Isolation of Heterotrophic Microalgae*

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Several reports are presented on the heterotrophic growth of unicellular green algae[1,2], and the application of them for treatments of organic waste waters, especially night soil, are investigated[3]. In every summer, unicellular green algae are often found to be propagating on the surface of alcohol distillers slops pool, abundant in organic compounds, in Chiba city. They seemed to multiply on carbon dioxide in light, and on organic compounds in darkness. Initial purpose of this investigation was to obtain heterotrophic microalgae to decolorize distillers slops of blackstrap molasses and to treat Shôchû distillers slops. One of the authors has isolated many strains of microalgae from the alcohol distillers slops pool in the Fermentation Research Institute, and from sewage in and about Ue-arata campus of Kagoshima University One of the algal isolates, *Chlorella vulgaris,* Al-ly, was found to accumulate a lot of starch granules extracellularly, when growing on glucose both in light and in darkness. The investigations were also carried out on various conditions of cultivation of this strain, especially with respect to extracellular starch accumulation.

This paper describes the isolation of heterotrophic microalgae from alcohol distillers slops pool and a sewage, and the extracellular starch accmulation of *Chlorella vulgaris,* Al-ly.

Isolation. Sample materials for isolation consisted of 3 slops samples, collected from the distillers slops pool, and 11 sewage samples, each including considerable amount of mud. The alcohol distillers slops pool is situated in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Chiba city (Fig. 1 a, and b). Sewage samples were collected in and about Ue-arata campus of Kagoshima University, Kagoshima city.

Isolation from the samples of the alcohol distillers slops pool was carried out by the following methods. The isolation medium was composed of: potassium nitrate, 2 g; yeast extract, 0.1 g; Calcium carbonate, 30 g; 1 liter of mineral salts solution[4]. 1 ml of sample material was inoculated into 50 ml of the isolation medium in 100 ml Erlenmeyer flask and the flask was incubated 2 meter away from the window in the south side for 2 weeks. After 2 weeks, a loop of culture was streaked on the isolation medium, from which calcium carbonate was removed. A streaked plate was incubated 30 cm away from a 20 W fluorescent lamp. Algal isolates were purified by repeated streaks (A1-1, and A1-5). On the purification of algal isolates, contaminating organisms were detected with subcultures on the nutrient agar, and on the yeast extract malt extract agar[4]. On the yeast extract malt extract agar plate streaked with the strain Al-l, some yellowish green colonies were found among deep green

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colonies of the original strain. This yellowish green strain grows much more on the yeast extract malt extract agar than on the isolation medium, and was indicated with A1-1y.

The next isolation medium was prepared as follows. The solution of blackstrap molasses was composed of distilled water, 200 ml, blackstrap molasses, 5g, and saccharose, 10g, and fermented by an alcohol yeast IFO 2058 at 30 for 1 week. Alcohol produced by the yeast was distilled off. 80 ml of supernatant was recovered out of the leaving solution, and added to a solution, composed of: potassium nitrate, 0.8 g; agar, 8 g; mineral salts solution, 320 ml. This distillers slops medium was adjusted to pH 7.0. Algal clods that appeared in sample materials, alcohol distillers slops, in a flask incubated 2 meters away from the window on the south side for a month, were streaked on the distillers slops agar plate, and inbucated under 20 W fluorescent lamp. The isolates by the method mentioned above were A1-41, 42, 43, and 44.

15 to 20 ml of the sewage sample in a test tube was incubated for a month, 2 meters away from the window on the south side, and algal clods appeared. Isolation from the sewage samples in and about Ue-arata campus of Kagoshima University was carried out by the method of streaking of algal clods on the acetate medium. The acetate medium is composed of: sodium acetate. 5 g; yeast extract, 1 g; polypepton, 2 g; mineral salts solution, 1 liter; pH 7.4. Pinhead colonies developing on the plate in 3 to 7 days incubation in light were repeatedly streaked and purified into the strains A1-10 to A1-40, and A1-45 to A1-54. *Chlamydomonas* sp. was picked up from algal clods by means of a capillary under the microscope, and cultivated on the acetate agar (A1-56).

The strain A1-55 was isolated from green water in a glass fish bowl, in which some killifish were kept by one of the authors, and was identified with *Scenedesmus obliquus* (Fig. 5 a, b, and c).

The authentic strains. *Chlamydomonas* sp. IAM C-12, *Chlorella ellipsoidea* IAM C-27, and *Scenedesmus basilensis* IAM C-66 were sent kindly from the type culture collection of the Institute of Applied Microbiology, University of Tokyo. These authentic strains were employed for comparison with the algal isolates.

The isolates and the authentic strains were maintained on the mineral salts medium in light at a room temperature and subcultured every two months.

The isolates.

Chlorella vulgaris Beijerinck

Strains: A1-1, 1y; 11, 12, 13, 14, 15, 16, 17, 18, 21, 22, 23, 24, 32, 33, 34, 50, 52; 31, 38, 39, 40, 45, 46, 47, 48, 49, 54; 19, 20, 25, 26, 27, 28, 29, 30, 51, and 53.

According to KESSLER and SOEDER, and SOEDER, *Ch. vulgaris* Beijerinck does not possess hydrogenase, turns pale due to the loss of both chlorophylls and carotenoids under conditions of nitrogen deficiency, and develops a brilliant red color with ruthenium red, while *Ch. ellipsoidea* Gerneck can be recognized by the strongly elliptical shape (even in agar cultures) of its cells. The latter does not contain hydrogenase, turns pale fairly slowly in nitrogen deficient cultures, and does not react with ruthenium red[5,6]. The above strains developed a red color with ruthenium red, possess a pyrenoid, and produce starch granules of considerable size. All the strains, except Al-ly, turns pale fairly slowly in nitrogen deficient cultures, but an average ratio of length by width was 1.08 (ratio of length by width ranging

from 1.11 to 1.27, 1.11 for A1-1 and A1-11, 1.08 for A1-25, 1.07 for A1-45, and 1.05 for A1-1y). According to SOEDER, average ratios of the autospore-mother cell and the autospore are 1.6 and 1.8 for *Ch. ellipsoidea,* and 1.0 and 1.2 for *Ch. vulgaris,* respectively. Therefore, the above strains should be considered as *Ch. vulgaris.* (Fig. 2 a, b, c, d, e, f, g, h, i, j, k, l, and m).

Chlamydomonas sp.

Strains: A1-10, 56; 41, 42, 43, and 44.

These strains belong to the genus *Chlamydomonas,* but the mating of these strains is unknown. (Fig. 3 a, b, c, d, e, f, g, h, i, j, k, l, m, and n).

