Development of a new chemically defined sporulation medium for the yeasts in the genus *Lipomyces*

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A chemically defined sporulation medium (AF medium) for the yeasts belonging to the genus *Lipomyces* was developed. The chemical composition was derived from chemical analyses of soybean extract. Some chemical modification of the AF medium indicated that the nitrogen sources (aspartic and glutamic acids) and zinc ion were essential for sporulation. The significance of medium pH was discussed.

Key Words—*Lipomyces kononenkoae*; *Lipomyces starkeyi*; *Lipomyces tetrasporus*; soybean extract; sporulation medium; *Waltomyces lipofer*

Under certain conditions, yeasts belonging to the genus *Lipomyces* form asci outside of the mother cells and each ascus contains 2–20 amber-colored spores (Sloof, 1970). The surface structure of the ascospores is an important criterion in the taxonomic identification of species within the genus (Nieudorp et al., 1974; Smith and Batenburg-van der Vegte, 1984). Some laboratory strains, however, deteriorate in their ability to sporulate and eventually lose the ability (Van der Walt and Yarrow, 1984). Sporulation of the type strain of the species (*Lipomyces starkeyi* CBS 1807) has not been observed on ethanol medium, a specific sporulation medium developed by Starkey (1946). We, therefore, tried to develop a new chemically defined sporulation medium, which is effective for the type strain.

The development was carried out in three steps: First, crude extracts of natural products were tested for sporulation medium; second, the chemical composition of a selected natural extract, soybean extract, was analyzed; and third, a chemically defined medium was derived from the analytical data and characterized.

Materials and Methods

Strain. *Lipomyces starkeyi* CBS 1807 (type strain) was mainly used.

Media. Media used were as follows: YM (1% glucose, 0.5% polypeptone, 0.3% yeast extract, 0.3% malt extract, 2% agar), 1/10 YM (0.1% glucose, 0.05% polypeptone, 0.03% yeast extract, 0.03% malt extract, 2% agar), Starkey's ethanol (1.5% ethanol, 0.08% K₂HPO₄, 0.02% KH₂PO₄, MgSO₄ · 7H₂O, 2% agar) (Iizuka and Goto, 1969; Starkey, 1946), 1/10 V8 (Iizuka and Goto, 1969) and V-8 (Iizuka and Goto, 1969).

All media mentioned above were used as the slant in test tubes after autoclaving at 121°C for 10 min.

Culture conditions. Sporulation experiments were carried out using the following method. A loopful of the surface growth from a YM slant cultured for 1 week at 25°C was transferred onto a slant of the sporulation medium and incubated at 22°C for 3 or 4 weeks.

Determination of sporulation. A loopful of the surface growth on a sporulation medium was suspended in 0.2 ml of 0.7 M mannitol solution. Asci and vegetative cells in the suspension were observed and their numbers counted separately using an Olympus AHS light microscope (Olympus Co., Ltd., Tokyo, Japan). Sporulation frequency was calculated by the following equation;

$$\text{Sporulation frequency (\%)} = \frac{\text{asci}}{[\text{asci} + \text{vegetative cells}]} \times 100.$$

Preparations of soybean extract. One-hundred grams of soybeans were macerated in a 100 ml of distilled water for 24 h, and the soybeans were crushed. The crushed soybeans were then mixed with the

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water used for maceration, and heated for 10 min at 100°C. The suspension was filtered by a filter paper (No. 2, Advantec, Tokyo, Japan), distilled water added to a volume of 100 ml and designated 'soybean extract.'

Dialyses of soybean extract. After the pH of the soybean extract was confirmed to be 6.5, 100 ml of soybean extract was dialyzed three times against 500 ml of distilled water (pH 6.5) at 4°C for 20 h. After dialysis, distilled water was added to the internal (non-dialyzable) fraction up to 100 ml volume and the solution designated 'high-molecular fraction.' The external dialyzable fraction was collected and evaporated to a 100-ml volume and designated 'low-molecular fraction.'

Analyses of low-molecular fraction. Concentrations of sugars, nitrogen compounds, phosphorus compounds and inorganic cations in the low-molecular fraction were quantitatively estimated. As Kawamura and Tada (1967) indicated that most of the saccharide in the soybean extract was sucrose, only glucose and sucrose were estimated as sugar in the work. The concentration of glucose was assayed by a diagnostic assay kit (Glucose C-Test Wako, Wako Pure Chemical Industry Co., Ltd., Osaka, Japan). Sucrose concentration was determined by the assay kit after hydrolysis of the sucrose by invertase.

