		-

Data are expressed as g per 100 g protein.

arrhizus was due to the deactivation of endogenous RNase by heat treatment during biomass prepration.

The amino acid composition of the mycelium is shown in Table 6. The amino acids were fairly well represented when compared to those of the FAO reference. The nutritional value, which awaits proper evaluation by way of animal feeding experiments, appeared theoretically comparable to that of other fungi used for single cell protein production (Anderson *et al.* 1975).

At present, the application of *R. arrhizus* biomass as animal feed is actively in progress in our laboratory. Research on the digestability and acceptability of the biomass by the test animals is also being performed. Based on the results gathered so far, we strongly conclude that the biomass which is non-toxic and non-pathogenic, can be a potential source of protein. Some of the highlights of the findings will be reported elsewhere.

ACKNOWLEDGEMENT

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