The strain A1-5 is characterized by a deep orange color on nitrogen deficient cultures. Accordingly, this strain seemed to be identical with *Chlorella pyrenoidosa,* but there are still

Table 1. Physiological groups, into which microalgae are distinguished by utilization of glucose, ethanol, and acetic acid.

strains	a ratio of length by width	cell size in μ	utilization glucose	ethanol	acetic acid	no carbon source*
Chlorella vulgaris A1-11,12, 13, 14, 15, 16, 17, 18, 21, 22, 23, 24, 32, 33, 34, 50, 52.	1.00~1.27	2.7~5.0 ×3.3~5.6	+	+	+	1.4~1.9
Ch. vulgaris A1-31, 38, 39, 40, 45, 46, 47, 48, 49, 54.	1.00~1.20	3.2~5.0 ×3.2~5.6	+	+	+	0.5~0.9
Ch. vulgaris A1-1, 1y.	1.00~1.17	3.3~5.0 ×3.3~5.2	+	−	+	0.6~0.9
Ch. vulgaris A1-19, 20, 25, 26, 27, 28, 29, 30, 51, 53.	1.00~1.21	2.8~3.9 ×3.1~4.2	+	−	+	0.6~0.9
Chlamydomonas sp. A1-10, 35. 36. 37, 56.		9.8~21.0 ×11.2~23.8	−	−	−	0.2
Chla. sp. A1-41, 42, 43, 44.		11.2~22.4 ×12.6~23.8	−	−	+	0.3~0.4
Scenedesmus obliquus A1-55.		2.1~6.1 ×2.8~12.4	+	−	−	0.6
Scenedesmus basilensis A1-1AM C-66.		4.2~8.4 ×7.7~11.2	+	−	−	0.7
A1-5.		5.5~8.3 ×6.1~9.5	+	−	−	2.0

* Optical density of 10 days culture at 660 mμ.

some problems on its morphology (Fig. 4 a, b, c, d, and e).

Algal growth in darkness. Utilization of carbon sources in darkness was investigated in the mineral salts solution, containing potassium nitrate, and one of carbon sources at a concentration of 2 % (W/V). Carbon sources used were as follows: arabinose, xylose, glucose, galactose, mannose, fructose, maltose, saccharose, lactose, raffinose, starch, inulin, ethanol, mannitol, sodium acetate, sodium succinate, citric acid, malic acid, lactic acid, pyruvic acid, sodium glutamate, casamino acid, and polypepton. *Chlorella vulgaris* A1-1 and A1-1y, *Scenedesmus basilensis* IAM C-66, *Chlamydomonas* sp. IAM C-21, and the strain A1-5 served for investigation. Of these carbon sources, glucose was utilized by all the strains employed, fructose, by *Sc. basilensis* IAM C-66 and *Chlamydomanas* sp. IAM C-21, galactose, by *Ch. vulgaris* A1-1, *Chlamydomonas* sp. IAM C-21, and the strain A1-5, maltose, by 4 strains em-

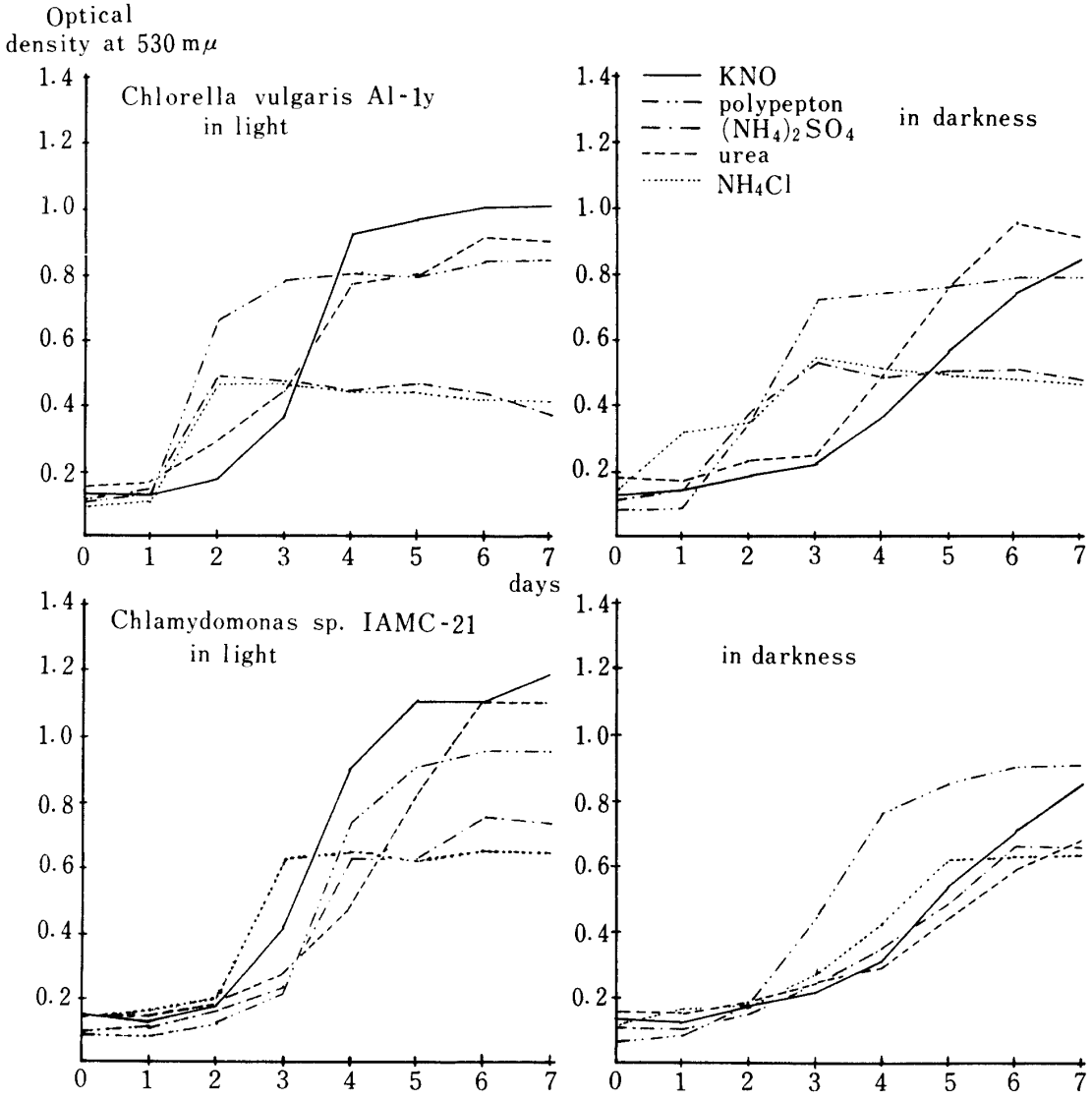


Fig. 6. Utilization of nitrogen sources of microalgae.

ployed, except *Ch. vulgaris* A1-1, and sodium acetate, by *Ch. vulgaris* A1-1 and A1-1y. Other carbon sources could support no growth of 5 strains employed. All the isolates were distinguished into physiological groups, according to utilization of glucose, ethanol, and sodium acetate in darkness, as shown in Table 1.