Total nitrogen concentration was determined by the micro-Kjeldahl method (Ballentine, 1957), and inorganic nitrogen compounds were determined as ammonium salts by methods reported previously (Korenaga et al., 1976).

The concentrations of iron, zinc, magnesium, manganese and potassium ions and inorganic phosphorus compounds were also determined by methods reported previously (Korenaga et al., 1976).

Results

Sporulation on 1/10 YM, 1/10 V8, Starkey's ethanol, and soybean media

L. starkeyi CBS 1807 was cultured for 4 weeks. As shown in Fig. 1, V8, 1/10 V8 and 1/10 YM slants induced yeast sporulation within 2%. No sporulation was observed on the Starkey's ethanol slant, and the soybean slant showed the highest sporulation frequency.

Comparison of sporulation on high-molecular and low-molecular fractions

In order to specify the active fraction for inducing sporulation, high-molecular and low-molecular fractions of soybean extract were separately solidified with 2% agar and their sporulation frequencies examined. The low-molecular fraction slant showed activity to induce sporulation but activity by the high-molecular

Fig. 1. Sporulation of *L. starkeyi* on soybean, 1/10 YM, 1/10 V8, V8 and Starkey's ethanol slants.

Vegetative cells of *L. starkeyi* were smeared on the surface of agar slants and incubated for 4 weeks at 22°C.

Fig. 2. Selection of active fraction in soybean extract for sporulation in *L. starkeyi*.

Vegetative cells of *L. starkeyi* were smeared on the surface of agar slants and incubated for 4 weeks at 22°C.

fraction was scarce (Fig. 2).

Analysis of the low-molecular fraction

In order to reproduce the active low-molecular fraction by commercially available reagents, the major carbon source and inorganic bioelements in the fraction were analyzed quantitatively. The low-molecular fraction contained 2,850 mg/l of sucrose but no glucose. An organic nitrogen compound was detected, but no ammonium ion was detected. The total nitrogen concentration was 73 mg-N/l. The metal elements contained were as follows: Fe, 0.35 ppm; Mn, 0.11 ppm; Zn, 1.07 ppm; Mg, 65 ppm; and K, 254 ppm. The concentration of phosphorus was 56 ppm. Based on these analytical results, a new chemically defined medium was composed and named *Ascospores Formation* (AF) medium (Table 1). As for the nitrogen source, glutamic and aspartic acids were used, because these amino acids are the major nitrogen components of soybean extract (Watanabe and Shibasaki, 1974).

Evaluation of the new chemically defined medium

Five strains in the genus *Lipomyces*, including *L. starkeyi* CBS 1807, and a strain of *Waltomyces*

Table 1. Composition of the chemically defined AF medium for sporulation of the genus *Lipomyces*.

Compounds	
Sucrose	2,850 mg
Aspartic acid	230 mg
Glutamic acid	510 mg
KH ₂ PO ₄	245 mg
MgSO ₄ · 7H ₂ O	660 mg
FeCl ₃ · 6H ₂ O	1,700 µg
MnSO ₄ · 6H ₂ O	510 µg
ZnSO ₄ · 7H ₂ O	4,500 µg
KOH	410 mg
Agar	20 g
Distilled water	1,000 ml
Initial pH	6.5

Table 2. Sporulation frequency on AF and soybean media.

Yeast	Strain	Sporulation frequency (%)	
		AF	Soybean
<i>L. starkeyi</i>	CBS ^a 1807	14.6	15.4
	CBS 1809	9.2	0.7
<i>L. tetrasporus</i>	CBS 1810	8.2	3.1
	CBS 6048	5.4	5.7
<i>L. kononenkoae</i>	KW ^b -3	3.1	3.4
<i>W. lipofer</i>	CBS 5841	0.1	0.1

Yeasts examined were incubated for 3 weeks at 22°C.

^a CBS, Central Bureau voor Schimmelcultures, Delft, Netherlands.

^b Supplied from Dr. M. Yamazaki (Yamazaki and Goto, 1985).

lipofer, formerly named *Lipomyces lipofer*, were examined for sporulation on the new synthetic medium. All the strains examined sporulated both in soybean medium and AF medium (Table 2). Two strains, CBS 1809 and CBS 1810, sporulated in the AF medium at a higher frequency than in the soybean medium. *W. lipofer* showed low sporulation frequencies both in soybean medium and AF medium.

