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Preliminary study on plasma membrane fluidity of Psychrophilic Yeast *Rhodotorula* sp. NJ298 in low temperature

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Abstract The ability of cell to modulate the fluidity of plasma membrane was crucial to the survival of microorganism at low temperature. Plasma membrane proteins, fatty acids and carotenoids profiles of Antarctic psychrophilc yeast *Rhodotorula* sp. NJ298 were investigated at – 3 ℃, 0 ℃ and 8 ℃. The results showed that plasma membrane protein content was greater at – 3 ℃ than that at 8 ℃, and a unique membrane polypeptide composition with an apparent molecular mass of 94.7 kDa was newly synthesized with SDS-PAGE analysis; GC analysis showed that the main changes of fatty acids were the percentage of unsaturated fatty acids (C18:1 and C18:2) and shorter chain saturated fatty acid (C10:0) increased along with the decrease of the culture temperature from 8 ℃ to – 3 ℃; HPLC analysis indicated that astaxanthin was the major functional carotenoids of the plasma membrane, percentage of which increased from 54.6 ± 1.5% at 8 ℃ to 81.9 ± 2.1% at – 3 ℃. However the fluidity of plasma membrane which was determined by measuring fluorescence anisotropy was similar at – 3 ℃, 0 ℃ and 8 ℃. Hence these changes in plasma membrane's characteristics were involved in the cellular cold-adaptation by which NJ298 could maintain normal plasma membrane fluidity at near-freezing temperature.

Key words psychrophilc yeast; plasma membrane fluidity; fatty acid; protein; carotenoids; cold-adaptation

1 Introduction

Antarctica sea ice is a relatively extreme environment with internal temperatures ranging from – 1 ℃ to as low as – 50 ℃ in winter[1]. In order to survive, microorganisms living in these severe environments must adapt structurally and physiologically. On cold-adaptation of Antarctic extreme cold environment microorganism previous studies have mainly addressed the production of cold-active enzymes, temperature-dependent synthesis of pigments, the maintenance of protein synthesis and the production of cold-acclimation proteins, and the mechanisms of freeze tolerance or avoidance[2-5]. But one important cold-adaptation strategy which was crucial to the survival of the cell, related to the ability of cell to

modulate the fluidity of plasma membrane at low temperature. Antarctic sea ice psychrophilic yeast *Rhodotorula* sp. NJ298 was able to survive due to its unique ability to cope with extreme conditions of Antarctic environment, and thus made itself an attractive eukaryotic model to understand the physiology and biochemistry aspect of cold-adaptation. In this paper the major plasma membrane fatty acids, carotenoids and protein compositions of NJ298 were investigated at near-freezing temperature, which would help to understand the cellular basis of Antarctic microbial cold adaptation.

2 Materials and methods

2.1 Strain and cultivation

Strain NJ298 was isolated from the sea ice in the Antarctica (68°30′E, 65°00′S) during 2001–2002 and it was identified as *Rhodotorula* sp. The strain was cultured in the sea water medium (peptone 0.5% and yeast extract 0.1%, pH 7.5) at different steady-state temperatures −3 ℃, 0 ℃ and 8 ℃. Cells were harvested at the logarithmic growth phase after 96 h of incubation and washed twice with distilled water.

2.2 Isolation of plasma membrane

NJ298 plasma membrane of each culture was isolated at high purity essentially as described previously[6] with modifications. All the steps were performed at 4 ℃. Briefly, 5 grams wet weight of cells were suspended in 20 ml of cold grinding medium (250 mM sucrose, 10 mM Tris-HCl buffer, pH 7.5), (and) containing a protease inhibitor mix (1.0 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml antipain, 2 μg/ml pepstatin, 2 μg/ml chymostatin). After homogenization with 20 g of glass beads (diameter 0.25–0.32 mm) were shaken vigorously on a homogenizer, the resulted homogenate was centrifuged at 2 000 g for 10 min to remove glass beads and unbroken cells. The pellet was washed once with the same grinding medium. The combined supernatant was centrifuged at 15 000 g for 45 min. The crude pellet obtained was resuspended in 5.0 ml of 10 mM Tris-HCl buffer (pH 7.5) and EDTA (1 mM), then titrated to pH 4.9 with 0.1 N acetic acid and quickly centrifuged at 11 200 g for 5 min. The pH of the supernatant was raised to 7.5 with 0.1 N NaOH and then centrifuged at 45 000 g for 15 min. Lastly the pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.5) containing glycerol (30%), phenylmethylsulfonyl fluoride (1.0 mM), and dithiothreitol (1.0 mM).

