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THE EFFECT OF CONCENTRATION OF NITROGEN AND PHOSPHORUS ON CELL CULTURE IN *PORPHYRA YEZOENSIS*

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ABSTRACT Nitrogen and phosphorus have a great influence on cell growth and of development in *Porphyra*. This paper discussed the effects of defferent sources of nitrogen, varied concentration of nitrogen and phosphorus, and different cell densities on cell survival ratio and cell growth when cells isolated from thalli on *Porphyra yezoensis* cultured in vitro. The results showed that the isolated cells grew better in NO_3-N than in NH_4-N media. For the first 3 days, the cultured cells had some endurance to vary concentration of N and P. After 3 days, the concentration of N/P would influence the cell survival ratio. The cell survival ratio was the highest when the concentration of N/P was 18—20/1.8—2.0 mg l^{-1}. And the cell division speed would increase when the concentration of N/P promoted. The cell density of $1-2\times10^5$ cells/ml was better to cell survival ratio and growth, if cell density was too higher, the survival ratio would be low and cell growth would be slow. The experiments had laid the foundation of the cell culture and breeding with large scale in *Porphyra yezoensis*.

KEYWORDS cell culture, nitrogen and phosphorus, survival ratio, division ratio, *Porphyra yezoensis*

0 INTRODUCTION

The breeding with single cells in *Porphyra* has been a more successded application research [Wang Sujuan and He Peimin, 1992], which applied the biotechnology on *Porphyra* cultivative production. In the early of 80's, the experiment of single cell seedlings cultivation at sea field in *Porphyra yezoensis* was made [赵焕登和张学成, 1984]. Then the breeding with cells in *P. yezoensis* and *P. haitanensis* was made and the cell seedlings were moved into sea field [方宗熙等, 1978; 王素娟等, 1987; 戴继勋等, 1988]. Further, the advanced methods, breeding with the spores from cell aggregates and breeding with immobilizated cells were carried out [王素娟和何培民, 1992; Wang Sujuan and He Peimin, 1992; He Peimin and Wang Sujuan, 1994]. But the cell survival ratio was still lower, so it was urgent

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to establish steady cell supension culture and increase the cell survival ratio, to provide much more cells for the cell breeding in *Porphyra*. Cell suspension culture conditions should be strict and optimum. Some extro conditions and important procedings have been reported in other papers [He Peimin and Wang Sujuan, 1992; 何培民和王素娟, 1992; Wang Sujuan and He Peimin, 1992], and this paper mainly discussed the effect of concentration of nitrogen and phosphorus on cell culture. The nutrient in medium, especially nitrogen and phosphorus, was very important medicine and economic plant cell suspension systems have been established, the nitrogen and phosphorus have been as the limiting nutrient to control cell growth and development [Dougall & Keith, 1980; King & Street, 1973; Martin *et al.*, 1977]. However, in *Porphyra* cell culture, there were few papers dealing with this aspect. For the future large scale culture, the establishing steady suspension culture should be studied.

1 MATERIAL AND METHODS

1.1 Material collection

The fresh thalli of *Porphyra yezcensis* were collected in Haifeng seafield, Jiangsu Province. After fast dried in the shade, the collection was put in deep freezer (−20℃) with air tight plastic sacks.

1.2 Preparation of isolated cells

Freezed thalli were recovered in seawater for 4−5 days. Brushed and rinsed them in sterile seawater for three times. Cut them into pieces less than 2 m^2 and put them into enzyme solution for 2−3 h at 26℃. The enzyme solution was prepared with 1.5% sea snail enzyme and 0.5% cellulase dissoved in 0.7M mannitol with sea water, pH=5.8.

1.3 Cell culture method

(1)*State culture* Cells were cultured in culture dishes (the dish 6cm measured in diameter) with 10ml media, the cell density was 1×10^5 cells/ml.

(2)*Suspension culture* Cells were cultured in 250ml conical flasks on a shaker (2R Modle) made by New Brunswik Company, the ration was 125rpm.

1.4 Culture conditions

The culture media was steriled seawater with MES (medium of enriced seawater)[王素娟等, 1986], and adding nitrogen from $NaNO_3$ and $(NH_4)SO_4$ and phpsphorus from KH_2PO_4. The cultures were performed at 17−21℃, 2500Lux, light period was 12L:12D. Counted the cell number every 3 days, then refresh the culture medium after each counting.

1.5 Counting method

Counting live cells and dead cells in 10 fields under microscope with 10×20 miltiplicity.

