Utilization of oils by a strain of *Candida tropicalis*

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The number of lipophilic microorganism occurs in the most natural environment high in oil and has been the object of considerable study as hydrocarbon utilizer through recent decade. Some of them have been valuable for reforming quite considerably the food production as microbial protein synthesized from hydrocarbon and worthless soybean source oil. The present report will also partly confront this problem.

On the other hand, the lack of knowledge about growth behavior and metabolic pathways of lipophilic and somewhat halophilic yeast under saline condition is quite surprising, despite the fact that marine microorganisms responsible for the breakdown of hydrocarbons are widespread in the sea as well as in the habitats of the salted foods such as soybean source and pickles, for example. *Candida pelliculosa* was found in tub of Japanese pickles during fermenting process, as reported by Hichiri (1968).

In these two physiological abilities of hydrocarbon utilizable yeast, *Candida tropicalis*, the utilizability of hydrocarbon or fatty acid and the ability to survive under the saline condition will be confirmed. These experiments constitute the initial steps into an investigation of why and how the present material can utilize lipid under saline condition.

Materials and Methods

Isolation and Identification: The present material was collected beneath the soil surface containing abundantly petroleum at Osaka City in 1967. A small amount of the fresh soil samples was suspended in 10 ml. of strilized water. A loop of this solution was placed on plate medium for isolation as shown in Table 1. In order to increase the chance of isolating lipophilic strains, each sample was treated with several passages through plate cultures, as described in detail previously (Ohara 1967).

After isolation of lipophilic yeast, according to the description which was published in Kreger-van Rij and Lodder monograph (1952), the present material was identified as *Candida tropicalis* on the basis of the characteristics though only few important ones among them were mentioned here as follows. After transfer of the present material to gypsum blocks or application of Gorodokowa's agar plating method, any sporulating ability was not observed, and this species generally occurs in vegetatively

Table 1. Composition of medium employed for isolation. Figures mean the concentration of g. per l. pH was adjusted to 5.5 initially.

Hydrocarbon (Kerosene)	20.0
NH_4NO_3	4.0
KH_2PO_4	4.7
$Na_2HPO_4 \cdot 12H_2O$	0.3
$MgSO_4 \cdot 7H_2O$	1.0
$FeSO_4 \cdot 7H_2O$	0.01
$CaCl_2 \cdot 2H_2O$	0.01
$MnSO_4$ 4-$6H_2O$	0.01
Ashed yeast extract	0.005
Tween-2O	0.1
Chloram phenicol	0.02
Agar agar powder	20.0

Table 2. Growth medium for experimental use. Carbon source was added to the respective medium in each experiment. Figures mean the concentration of g. per l. pH was adjusted to 5.0 initially.

NH_4NO_3	5.0
KH_2PO_4	2.5
$MgSO_4 \cdot 7H_2O$	1.0
Tween-20	0.5

one-cell phase in the medium as shown in Table 2, though it forms also occasionally filaments. This species is only slightly capable of assilmilating nitrate and is also good fermenter, at least of glucose, and further as carbon source other than hydrocarbons and fatty acids shown positive splitting of arbutin.

Finally, the present species was classified in genus *Candida* and identified as *C. tropicals* which is designated as a strain *OT-65* in our laboratory.

Preparation of worthless soybean source oil: Crude soybean source oil was obtained from crude soybean source after brewing (Moromi in Japanese) which was manufactured in the factory of Maruten Syoyu Co., Ltd. in Tatsuno City, Hyogo Prefecture, 1967. For purification of this crude oil, after washing several times with distilled water to desalt the crude oil till chloride reaction with silver nirtrate has not been observed, and further extracting with ether. Extracted oil solution was dehydrated by addition of sodium sulfuric anhydride and then the oil was subjected to the experimental use after ether in oil was evaporated.

Column chromatography: A column chromatography was poured at 20°C. Silica gel in the sodium form, was poured to 10 cm in depth into a column (40×2 cm). Sample was added to the column, followed by a benzen rinse. Thereafter samples in ether solution were placed on the column, and eluted with petroleum benzene, 2% methanol, benzene and ether, successively. After fractions were collected, they were titrated by alcohol with 0.01 N NaOH Sol.