The purity of the plasma membranes was checked by measuring the ATPase activity which was a marker of isolated plasma membrane vesicles[6], according to the work previously published on yeast strain[7]. Briefly, plasma membrane ATPase activity was measured as the liberation of inorganic phosphate in the following mixture (120 μl): 50 mM Mes-Tris, pH 6.0, 330 mM sucrose, 50 mM KNO_3, 3 mM $MgSO_4$, 6 mM ATP, 0.1 mM EDTA, 1 mM sodium azide, 0.1 mM ammonium molybdate. The reaction was initiated by the addition of 10 μl of plasma membrane preparation containing 10–15 μg of protein at 30 ℃ for 30 min, then terminated by the addition of 120 μl of 10% TCA and the amount of phosphate released was estimated[8]. The difference between activities in the presence and

absence of 100 μm vanadate was considered the true value for the activity of this maker, and the purity of the plasma membrane preparations was assessed by the measurement of the activities of the marker enzymes NaN_3-sensitive mitochondrial ATPase at optimum pH 8.5 and vanadate-sensitive plasma-membrane ATPase at optimum pH 6.0.

2.3 *Protein determination*

Protein concentration was determined in the presence of 0.01% Triton X-100 by the Bradford method[9], with bovine serum albumin (BSA) as standard.

2.4 *SDS-polyacrylamide gel electrophoresis*

The polypeptide composition in the plasma membrane preparations of each culture were determined by SDS-polyacrylamide gel electrophoresis utilizing a 12.5% (w/v) separating and 3% (w/v) stacking gel by the technique of Laemmli[10].

2.5 *Preparation and analysis of fatty acid methyl esters*

Lipids of plasma membrane preparations (10 μl) of each culture were converted to fatty acid methyl esters by treatment with 5% HCl in methanol solution at 75 ℃ for 1 h. Resultant fatty acid methyl esters were extracted with hexane and analyzed as described[11] with a GLC Hewlett Packard 5890 gas chromatograph equipped with a flame ionisation detector and a HP-5 column (30 mm × 0.32 mm × 0.25 μm). The separated fatty acid methyl esters were identified by comparing their retention times with standard methyl esters and were quantitated with a datum analyzer. The experiments were performed in triplicate.

2.6 *Carotenoids extraction and quantification*

Plasma membrane preparations (20 μl) of NJ-298 of each culture was treated with 100% acetone (3 ml), vortexed and centrifuged until they became entirely colourless. The coloured phases were diluted with distilled water (10 ml), and then carotenoids were extracted with light petroleum (5 ml) until disappearance of any pigmentation from the lower phase. The pooled organic phases were washed repeatedly with distilled water, dried over Na2SO4 and evaporated under a stream of nitrogen gas. All above processes were conducted in darkness in order to avoid pigment degradation. Later the major carotenoids which was astaxanthin (unpublished) was quantified by high performance liquid chromatography as described previously[12], the experiment of which was conducted on the Agilent 1100 analytical system equipped with an photodiode-array detector and a reversed-phase C18 column (Apex ODS Column 4H25310, 4 × 250 mm 5 μm). The injected sample volume was 20 μl, and eluting solvent was methanol/water/n-hexane (95∶4∶1 v/v/v) with a flow rate 0.5 ml/min and the effluent was monitored at 478 nm. Before analysis, the mobile phase and samples were filtered through 0.45 μm filter membrane. All of the solvents used were of HPLC grade. Content of each carotenoids in the extract was calculated using the area percentage obtained from HPLC chromatography analysis. All tests were determined in trip-

licate.

2.7 *Plasma membrane fluidity*

Membrane fluidity in whole cells of *Rhodotorula* sp. NJ298 which cultured in seawater medium at −3℃, 0℃, 2℃, 4℃, 6℃ and 8℃, respectively, was determined by measuring fluorescence anisotropy using the membrane probe DPH (1, 6-diphenyl-1, 3, 5-hexatriene) on a Hitachi 650−10 fluorescence spectrophotometer based on Laroche's[13]. Excitation and emission wavelengths were 340 nm and 432 nm, respectively. The degree of fluorescence polarization (p) was calculated using the following equation: $P = (I_{VV} - G \cdot I_{VH}) / (I_{VV} + 2G \cdot I_{VH})$ with $G = I_{HV} / I_{HH}$, where I is the corrected fluorescence intensity, and subscripts V and H indicate the orientation (vertical or horizontal) of the excitation and analyzer polarizers. Fluorescence anisotropy was calculated by the following equation[14]: $r = 2P / (3 - P)$

3 Results

3.1 *Purity of isolated plasma membrane*

In order to estimate the purity of isolated plasma membranes, ATPase activity was measured. The results showed that the ATPase activity at pH 6.0 was inhibited more than 90% by 0.1 mM vanadate and less than 5% by 1 mM NaN_3. At pH 8.5, there was also no significant inhibition by NaN_3, suggesting that the isolated plasma membrane preparations were not significantly contaminated with mitochondrial membranes[15].