Utilization of nitrogen sources was investigated in the mineral salts solution, containing glucose and one of nitrogen sources. Nitrogen sources used were as follows; ammonium chloride (0.2 %), ammonium sulfate (0.2 %), polypepton (0.5 %), potassium nitrate (0.2 %), and urea (0.2 %). Potassium nitrate, urea, and polypepton supported good growth of all the strains employed. (Fig. 6). Ammonium salts, including ammonium nitrate, seemed to be used with greater ease than nitrates at the early stage of cultivation, but at the middle of logarithmic phase of growth, the strains employed ceased from multiplication with rapid reduction of pH value of the cultures, and decolorization of algal cells followed.

HUGH-LEIFSON's test for bacteria and its modification were employed to determine whether sugar utilization of the algae was fermentative, or oxidative[7]. *Chlorella vulgaris* A1-1, A1-1y, and *Ch. ellopsoidea* IAM C-27 did not produce acid, but *Scendesmus basilensis* IAM C-66 and the strain A1-5 produced acid both in an open tube and in a paraffine-covered tube, when incubated both in light and in darkness, and *Chlamydomonas* IAM C-21 produced acid in both tubes, only in light. Acid production both in an open tube and in a paraffine-covered tube indicates oxidative sugar utilization of these algae.

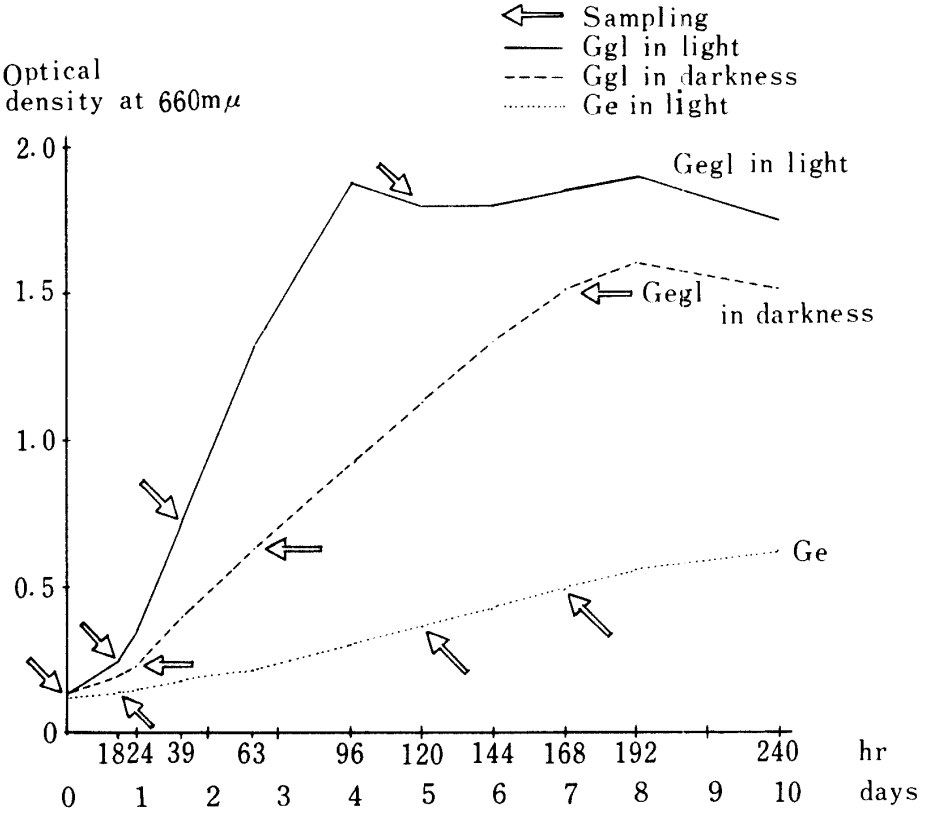
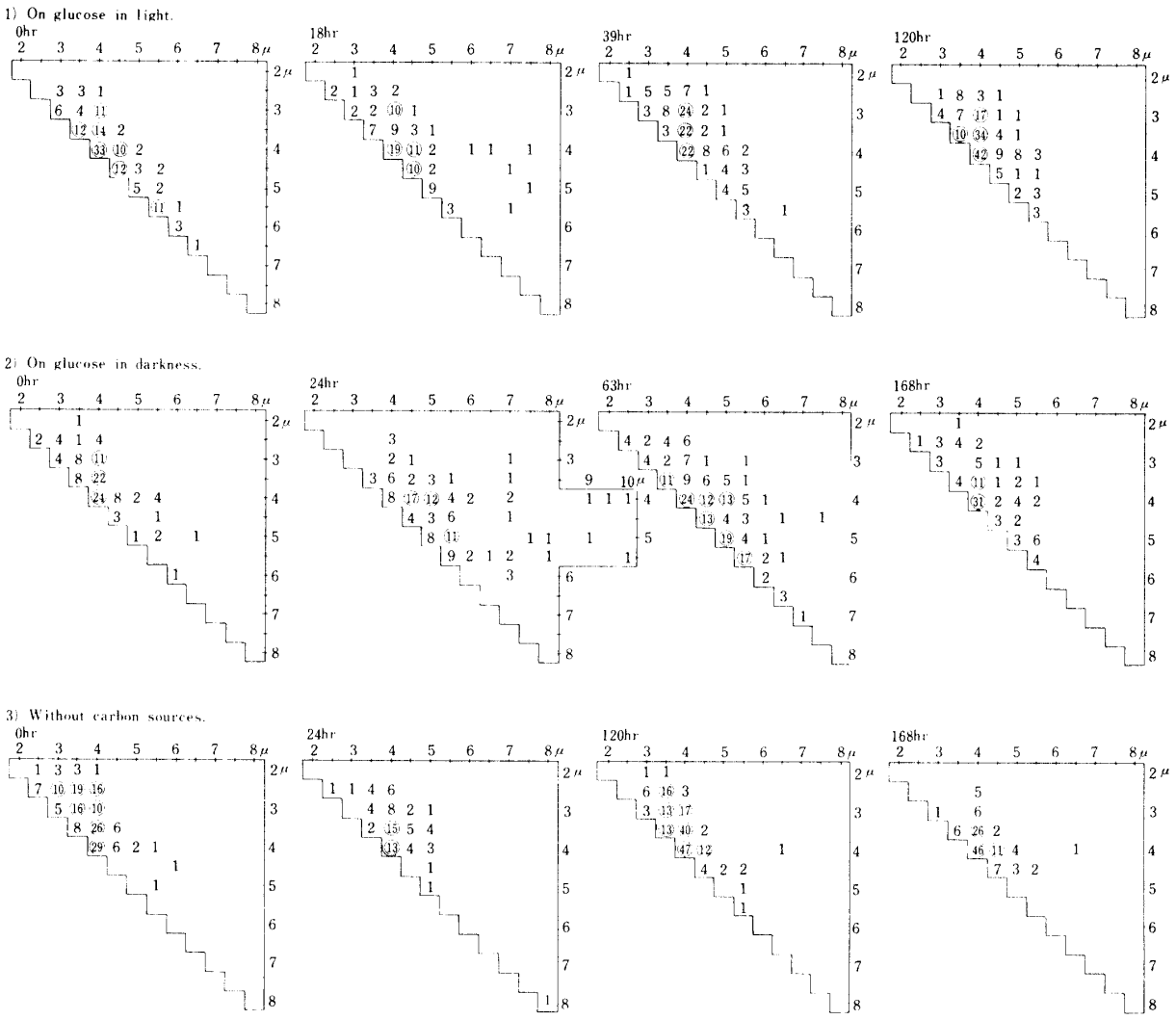