Gas chromatography: All gas chromatographic analysis were made with gas chromatograph equipped with TCD detector. This was coupled with a recorder fitted with a disc integrator. Columns were packed with Chromosorb-W, 80 to 100 mesh coated with 25% di-ethglene glycol succinate (DEGS). All columns were conditioned before use at 230°C for at least 24 hours; helium flow rate 30 ml per minute; sample size, 0.5 to 1.0 μl.

A qualitative analysis was made by injection of a standard mixture of fatty acids

either before or after test. Quantitative analysis were performed with use of the disc integrator.

Preparation of culture medium for experimental use: As carbon and energy sources, kerosene, which is a mixture of petroleum hydrocarbons, chiefly of the methane series having from 10 to 16 carbon atoms per molecule, worthless soybean source oil and rancid food oil were mainly employed, respectively. As emulsifying agent Tween 20 (polyetylene sorbitan monolaurate) was added to medium, after it has been proved that Tween-20 itself can be hardly utilized by the present material.

Growth experiments: Cultures of *C. tropicalis OT-65* were grown in 500 ml. flaskes containing 50 ml. of medium as shown in Table 2. The carbon source was added as shown in each experiment. The pH of the culture medium was adjusted to 5.0 prior to steam sterilization and did not change appreciably with this treatment. Suspensions contained in flasks were incubated on a reciprocating shaker which was operated at approximately 120 oscillations per minute at a stroke length of 20 cm. or under stationary condition at 29°C. At appropriate time intervals the cells were harvested to determine the growth which was expressed as cell volume per 50 ml culture medium packed in modified haematocrit after centrifugation at 3500 r.p.m. for 10 minutes, because of the difficulty of strict measurement of dry weight of the cells by contamination of oil which may be adhered to cell wall extracellularly.

Result

Growth in worthless soybean oil, rancid salad oil and soybean oil media Fig. 1 compares the effect of soybean oil and worthless soybean oil as sole carbon source, respectively on growth.

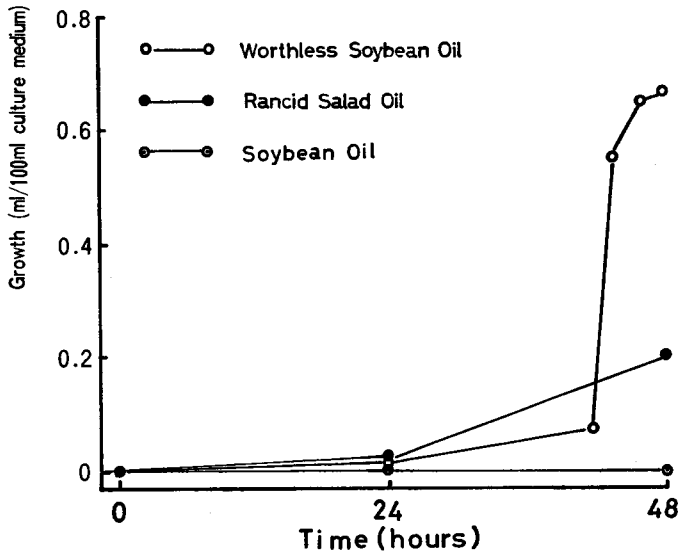

Fig. 1. Growth in worthless soybean oil, rancid salad oil and soybean oil media.

Since the composition of worthless soybean source oil must be originated from soybean oil, the comparative analysis of fatty acids between both soybean and worthless soybean source oils were made by column chromatography, when natural and domescated oil were used as carbon source for growth of the present material, fatty acid which formed from oil by interaction of either cell metabolism or excreted

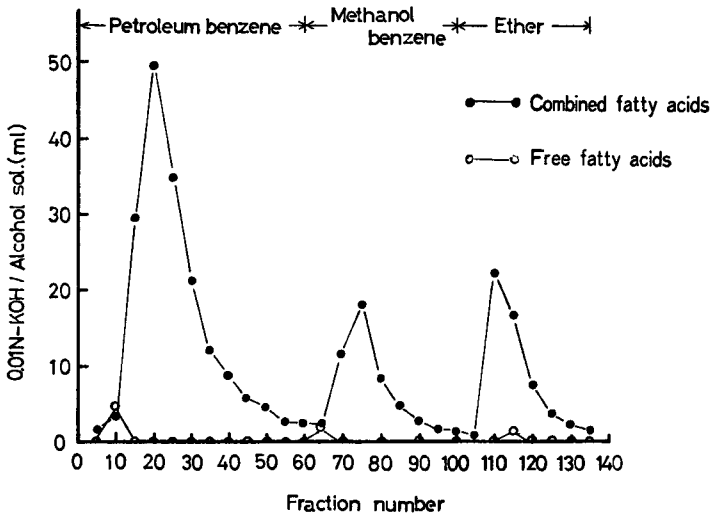

Fig. 2. Column chromatogram of combined fatty acids and free fatty acids in saponified soybean oil on silica gel.