Effect of sugar source on sporulation

Sporulation was examined using modified AF media in which the sugar source was replaced with other sugars. In this experiment, various sugar sources were each added to medium at the concentration of 0.6 g/l as carbon. As shown in Table 3, all sugars examined could be used as carbon sources, but glucose and mannose decreased sporulation frequency slightly.

Effect of nitrogen source on sporulation

The effect of nitrogen source on sporulation fre-

Table 3. Effect of carbon sources on sporulation.

Carbon source	Sporulation frequency (%)
Monosaccharide	
Glucose	11.1
Fructose	23.6
Galactose	19.6
Mannose	13.7
Oligosaccharide	
Sucrose (control)	18.0
Maltose	19.8
Trehalose	16.7
Cellobiose	17.3
Raffinose	21.2
Maltopentaose	17.5

L. starkeyi was smeared on the standard AF medium and other media, and incubated for 3 weeks at 22°C.

Table 4. Effects of nitrogen sources on sporulation.

Nitrogen source	Sporulation frequency (%)
L-Asp and L-Glu (control)	19.1
L-Glu	14.9
L-Asp	19.4
A neutral or basic amino acids (average) ^a	0.9
(NH ₄) ₂ SO ₄	0.0
(NH ₄) ₂ CO ₃	2.1
NH ₄ Cl	0.3

Cells of *L. starkeyi* were inoculated on the standard AF medium or media containing one of the other nitrogen sources and incubated for 3 weeks at 22°C.

^a Average of sporulation frequency obtained from individual L-amino acids as nitrogen source: L-amino acids used; Ala, Val, Leu, Ile, Pro, Phe, Trp, Met, Gly, Ser, Thr, Cys, Gln, Asn, Tyr, Lys, Arg and His.

quency was examined. Total nitrogen concentration in the examined medium was equal to that in the standard AF medium. As shown in Table 4, ammonium salts and neutral or basic L-amino acids inhibited sporulation strongly.

Effects of concentration of components included in AF on sporulation

A lower sucrose concentration decreased both sporulation and apparent cell growth, observed as the thickness of surface growth (Table 5). A high sucrose concentration promoted apparent cell growth but not sporulation frequency. A change in nitrogen concentration did not affect apparent cell growth, but its deficiency suppressed sporulation. Especially, when no nitrogen source was added sporulation was not induced (Table 5).

A pronounced decrease in potassium or phosphorus concentration suppressed both apparent cell growth and sporulation frequency (Table 5). A de-

Table 5. Effects of component concentration on sporulation frequency and apparent growth.

Factor [Compound]	Relative concentration ^a	Sporulation frequency (%)	Apparent growth ^b
Sugar source [Sucrose]	0 (no addition)	5.3	I
	1/100	4.2	I
	1/5	6.5	I
	1 (standard)	19.6	III
	2	18.8	IV
Nitrogen source [Asp+Glu]	0 (no addition)	0.0	III
	1/100	0.7	III
	1/5	3.8	III
	1 (standard)	19.6	III
	2	11.0	III
K ⁺ [KCl] ^c	0 (no addition)	6.1	II
	1/100	11.0	II
	1/5	19.0	III
	1 (standard)	16.7	III
	2	17.7	III
Mg ²⁺ [MgSO ₄ · 7H ₂ O]	0 (no addition)	17.0	II
	1/100	13.6	II
	1/5	12.6	III
	1 (standard)	16.7	III
	2	17.4	III
PO ₄ ³⁻ [NaH ₂ PO ₄ · 2H ₂ O] ^d	0 (no addition)	2.5	II
	1/100	8.9	II
	1/5	16.3	III
	1 (standard)	16.7	III
	2	18.3	III
Zn ²⁺ [ZnSO ₄ · 7H ₂ O]	0 (no addition)	3.4	III
	1/100	5.9	III
	1/5	15.1	III
	1 (standard)	16.7	III
	2	16.8	III
Fe ³⁺ [FeCl ₃ · 6H ₂ O]	0 (no addition)	15.9	III
	1/100	17.2	III
	1/5	16.1	III
	1 (standard)	16.7	III
	2	16.2	III
Mn ²⁺ [MnSO ₄ · 6H ₂ O]	0 (no addition)	19.7	III
	1/100	16.6	III
	1/5	16.3	III
	1 (standard)	16.7	III
	2	16.1	III

L. starkeyi was incubated for 3 weeks at 22°C on media containing nutrient at the concentration indicated in the table.

^a Unit indicates standard concentration.