2 RESULT AND DISCUSSION

2.1 Effcts of different nitrogen sources on the ratio of survival cells

In plant cell culture, MS medium was a general medium. The NH_4—N concentration in the medium was about as high as 280 mg l^{-1}, and many researches showed both of NO_3 and NH_4 could be as the nitrogen source. Some algae absorbed the NH_4—N faster than the NO_3—N, and so was *Porphyra*[李纫芷,1980; 严昌敬,1990; 张自立和俞新大, 1990], so the experiment of effect for NO_3—N and NH_4—N on cell survival ratio was done.

From Tab. 1, it showed that the cell survival ratio had a tendence of decreasing as culture time increase. At the 3rd day, there was no difference between the two groups, both ratio was about 80%. At 6th day, there was a little difference, the ratio was about 65—80%, and the relative ratio was about 92.2—103.6%. At 9th day, it showed that the higher the concentration was, the lower the rato was, when concentration of N was over 20 mg l^{-1}. And there was a big difference of ratio between the two nitrogen source with the 10—20 mg l^{-1} concentration range. The survival ratio with NO_3—N was obviously higher than with NH_4—N. The result showed NO_3—N was better than NH_4—N as nitrogen source.

Tab. 1 Comparison between the different effects of NO_3—N and NH_4—N on ratio of cell survival (%)

concentration of N/P (mg l^{-1})		10.0/1.0	20.0/2.0	40.0/4.0	60.0/6.0
3rd day	NO_3—N	80.20	83.37	84.08	79.08
	NH_4—N	81.09	80.63	82.74	81.93
6th day	NO_3—N	76.68	79.41	77.65	70.89
	NH_4—N	77.64	77.41	71.63	69.17
9th day	NO_3—N	60.64	63.13	48.89	42.19
	NH_4—N	42.15	51.65	48.51	40.56

From Fig. 1, it showed that when cell cultured at 9th day, the ratio of survival cells with the concentration of 20 mg l^{-1} NO_3—N was the highest, about 63%, and the 10 mg l^{-1} was the second; As for NH_4—N, the best concentration was also 20 mg l^{-1}, ratio about 52%, then the 40 mg l^{-1} second. When concentration was more than 40 mg l^{-1}, the ratio decreased.

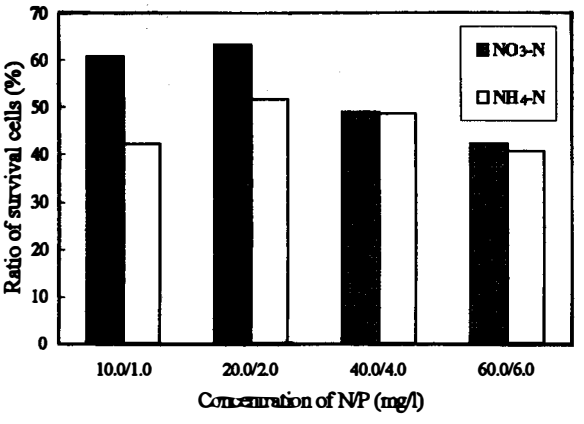

Fig. 1 Effect of NO_3—N and NH_4—N on cell survival ratio at 9th day

Generally, in plant cell culture, the medium contains about 350 mg l^{-1} NO_3—N, the NH_4—N concentration should be in the range of 28—280 mg l^{-1}[严昌敬,

1990；张自立和俞新大，1990], and if NO_3 and NH_4 are used with mixture, the NH_4 is absorbed by cells faster than NO_3.

From above results, the NO_3—N should be the main nitrogen source, and adding low concentration NH_4—N would be beneficial to cell culture in *Porphyra*. For the concentration of NO_3—N and NH_4—N in seafield are about 20 $\mu g\ l^{-1}$ and 30—240 $\mu g\ l^{-1}$, the *Porphyra* can not grow in the medium with so high nitrogen concentration as plant cells. So in the *Porphyra* cell culture medium, the NO_3—N concentration should be 20 mg l^{-1}, and the NH_4—N may be about 5 mg l^{-1}.

2.2 Effect of Concentration of N/P on the cell survival ratio with suspension culture

After studied cell culture with static method, we also studied the change of cell survival ratio among different N/P concentration of NO_3—N and PO_4—P in cell suspension culture. Tab. 2, Fig. 2 and Fig. 3 showed the results.