3.2 *Plasma membrane proteins analysis*

It was found that the plasma membrane protein content from NJ298 was greater at −3 ℃ than that at 8 ℃ (Fig. 1). Analysis by SDS-PAGE showed that the polypeptides pattern of plasma membrane obtained from NJ298 cells grown at −3 ℃ was different from that obtained at 0 ℃ and 8 ℃ (Fig. 2). One polypeptide band with an apparent molecular mass of 94.7 kDa was absent at 8 ℃ and 0 ℃, but present at -3 ℃ which was indicated by arrow (Fig. 2 lane 3).

3.3 *Fatty acids analysis*

The fatty acid profiles of plasma membrane from NJ298 revealed decanoic (C10:0), hexadecanoic (C16:0), octadecanoic (C18:0), octadecenoic (C18:1) and octadecadienoic (C18:2) acid to be the predominant fatty acids when it was cultivated at different temperature, and as a function of temperature the changes in fatty acids content presented in Table 1. As the temperature decreased from 8 ℃ to −3 ℃, the main changes were the percentage of unsaturated fatty acids (C18:1 and C18:2) and shorter chain saturated fatty acid (C10:0) increased, especially C18:2 and C10:0 promoted a tremendous increase which was more than double change, but resulted in a decrease in the proportions of C18:

0, while C16:0 remained unchanged on the whole. The plasma membranes contained a high concentration of saturated fatty acids at all growth temperatures, but the ratio of saturated/unsaturated fatty acids changed from 2.72 at 8 ℃ to 1.74 at −3 ℃.

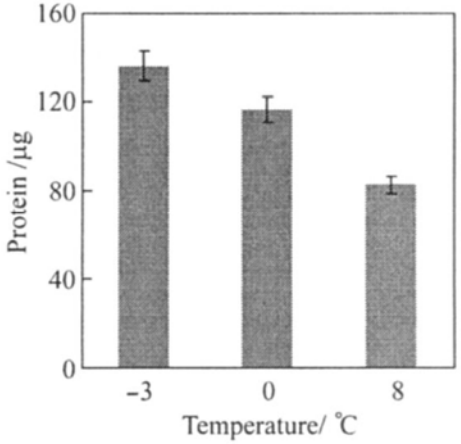

Fig. 1 Effect of temperature on the Plasma membrane protein production of *Rhodotorula* sp. NJ298. The data was the mean of three independent experiments. Standard deviations were depicted by the error bars.

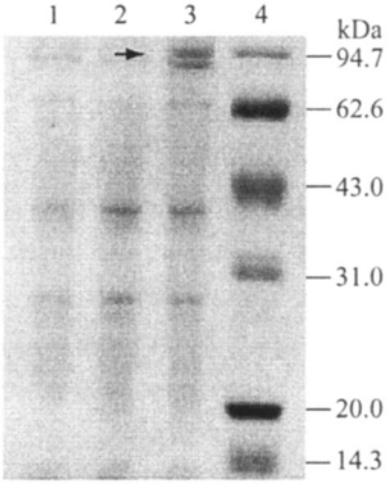

Fig. 2 Comparison of SDS-PAGE profiles of polypeptides from the plasma membrane fraction of *Rhodotorula* sp. NJ298 cultivated at 8 ℃ (lane 1), 0 ℃ (lane 2), −3 ℃ (lane 3). Each lane was loaded with 5 μg sample. Protein molecular mass markers (expressed in kDa) were shown on the lane 4.

Table 1. Fatty acids composition and content change (% of total) of plasma membrane from NJ298 cells grown at 8 ℃, 0 ℃ and −3 ℃ ($\bar{x} \pm s$)

Fatty acid	% of total fatty acid		
	8 ℃	0 ℃	−3 ℃
C10:0	8.9±0.3	10.1±0.4	18.2±0.4
C16:0	15.8±0.6	15.5±0.4	15.7±0.5
C18:0	29.6±0.8	26.3±0.9	22.6±0.8
C18:1	10.7±0.4	11.6±0.3	12.8±0.4
C18:2	9.3±0.4	12.4±0.4	19.7±0.5
Unidentified	25.7±0.8	24.1±0.7	11.0±0.5