Fig. 8. The time courses of multiplication of *Chlorella vulgaris* Al-ly on glucose (Gegl) both in light and in darkness, and without organic carbon sources (Ge).

Extracellular accumulation of starch granules by Chlorella vulgaria A1-1y. A number of starch granules were observed in the culture broths of *Ch. vulgaris* A1-1y on glucose, or on sodium acetate under a microscope (Fig. 7). The time courses of multiplication of *Ch. vulgaris* A1-1y in shaking culture on glucose both in light and in darkness, and without organic carbon sources are shown in Fig. 8. Cell size of this strain was observed at the time indicated by an arrow in the time courses of multiplication, as shown in Fig. 8 and Table 2. Most of the cells of

Table 2. Cell sizes of *Chlorella vulagris* Al-1y, observed at various hours in the time courses of multiplication.



this strain in autotrophic growth is ranging from 3.5 to 4.0μ in the width and 4.0μ in the length. When grown on glucose, even if in light, or in darkness, it has a wide range of cell size, and especially at the early stage of logarithmic phase of growth, cells are elongated by 50 to 100 % along the long axis, as shown in Fig. 9. Extracellular accumulation of starch granules was observed in both cultures on glucose in light, and in darkness, but not in autotrophic cultures (Fig. 9). An amount of extracellular starch was determined by iodine-starch reaction

Table 3. Extracellular accumulation of starch granules by *Chlorella vulgaris* Al-1y for 7 days in light on various kinds of nitrogen sources in the mineral salts solution, containing 1% of glucose.

nitrogen source	final pH	dried cell weight in mg/ml	extracellular starch in μg/ml (as potato starch)
no nitrogen	5.6	0.56	7.8
KNO_3, 0.2 %	7.8	2.44	296.5
〃 , 0.1 %	6.3	2.26	216.5
〃 , 0.02%	5.6	1.29	84.0
$NaNO_3$, 0.2 %	7.8	2.28	351.0
NH_4NO_3, 0.2 %	4.2	0.79	11.7
$(NH_4)_2SO_4$, 0.2%	4.2	0.66	9.8
NH_4Cl, 0.2 %	4.4	0.81	23.4
urea, 0.2 %	4.5	2.08	84.0
polypepton, 0.5 %	4.8	1.54	84.0
Na-glutamate, 0.5 %	5.8	0.61	9.8
NH_4-acetate 0.2 %	5.8	3.74	241.8

of the supernatants at 580 mμ after boiling for 5 minutes and centrifugation of the cultures. When algae were grown on glucose in light, an amount of extracellular starch was 36.95 mg in 100 ml of the culture of *Ch. vulgaris* Al-1y for 7 days, eight times as much as that on sodium acetate, while it was 1.68 mg in the culture of *Ch. vulgaris* Al-1. Extracellular accumulation of starch granules by *Ch. vulgaris* Al-1y on various kinds of nitrogen sources is shown in Table 3. Good accumulation was observed on sodium nitrate, potassium nitrate, and ammonium acetate. The fact indicates that ammonium also is a good nitrogen source for growth and extracellular accumulation of starch granules of *Ch. vulgaris* Al-1y, if its anions are rapidly easten up. Extracellular accumulation of starch granules by *Ch. vulgaris* Al-1y at various concentrations of yeast extract is shown in Table 4. The temperature ranges of growth, and extracellular accumulation of starch granules of *Ch. vulgaris* Al-1y are presented in Fig. 10. It indicates

Table 4. Extracellular accumulation of starch granules by *Chlorella vulgaris* Al-1y for 7 days in light at various concentrations of yeast extract in the mineral salts solution, containing 0.2 % of KNO_3.

ingredients		dried cell weight in mg/ml	extracellular starch in μg/ml (as potato starch)
yeast extract in %	1 % of glucose		
0.01	not added	0.64	5.9
0	added	3.03	193.0
0.01	〃	4.73	302.0
0.1	〃	4.09	372.5
1	〃	1.40	46.8