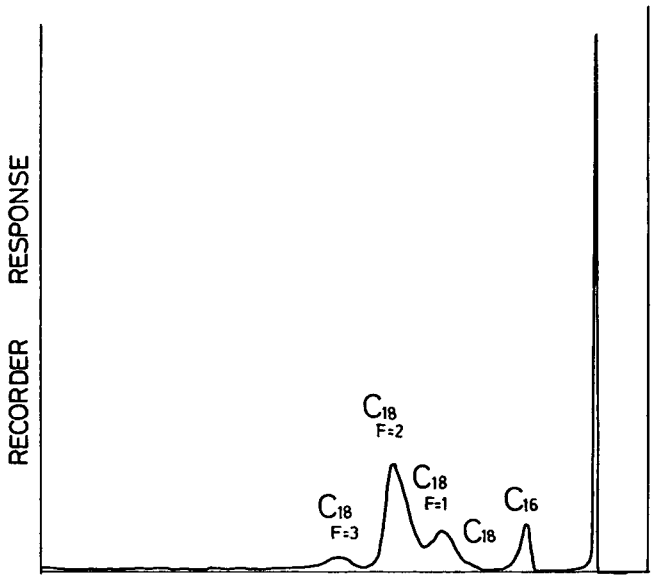

Fig. 3. Gas chromatogram of fatty acids in saponified soybean oil.

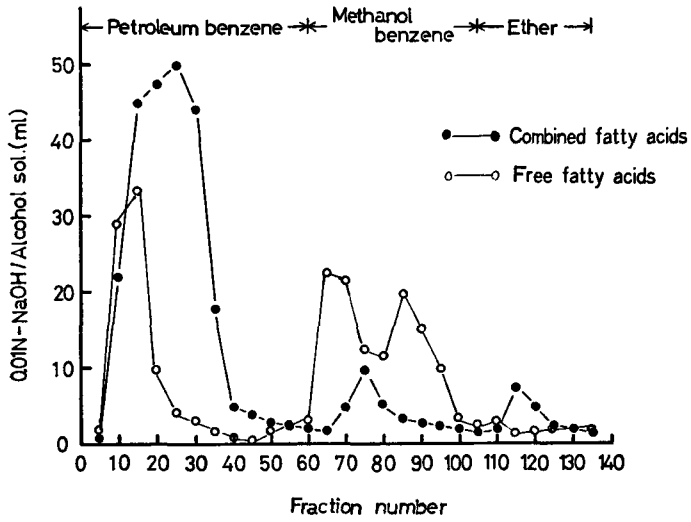

Fig. 4 Column chromatogram of combined fatty acids and free fatty acids in saponified worthless soybean source oil on silica gel.

enzymes from the cells with medium has been thought to be contributed to the changes in composition of the fatty acids as constituents of the oils. After saponification of soybean oil, we measured the combined fatty acids and free fatty acids on a column. The results were shown in Fig. 2 as shown in previous papar (1968). During the various gradient elutions, there was a large amount of monomer fatty acids in early number of tubes. Three peaks came off as a band. In Fig. 3. evidence obtained, by gas chromatography after saponification of soybean oil, for existence of C_{18}-compounds with two or three double bonds, is presented as shown in previous papar (1968).

After the soybean soyrce, the composition of combined and free fatty acids were somewhat changed, it being shifted to some extent to lower molecule of fatty acid from higher ones, as shown in Figs. 4 and 5. Therefore, by brewing the soybean source, the conversion of high molecular fatty acids or combined fatty acids to lower molecular fatty acids has been demonstrated in worthless soybean oil.