3.4 *Carotenoids analysis*

HPLC analysis showed evidently the content of astaxanthin (peak a) increased progressively along with the decrease of the culture temperature from 8℃ to - 3 ℃, while another two carotenoids content decreased (Fig 3). The astaxanthin content was 54.6 ±1.5, 66.6 ±1.8 and 81.9 ±2.1% at 8 ℃ (Fig 3A), 0℃ (Fig 3B) and - 3 ℃ (Fig 3C), respectively. The results, for carotenoids in the pigment extracts from the plasma membrane of NJ298 cells, indicated that the different percentages of carotenoids depended on the culture temperature and lower temperatures seemed to promote syntheses of astaxanthin.

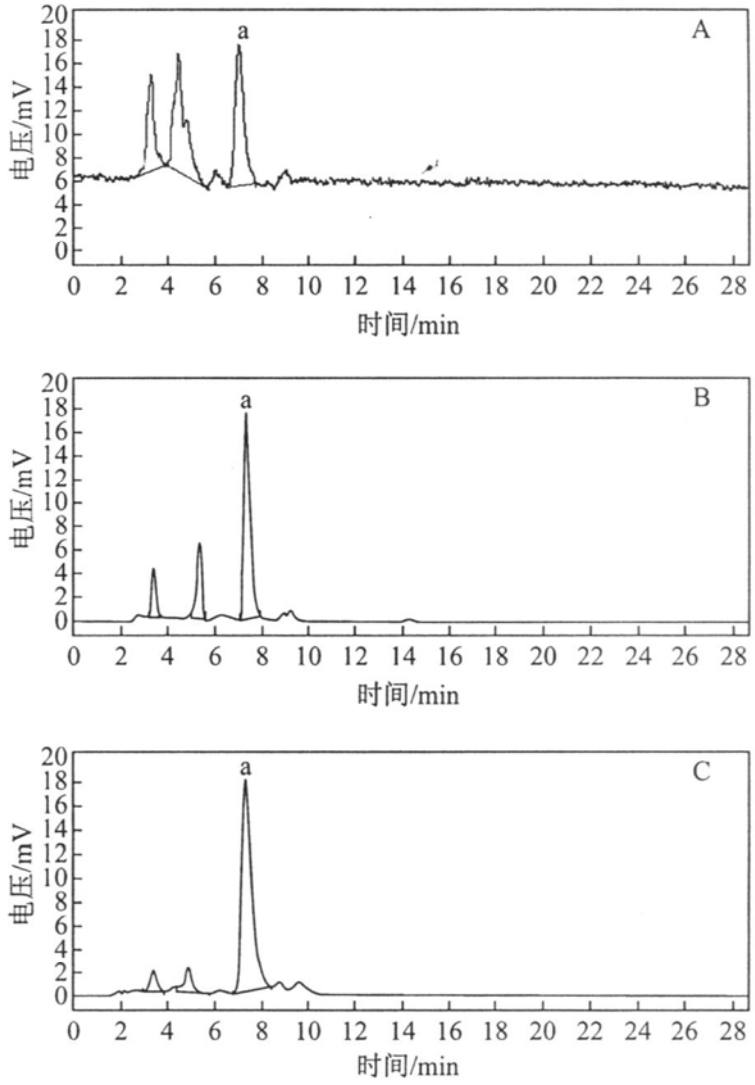

Fig 3 HPLC chromatograms showing carotenoids fractions and content separated from the plasma membrane of NJ298 cultured at different temperature; A: 8 ℃, B: 0 ℃, C: - 3 ℃; peak a astaxanthin

3.5 Cell plasma membrane fluidity

Usually, the fluidity of plasma membrane is measured as anisotropy by fluorescence polarization of probes, which decreases when membrane fluidity increases. The fluorescence polarization values of whole cells plasma membrane which corresponded to fluidity of plasma membranes of NJ298 were all steady at different temperatures (Fig 4). The results indicated the relative fluidity of the NJ298 cells was similar during cells growth from - 3 ℃ to 8 ℃.

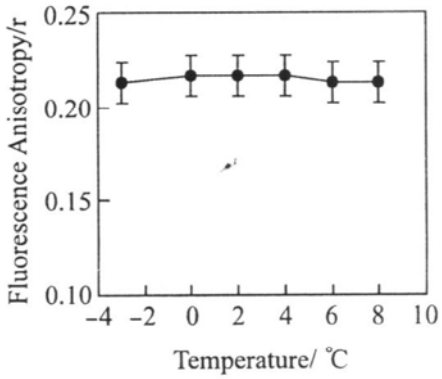

Fig 4 Fluorescence polarization of DPH probe in whole cell plasma memebrane of *Rhodotorula* sp. NJ298 grown at different temperature. Each datum point represented the mean of three independent experiments. Standard deviations were depicted by the error bars.