^b Degree of cell growth on each medium was estimated as follows: I, seldom growth; II, less than growth in standard concentration; III, as well as in standard concentration; IV, more than growth in standard concentration.

^c KOH and KH₂PO₄ were excluded from standard medium. NaH₂PO₄ was added as PO₄³⁻ source. Medium pH was adjusted to 6.5 by 0.1 M NaOH.

^d KH₂PO₄ was excluded from medium. KCl was added as K⁺ source. Medium pH was adjusted to 6.5 by 0.1 M NaOH.

crease in magnesium concentration suppressed apparent cell growth, but did not affect sporulation frequency (Table 5).

The concentrations of iron and manganese affected both apparent cell growth and sporulation frequency. In the case of zinc, a pronounced decrease affected

only sporulation (Table 5).

Discussion

Commonly recommended sporulation media, such as 1/10 YM, 1/10 V8 and V8 (Iizuka and Goto, 1969),

induced low sporulation frequency (below 2%), but AF medium was more effective (Tables 1 and 2). Moreover, the AF medium is effective for inducing sporulation for almost all yeasts belonging to the genus *Lipomyces*. The medium is expected to be useful in taxonomic studies of newly isolated soil yeasts.

The major amino acids in soybean extract were aspartic and glutamic acids, and the amount of glutamic acid in the soybean extract was about two times that of aspartic acid (Watanabe and Shibazaki, 1974). Most of the soluble carbohydrate contained in soybeans was sucrose (Kawamura and Tada, 1967). Additionally, concentrations of inorganic elements affected both cell growth and lipid content significantly in *L. starkeyi* (Naganuma et al., 1985). Considering these investigations and the analytical results, the reagents used to compose AF medium were selected and their concentrations decided as shown in Table 1.

Potassium ion concentration in the AF medium, however, was not close to that in the soybean medium, because potassium hydroxide was used to adjust the initial pH of the AF medium to 6.5. Consumption of the nitrogen source (Asp and Glu) by cells in the AF medium probably brought the medium pH to a higher value. An imbalanced consumption between the acidic amino acids and potassium ions is believed to have taken place during cell growth prior to sporulation. High sporulation frequencies were observed only when acidic amino acids were used as the nitrogen source (Table 4). Even asparagine or glutamine could not be replaced. The results support the speculation that an imbalanced uptake between acidic amino acids and potassium ions took place, and the resultant pH change may be a possible factor for sporulation. Inorganic ammonium salts were not a suitable nitrogen source. After the ammonium ions were taken up by the cells, the remaining anions, especially sulfate and chloride ions, were believed to acidify the medium. These results and prediction lead to the hypothesis that a gradual change in medium pH to the alkaline side may be an important factor to induce sporulation in *L. starkeyi*. A minimal glucose-mineral medium (a modified chemically defined medium proposed by Wickerham) has long been used as a standard medium for growth studies of *L. starkeyi* (Uzuka et al., 1975). The minimal medium has an initial pH of 5.3 and medium pH gradually decreases with cell growth. No sporulation has been observed on the solidified medium, and the pH of the liquid medium usually decreased to about 2.0 at the stationary phase of growth. These observations do not contradict the prediction mentioned above. As long as solidified AF medium is used, it is, unfortunately, quite difficult to determine both medium pH and its effect on sporulation.

Elimination of the nitrogen source or phosphate source certainly decreased sporulation frequency (Table 5). These facts suggest sporulation requires the additional supply of some metabolites which are not sufficiently accumulated in vegetative cells. In the absence of a carbon source, on the other hand, a 5.3% sporulation frequency was observed (Table 5). The yeast *L. starkeyi* has a specific property to accumulate neutral lipids intracellularly, and the lipids were probably used as the carbon source.

A lack of zinc salt in the AF medium resulted in a low sporulation frequency (Table 5). Zinc concentration has been known to play an important role to switch cellular metabolite direction from lipid accumulation to cell growth. Therefore, zinc seems to be an essential factor to induced sporulation as well.

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