When the cells were inoculated to such worthless soybean oil medium under shaking culture condition, the changes in composition of fatty acids in worthless soybean oil medium after culture were demonstrated as shown in Figs. 6 and 7. By parallel experiment on uninoculated medium for the same period, the simultaneous changes in composition of fatty acids in worthless soybean source oil were also shown in Fig. 6. By inoculated and grown cells the monomer of fatty acids decreased in quantity, while the amounts of dimer and trimer did not so much increased as monomer did. Since quantitative fatty acids in uninoculated and inoculated media were approximately equal about 3 elution areas were selected for analysis. The fatty acids with relatively low molecule increased more significantly in total amount in uninoculated than

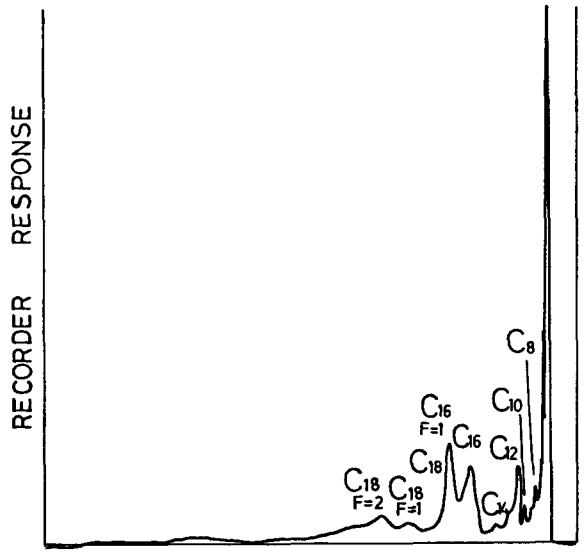

Fig. 5. Gas chromatogram of fatty acids in sapomified worthless soybean source oil.

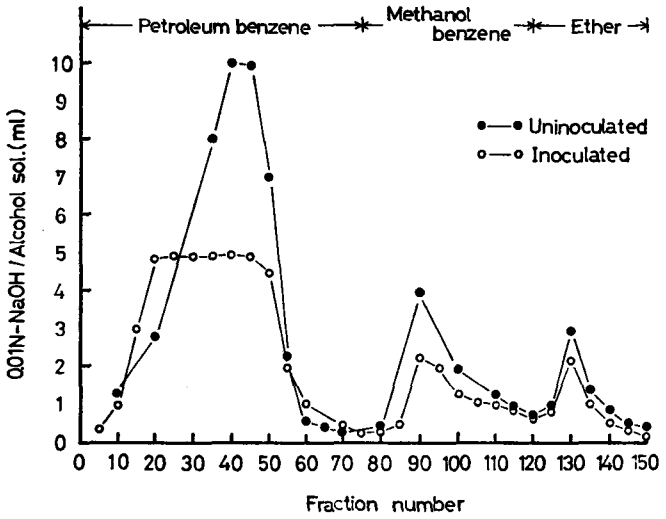

Fig. 6. Column chromatogram of combined fatty acids and free fatty acids in saponified worthless soybean source oil on silica gel.

inoculated medium. During growth period such changes occur in distribution of low molecular fatty acids, whereas there was a little differences in higher molecular fatty acids between uninoculated and inoculated media.

Especially a large amount of lower fatty acids appeared in soybean source oil after brewing, perhaps by degradation of oil and higher fatty acid esters by enzymatic

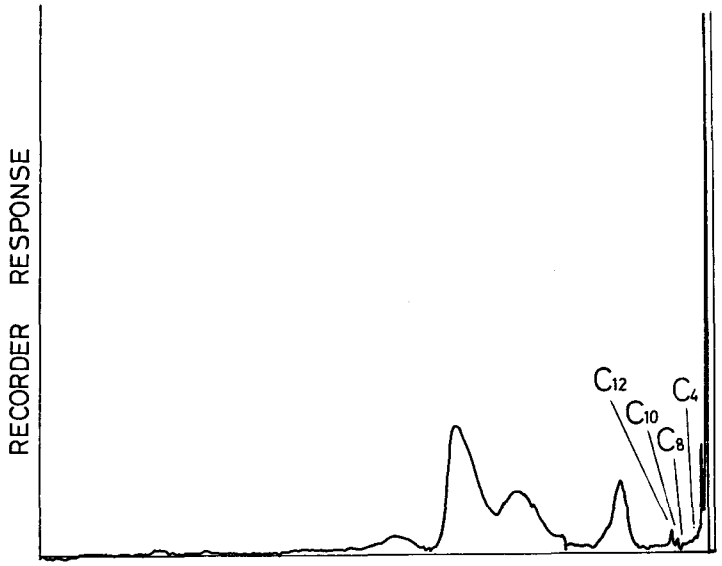

Fig. 7. Gas chromatogram of fatty acids in saponified worthless soybean source oil soparated from the culture under shaking culture condition.