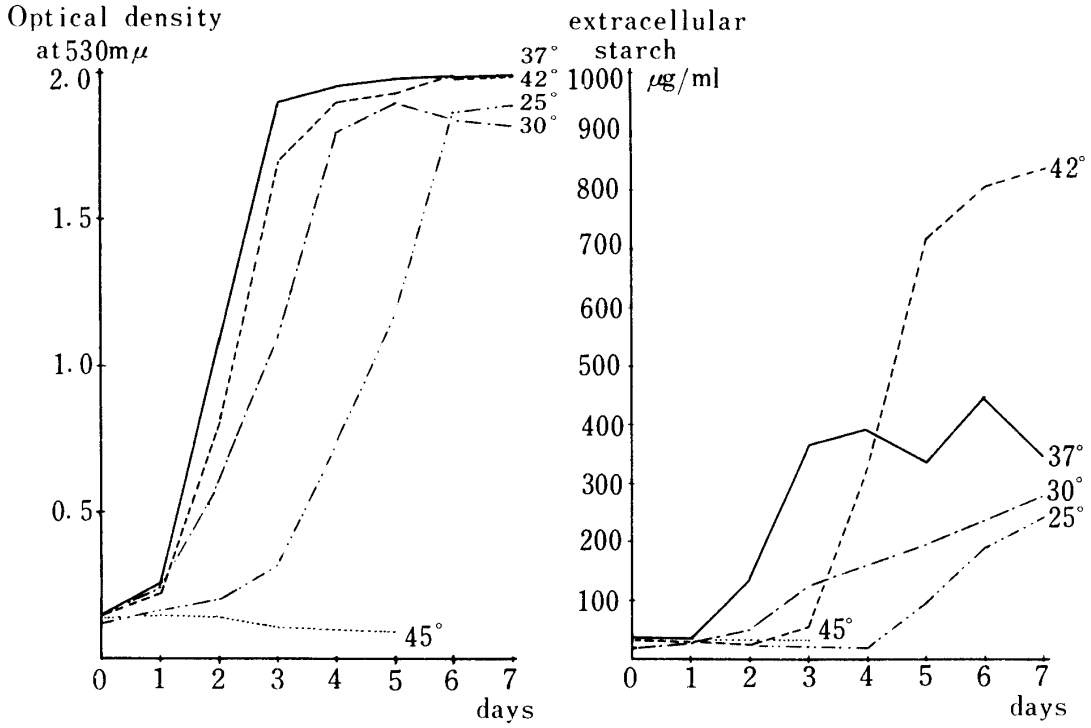


Fig. 10. Temperature ranges of growth, and extracellular accumulation of starch granule of *Chlorella vulgaris* Al-ly.

an optimum temperature for growth at between 37° and 42°, and for starch accumulation at 42°.

SUMMARY

In order to decolorize distillers slops of blackstrap molasses and to treat Shôchû distillers slops, 47 strains of heterotrophic microalgae were isolated. They were identified each with *Chlorella vulgaris*, 39 strains, *Chlymadomonas* sp., 6 strains, *Scenedesmus obliquus*, 1 strain, and unidentified, 1 strain, respectively. *Chlorella vulgaris* A1-1y, separated from *Ch. vulgaris* A1-1 on the yeast extract malt extract agar, was found to accumulate lots of starch granules extracellularly. With this strain, good growth and extracellular starch accumulation also were observed at 42 C on glucose in light.

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Fig. 1 a. The alcohol distillers slops pool in the Fermentation Research Institute, Chiba city.
1 b. Colony of microalgae on the surface of distillers slops.
Fig. 2. Cells of *Chlorella vulgaris*.
2 a, b, and c. The strain Al-1.
2 d, e, and f. The strain Al-1y.
2 g, h, and i. The strain Al-11.
2 j, and k. The strain Al-45.
2 l, and m. The strain Al-25.
Fig. 3. Cells of *Chlamydomonas* sp.
3 a, b, and c. Ue-arata strain (Al-10, 56), before isolation.
3 d, e, and f. The strain Al-10.
3 g, h, and i. The strain Al-56.
3 j. Chiba strain (Al-41), before isolation.
3 k, l, and m. The strain Al-41.
Fig. 4. Cells of the strain Al-5.
Fig. 5. Colonies of *Scenedesmus obliquus* Al-55.
Fig. 7. Starch granules in the culture broth of *Chlorella vulgaris* Al-1y on glucose.
Fig. 9. Cells of *Chlorella vulgaris* Al-1y.
9 a. Inoculated cells, at 0 hr after inoculation.
9 b, c, and d. Cells growing on glucose in darkness, at 24, 39, and 120 hr after inoculation, respectively.
9 e, and f. Cells growing without organic carbon sources, at 39, and 12 hr after inoculation, repectively.
9 g, and h. Cells growing on glucosse in light, at 24, and 120 hr after inoculation, respectively.