4 Discussion

Antarctic microorganism cellular adaptations to low temperatures and the underlying cold-adaptation mechanisms are not fully understood yet and are still being investigated. Previous studies have showed that fatty acids were crucial to the survival of the microorganisms at low temperature[16, 17]. In this paper, fatty acid analysis of NJ298 plasma membrane revealed a classical type of cold-adaptation by increasing the content of unsaturated fatty acids with decreasing temperature. Unsaturated fatty acids were significant to the survival of the microorganisms which increased membrane fluidity at low temperatures[18]. On the other hand, the content of shorter chain (C10: 0) fatty acids increased which was different from some Antarctic psychrophilic bacterium which adapted to extreme conditions by changing in the fatty acid composition with a preponderance of branched chain fatty acids[19-21]. Shorter chains fatty acids, especially those with less than 12 carbons, were unable to span the bilayer and could not form hydrophobic interactions with other lipids and proteins, and thus conduced to maintain the fluid state of the plasma membrane. And the marine bacterium *Shewanella putrefaciens* adapted to fluctuating temperature conditions by varying the content of short-chain fatty acids[22]. Furthermore, short chains fatty acids had lower melting points than those of longer chain (↓ length)[23]. So the significance of the two main changes of fatty acids confirmed NJ298 cells grown at near-freezing temperature by increasing the fluidity of the plasma membrane. The effect of growth temperature on the outer membrane proteins of *E. coli* has been well documented[24]. The results of plasma membrane protein analysis revealed that NJ298 responded to the low temperature by inducing the synthesis of a set of proteins, and it was noteworthy that the psychrophilic yeast NJ298 showed the presence of cold inducible protein of 94.7 kDa which was only present in cells grown at near freezing temperature – 3 ℃, indicating that expression of the membrane protein was under temperature specific regulatory control which may play a role in cold-adaptation. In *E. coli* the phenomenon had been studied and shown one novel membrane protein of 93.0 kDa response to low temperature[25]. Furthermore it was reported membrane proteins could interact with the lipids, and contributed to the overall stability of the bilayer[26, 27]. Meanwhile pigment analysis firstly indicated polar carotenoids astaxanthin was the major carotenoids of the plasma membrane from NJ298, and its content increased with the culture temperature decreased

which was consistent with temperature-dependent synthesis of pigments in Antarctic psychrotrophic *M. roseus* revealed higher yield of polar carotenoids at low temperature, compared to the production of non-polar carotenoids[4]. A number of Antarctic bacteria have been found to contain carotenoid type of pigments in their membrane, and it had been speculated that these pigments may play a role in buffering membrane fluidity due to their ability to localize in the membrane[11, 28]. Furthermore, it has been suggested that the survival of the Antarctic microorganisms at low temperature may be enhanced by the ability of carotenoids to stabilize membranes[29]. However carotenoids structural features influenced the chemical properties of the carotenoids as well as their location and orientation within lipid bilayers in biological environments. In vitro studies based on the interaction of carotenoids with model membranes had clearly demonstrated that polar carotenoids stabilize the membrane and non-polar carotenoids increase the fluidity of membranes[30]. In addition it was reported astaxanthin could avoid extreme high levels of lipid damage, change plasma membrane permeability and scavenge radicals inside the membrane both by the conjugated polyene chain and the terminal ring moiety[31, 32]. Hence astaxanthin was possible playing an important role in regulation of plasma membrane fluidity at near-freezing temperature.

Taken together, the cold-adaptation response of NJ298 cell to extreme low temperature was possible to postulate that at near-freezing temperature some of the normally fluid components of plasma membrane became gel-like; however, in plasma membrane the unsaturated and shorter chain fatty acids increased, resulting in maintaining the plasma membrane in a fluid state; and meantime cold-active and inducible proteins, polar carotenoids astaxanthin combined which counterbalanced this effect and helped to stabilize the plasma membrane in a fluid state, and then the gel formation was prevented, in turn maintained the normal plasma membrane fluidity and preserved the function inside the cell, as a consequence NJ298 was able to adapt and still survived at near-freezing temperature. In conclusion, the understanding of these multiple changes of the plasma membrane components in NJ298 throw new light on the importance of the plasma membrane in the physiology of highly cold-adapted microorganism and provide significant new information relating to a characteristic of importance in various biotechnology applications.

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