Tab. 2 Effects of different concentration of nitrogen and phosphorus on ratio of survival cells with suspension culture

concentration of N/P (mg l^{-1})		10.0/1.0	20.0/2.0	40.0/4.0	60.0/6.0
	cells/ml	ratio of survival cells (%)			
3rd day	1×10^5	91.01	89.08	84.03	81.86
	2×10^5	89.1	82.3	90.7	88.9
	5×10^5	100.0	100.0	100.0	87.2
	1×10^6	95.0	87.5	90.0	83.5
6th day	1×10^5	47.8	56.6	20.8	10.4
	2×10^5	38.65	43.10	31.12	28.88
	5×10^5	84.0	83.2	78.4	50.4
	1×10^6	52.0	50.0	45.0	28.0
9th day	1×10^5	12.1	13.8	13.2	2.2
	2×10^5	26.34	22.47	9.5	15.3
	5×10^5	76.8	79.2	58.4	40.8
	1×10^6	47.5	47.5	19.8	17.8

Note: The cells in groups of cell density were not come from the same batch, so the comparison between groops of cell density could not be made.

Fig. 2 showed that the effect of concentration of N/P on cell culture with suspension culture was the same as with static culture. At the 3rd day, there was very little difference between each concentration groups, especially in 10/1.0, 20/2.0 and 40/4.0 mg l^{-1} groups. At the 6th day, the cell survival ratio of 10/1.0 and 20/2.0 mg l^{-1}groups was about 85%, decreasing about 15%. The 40/4.0 mg l^{-1} group decreased about 20%, and the 60/6.0 and 80/8.0 mg l^{-1} groups decreased about 25—30%, the ratio was about 50—65%. It showed the effects of N/P concentration was very obvious, the higher the concentration was, the

lower the survival ratio was. From Fig. 3, it showed the 20/2.0 mg l^{-1} was the best one, the survival ratio was about 80%. And the 10/1.0 mg l^{-1} group was the second, about 78%. The 40/4.0, 60/6.0 and 80/8.0 mg l^{-1} groups were 60%, 40% and 20% respectively. It showed higher concentration of over 40/4.0 mg l^{-1} was harmful to cell culture.

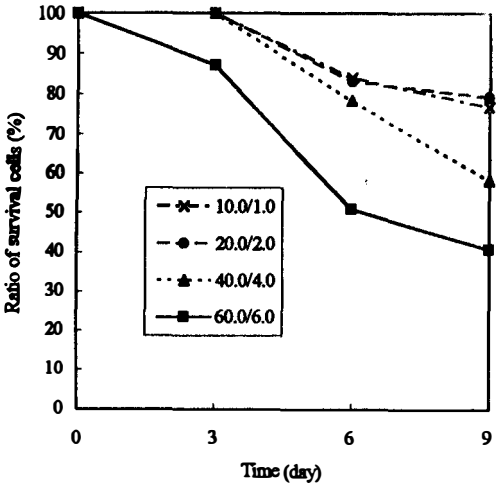

Fig. 2　Relation of culture time and different nitrogen concentration on cell survival ratio

The experiment of the small class of N/P concentration in the range of below 40/4.0 mg l^{-1} was made. From Tab. 3 and Fig. 4, the best concentration of N/P was 18/1.8 — 20/2.0 mg l^{-1}, and in the range of no more than 25/2.5 mg l^{-1}, the concentration of all groups was stuitable to cell culture. When the concentration was higher than 30/3.0 mg l^{-1}, the ratio of cell survival began to decrease, it showed when concentration was over 25/2.5 mg l^{-1}, the cell grew not well.

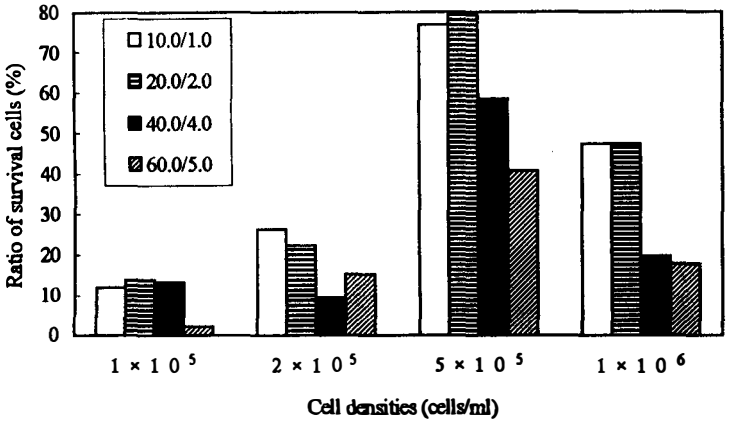

Fig. 3　Effects of different N/P concentration on ratio of survival cells with suspension culture for 9 days