Fig. 1 a

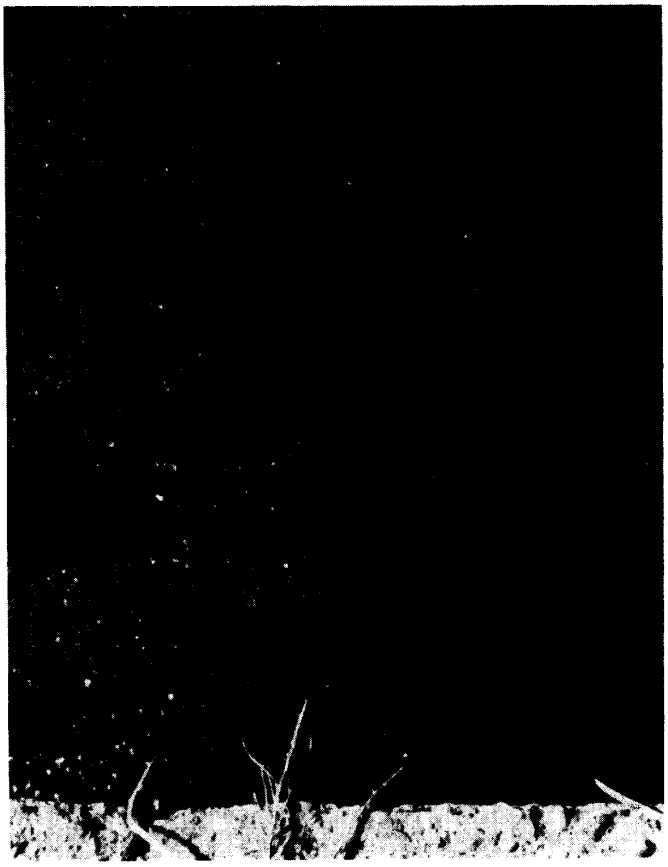


Fig. 1 b

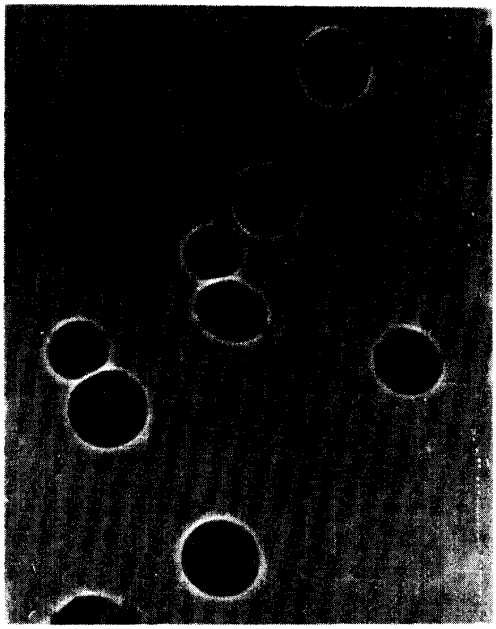


Fig. 2 a

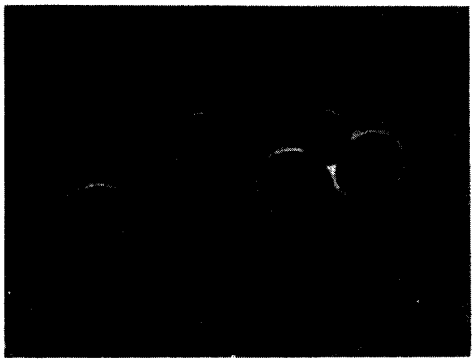


Fig. 2 b

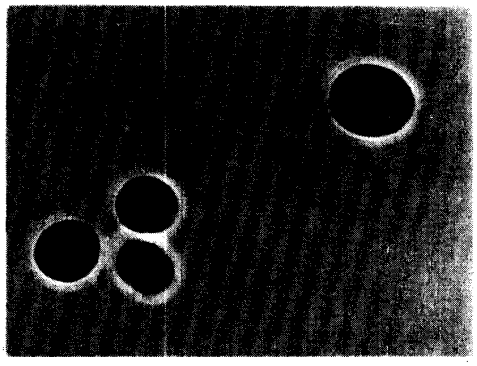


Fig. 2 c

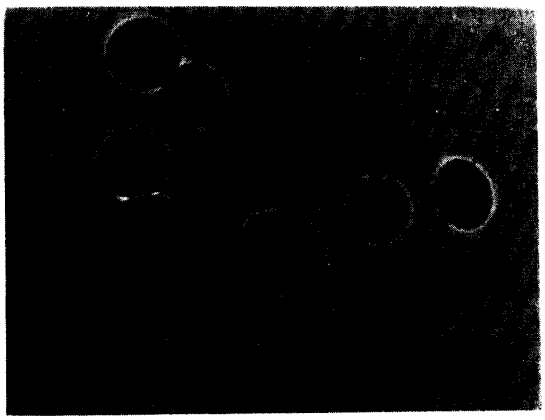


Fig. 2 d

10 μ

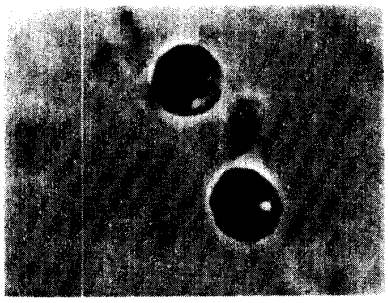


Fig. 2 e

Fig. 2 f

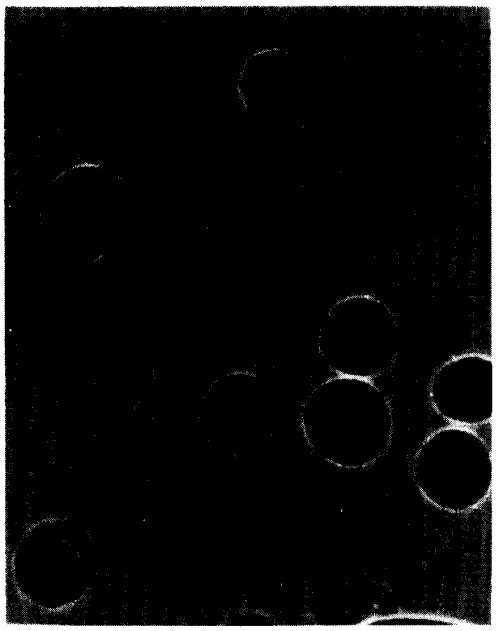


Fig. 2 g

Fig. 2 h

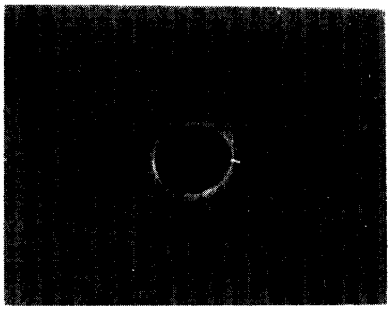


Fig. 2 i

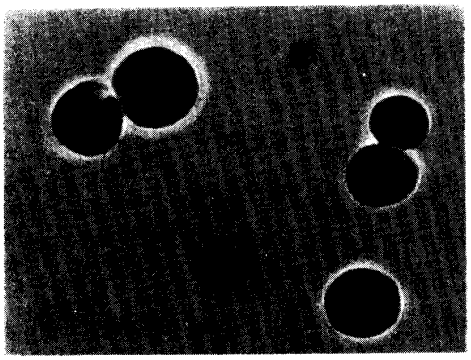


Fig. 2 j

Fig. 2 k

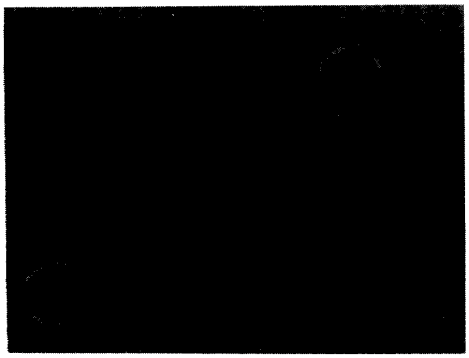


Fig. 2 l