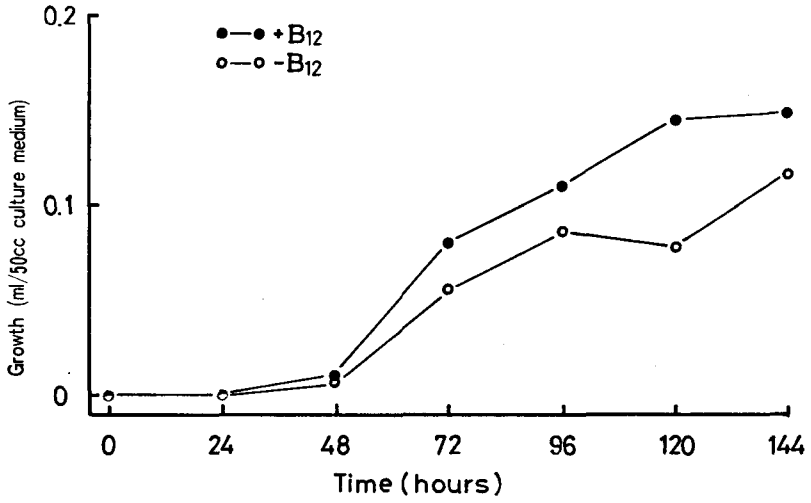

Fig. 8. Growth in fresh olive oil media in the absence of Vitamin B_{12}, or media containing Vitamin B_{12}.

attacks by several microbes during brewing period. Furthermore, after inoculation and culture of the present material in worthless soybean oil medium for 144 hrs under shaking condition, samples together with uninoculated medium were subjected to gas-liquid chromatography, some lower fatty acid esters, such as C_4-, C_8-, C_{10}- and C_{12}- compounds than those before inoculation were characterized to be detected in cultured medium supplied with worthless soybean oil as carbon source by culturing

Table 3. Effect of Vitamin B_{12} on growth of *Candida tropicalis* in the Kerosene medium.

	P. C. V. (ml)/100 ml	
Time (hrs)	$-B_{12}$	$+B_{12}$
48	0.09	0.25
96	0.14	0.38

the cells during growth period. These results suggested that the unknown compounds of lower fatty acids seemd to be butyric acid, caprylic acid, capric acid, and lauric acid when known compounds were compared with unknown samples by the retention times.

Effect of vitamin B_{12} on growth: In a further trial to discover whether growth could be enhanced by some cofactors and according to this S-shaped death curve after 144 hrs, as shown in the case of fresh olive oil as carbon source in Fig. 8 will be overcome, the medium was supplemented with vitamin B_{12} at the concentration of 3μMl. After the approximately same initial lag phase of growth in medium in the absence of vitamin B_{12} with those in medium containing vitamin B_{12} up to 48 hrs and the subsequent growth phase with steep logarithmic growing curve, any S-shaped death curves in medium containg vitamin B_{12} were not observed even after 96 hrs. The requirement of the present material for vitamin B_{12}, together with the example as mentioned above, was also recognized in the case of the growth in the kerosene medium, as seen in Table 3. Availability of hydrocarbons by the present material must also influence the stimulatory effect on growth through vitamin B_{12}.

Discussion

Numerous strains of these microbes have now been isolated, and although few studies on utilization of their carbon sources under saline condition have yet been made. As reported above in the present report, firstly the effects of natural oil and its worthless soybean oil after brewing were examined as each carbon source, prior to further studies concerning whether there is a correlation between lipid ut lizability and the enviromental hypertonicity with NaCl.