In plant cell culture, N and P concentration is very high. Such as, in MS medium, the total nitrogen (NO_3—N and NH_4—N) was about 840 mg l^{-1}, and PO_4—P was about 32 mg l^{-1}. But in the *Porphyra* cell culture, the cells only could grow well in very poor N/P concentration which was not higher than 25/2.5 mg l^{-1}. It seemed to be decided by the *Porphyra* nature and genetic speciality, for the *Porphyra* grew in seawater in which the N and P concentration was very low. On another hand, for the culture was not sterile culture, poor concentration of N and P could protect bacteria from growing. So the cells only could grow in low concentration of N and P better.

Tab. 3 Effects of different concentration of N/P on ratio of survival cells, cell division ratio, cell differentiation

concentration of N/P (mg l^{-1})	ratio of survival cells (%)	ratio of cell devision (%)	number of cell seedlings	number of cell aggregates	seedlings: aggregates
control	62.43	20.6	49	192	0.26
2.0/0.2	55.67	31.9	61	151	0.40
4.0/0.4	55.67	30.0	57	98	0.58
6.0/0.6	53.60	48.7	42	101	0.42
8.0/0.8	53.86	28.2	50	104	0.48
10/0.1	65.21	27.0	32	73	0.44
12/1.2	55.35	34.7	20	61	0.33
14/1.4	52.28	38.5	32	80	0.40
16/1.6	58.36	43.72	26	44	0.59
18/1.8	63.72	43.95	40	56	0.71
20/2.0	63.33	30.85	11	27	0.41
22/2.2	55.67	32.75	11	40	0.28
25/2.5	49.57	47.90	25	61	0.41
30/3.0	30.63	64.05	22	76	0.29
35/3.5	16.45	83.57	29	43	0.67

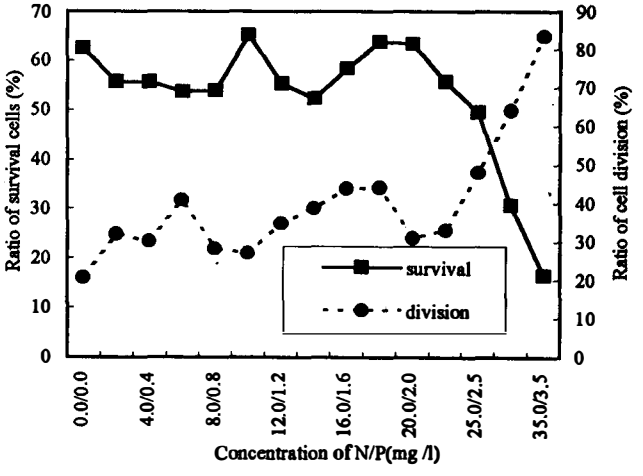

Fig. 4 Effects of different N/P concentration on ratio of survival cells and cell division

2.3 Effect of N/P concentration on cell growth and development

The N and P concentration was influence not only on the cell survival ratio but also on the cell division, growth and development.

From Tab. 3 and Tab. 4, in state culture, it showed that when concentration of N/P

promoted, the cell division ratio would increase, especially when N/P concentration was 35/3.5 mg l^{-1}, the division ratio reached as high as 83.5%. It seemed high concentration and was helpful to cell division.

From Tab. 4 the result showed when the N/P concentration was 40/4.0 mg l^{-1} the cell division ratio was the highest, which was 13.7%, but when the N/P concentration was 80/8.0 mg l^{-1}, the divsion was zero. So if concentration was too high, cell division would stop. But in some range, the higher the N/P concentration was, the more helpful the cell division and growth was. It showed when cell divided, the cell needed more nitrogen and phosphorus than when cell initially cultured.

Tab. 4 Effects of different N/P concentration and cell densities on division ratio with suspension culture

concentration of N/P (mg l^{-1})	10.0/1.0	20.0/2.0	40.0/4.0	60.0/6.0	80.0/8.0
ratio of cell divition (%) (5×10^5 cells/ml)	7.0	6.0	13.7	5.8	0
cell density (cells/ml)		1×10^5	2×10^5	5×10^5	$\times10^6$
cell divition ratio (%) (at 9th day)		65.40	53.34	4.97	0

There are two different cell developmental ways after cells divide and grow. The first one is to develop directly into seedlings, the another is to divide into cell aggregates [何培民和王素娟, 1992a]. Tab. 3 and Fig. 5 showed that the cell developmental way was not influenced by the N/P concentration changes, although cell survival ratio and cell division ratio were influenced by the N/P concentration. The way of cell differentiation and development may be decided by the cell locale and cell development stage in the thalli of *Porphyra*.