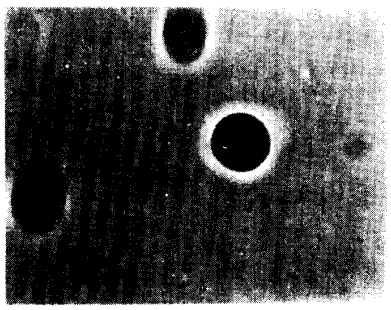


Fig. 2 m 10 μ

Fig. 3 a

Fig. 3 b

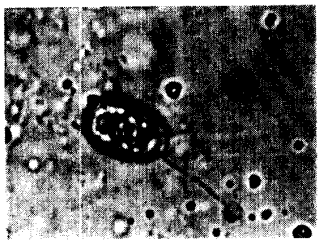


Fig. 3 c

10 μ

Fig. 3 d

Fig. 3 e

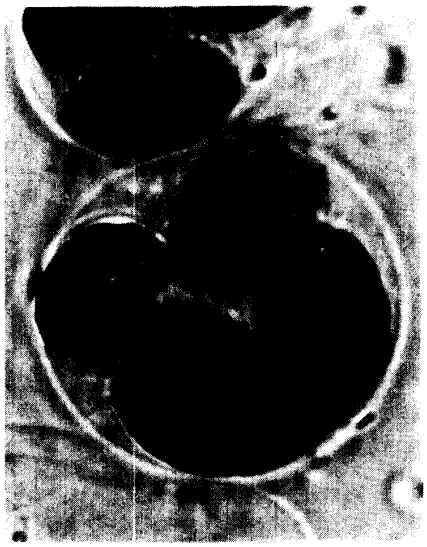


Fig. 3 f

10 μ

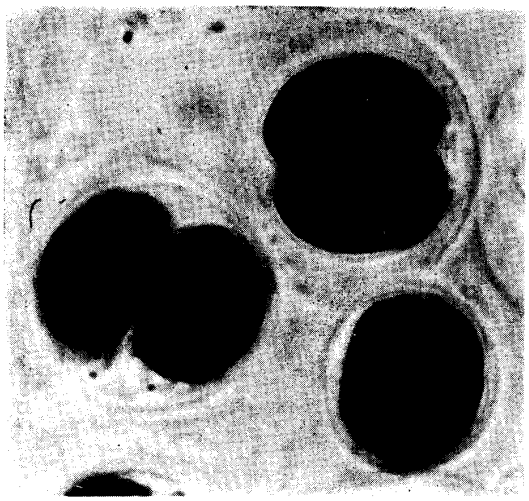
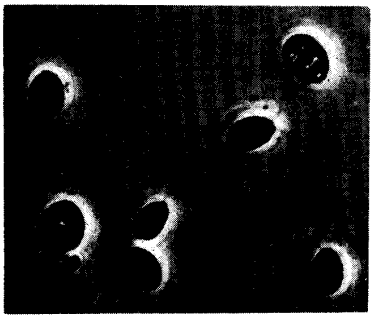


Fig. 3 i

10 μ



10 μ

Fig. 3 j

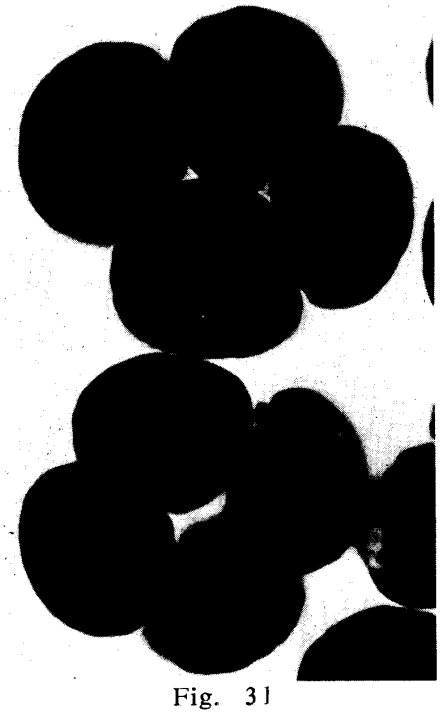


Fig. 3 l

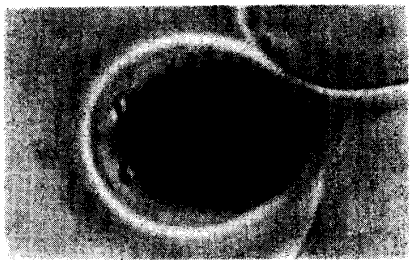
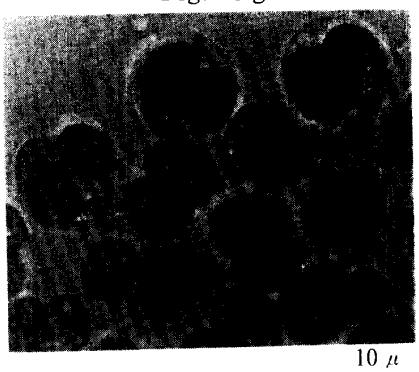


Fig. 3 g



10 μ

Fig. 3 h

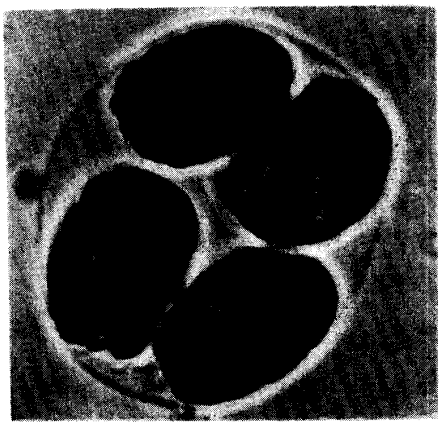
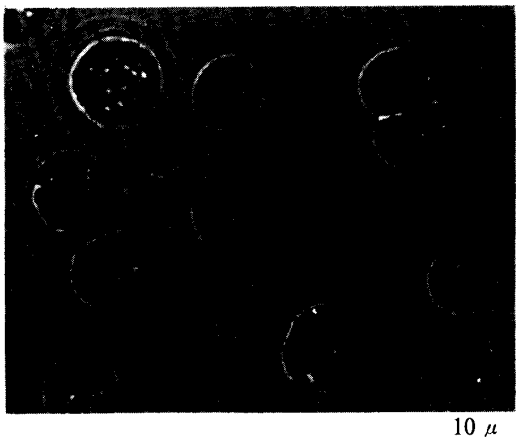