Non-halophilic yeast in general assimilates an average of 12.8 carbon compounds, whereas the marine species assimilates an average of 19.2. The difference between the two means is statistically significant (Van Uden et al. 1968). On the other hand the capacity some of non-halophilic yeast such as the present material, for assimilation of 21 to 22 carbon compounds under saline condition as well as normal condition may be an expression of assimilative versatillity to survive in the sea or in the saline environment. Therefore, the present material as one of the hydrocarbon utilizable yeasts are of interest both on account of their great economic importance and of their unique type of oxidative metabolism, which involves the mechanism of uptake and further transport of lipid under saline condition.

The present article is an attempt to bring a first information that will be gained during the searching course of utilization of lipid in saline, and the present study has been made of the mechanisms developed by the present material to deal with large molecules of oil. We shall consider that very large molecules such as kerosene and oil are reduced to assimilable and therefore presumably small molecules without any extracellular enzyme ever being demonstrable, because the very contact between the substrate and the organism seems to be enough. It is clearly impossible to deal in detail with the work on the existence of extracellular enzymes in the present work and there seems very need to elaborate further the importance of mechamism of penetrability of the present material to hydrocarbon and oil. As far as we know the evidences has been written before, the present material that is capable of utilizing hydrocarbon and oil of high molecular weight, may do so by librating into medium enzymes which break the oil down to very small assimilable molecules, which are probably oleic and stearic acids. On the other hand kerosene and oil can be broken down by close contact between the organism and the substrate. Whether or not the enzyme or enzyme system concerned with the breakdown of kerosene or oil is extracellular, intracellular, or residing on the cell surface, it may still remain obscure. This disagreement is unresolved and may, when further studied, tell us more about the conditions which decide whether some enzymes are intracellular or extracellular. When vitamin B_{12} was coexisted with carbon source, a possible example may be to be found in the present work on the problem mentioned above.

The oxidation of hydrocarbon as well as worthless soybean oil which has been made during brewing soybean source will provide an excellent example of the use of unique carbon compounds which permit the recognition of enzymes independently of enzymes which are always present to transfer essential metabolites. These oxidative reactions become particularly useful if some enzyme system is present to dispose of vitamin B_{12} and provide a basis for future studies of alterations of metabolism of different fatty acids. On the other hand, gibberellic acid (GA_3) will be more interest future studies. (A. Wood and L. G. Paleg)

Summary

A strain of yeast which was isolated from soil containing oil was identified as *Candida tropicalis OT-65*. This strain can utilize kerosene as carbon source. This attempt was made by culturing a suspension of cells in soybean source oil and worthless soybean source oil. These results would appear to be a satisfactory demonstration of a good growth. After growth, the substances appeared in culture medium were very much smaller in size than original oil, probably being fatty acids. The addition of vitamin B_{12} promoted the growth in medium supplied kerosene as well as olive oil as carbon source.

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References

Diskson, L. C., Galloway, R. A. and Patterson, G. W.: Enviromentally-induced changes in the fatty acids of Chlorella. Pl. Physiol. 44; 1413–1416, 1968.

Hichiri, H.: Yeasts associated with pickles and brinnes I. Yeasts isolated from "Takana" brine. Physiol. and Ecol. 15; 19–25, 1968.

Lodder, J. and Kreger-van Rij, M. J. W.: The yesats, a taxonomic study. Interscience Publishers Inc., New York. 1952.

Oginsky, E. L.: Vitamin B_{12} and methionine formation. Arch. Biochem. Biophys. 26: 327–329, 1950.

Ohara, K. and Tamaki, M.: Studies on hydrocarbon utilizable yeast KY-11. Identification. Proc. of Soai Women's Junior College 17; 25–31, 1967.

Ohara, K. and Tamaki, M.: Studies on the nature of Soyoil. The Japanese Journal of Nutrition 26; 84–89, 1968.

Shiio, I. and Uchio, R.: Microbial production of long chain dicarboxylic acids from n-alkanes. I. Screaning and properties of microorganisms. Producing dicarboxylic acid. Agr. Biol. Chem. Japan 35; 2033–2042, 1971.

Spoehr, H. A. and Milner, H. W.: The chemical composition of Chlorella; effect of environmental conditions. Pl. Physiol. 24; 120–149, 1949.

Van Uden, N. and Fell, J. W.: Marine yeasts in Adv. in microbiol, in the Sea 1; 167–211, 1968.

Wood. A and L. G. Paleg: Alteraction of liposomal membrane fluidity by gibberellic acid. Aust. J. Plant Physiol; 1974, 1, 31–40.

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