Fig. 3 m



10 μ

Fig. 3 k

Fig. 4 a

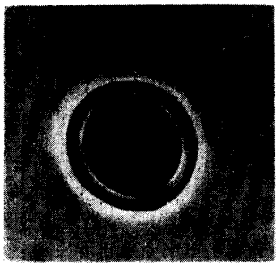


Fig. 4 b

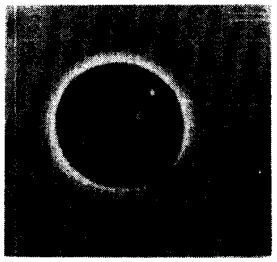


Fig. 4 c

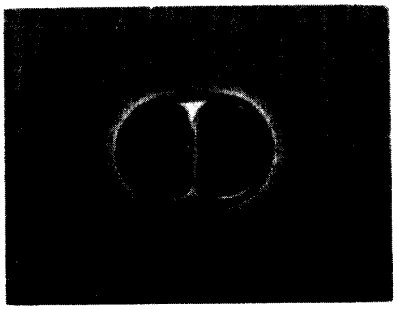


Fig. 4 d

Fig. 4 e 10μ

Fig. 5 a

Fig. 5 b

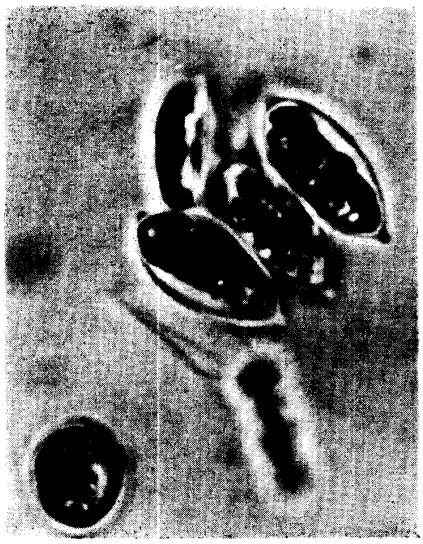


Fig. 5 c

10 μ

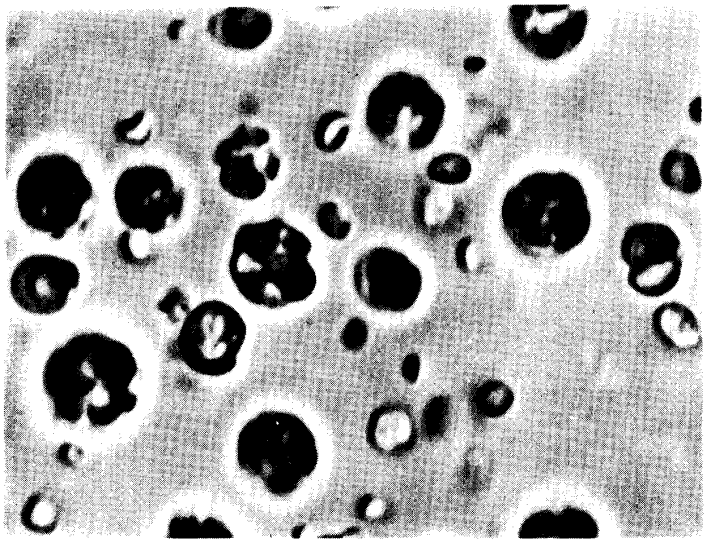


Fig. 7 10 μ

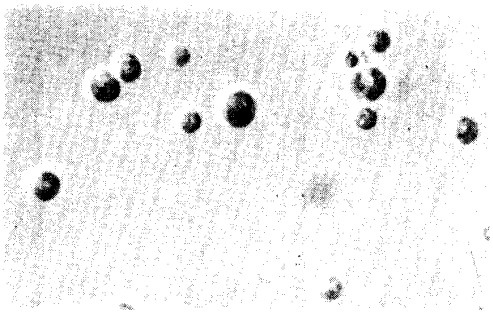


Fig. 9 a

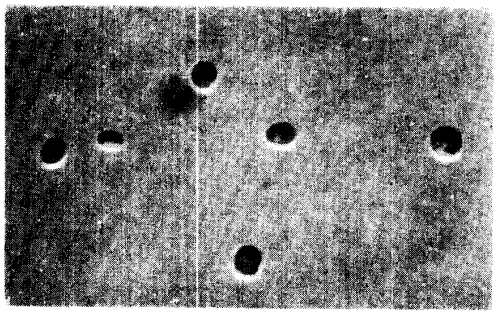


Fig. 9 e

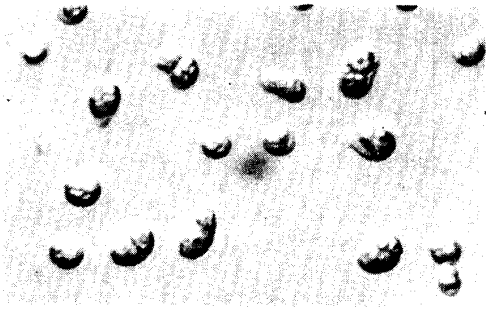


Fig. 9 b

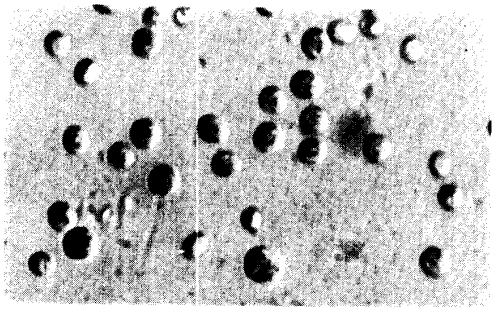


Fig. 9 f

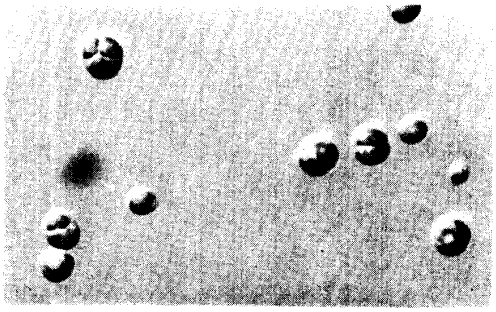


Fig. 9 c

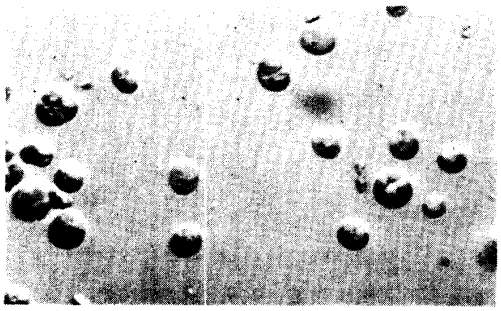


Fig. 9 g

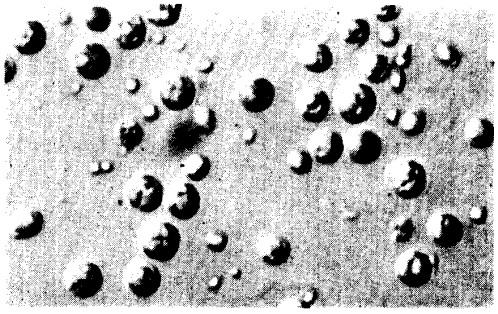


Fig. 9 d

Fig. 9 h

10 μ