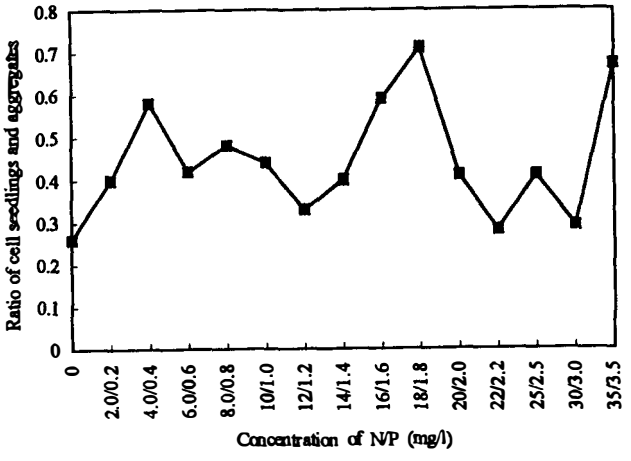

Fig. 5 Comparison among different cell densities after suspension culture for 9 days

2.4 Effect of cell densities to cell survival ratio and cell division ratio

In cell suspension culture system, the cell survival ratio and division ratio are closely ralated with cell densities. With the same batch of isolated cells, and with 20/2 mg l^{-1} N/P concentration, the experiment of effects of different cell densities was made.

Tab. 4, 5 and Fig. 6 showed the results. At the 3rd day, there was little difference of cell survival ratio between differen density groups, the survival ratio was high, about 90—95%. At the 6th day, the survival ratio decreased and there was a great difference among different densities. The higher the density was, the lower the survival ratio was. At the 9th day, the group of 1×10^5 cell/ml density was the best one, the survival ratio was the highest, about 62.7%, and the division ratio was also the highest, about 65.40%. It showed the higher the density was, the lower the division ratio was. When the density was 1×10^6, the division was zero. It seemed too high density was harmful to cell divison. For too high density, cell could not get enough illumination, so cells could not grow well and divide, If culture cells with high density, the illumination and nutrition must be improved, because the cells in *Porphyra* need higher light intensity than plant cells whose density can reach about 10^8 cell/ml. Higher N and P concentration can keep higher division, but lower survival ratio, so in the initial culture, cells would be cultured with low N/P concentration, after

Tab. 5 Comparison between different cell densities with suspension culture

Cell densities (cell/ml)	1×10^5	2×10^5	5×10^5	1×10^6
		ratio of survival cells (%)		
Culture at 3rd day	95	94	95	90
Culture at 6th day	76	56	42	39
Culture at 9th day	62.7	41	37	33

Nate: Culture with 20 mg l^{-1} nitrogen and 2 mg l^{-1} phosphorus.

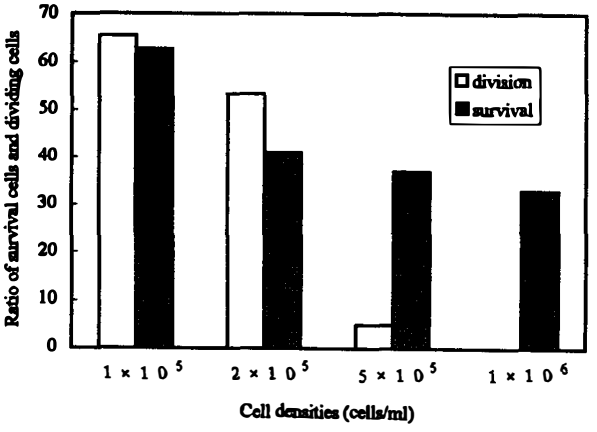

Fig. 6 Ralation of N/P concentration and way of cell differentiation and development

3 — 6 days, they could be cultured with higher N/P concentration for cells division, especially, the continuous culture should be established earlier as plant cells cultured in chemostat culture to control cell survival ratio and cell division ratio with N and P concentration changes.

3 CONCLUSION

From above results, it was concluded that NO_3—N was a good source of nitrogen, and the best N/P concentration was 20/2.0 mg l^{-1} in *Porphyra* cell culture. In the initial culture, lower N/P concentration was beneficial to cell survival. After 3 — 6 days, the N/P concentration should be increased for cell division. Too high cell density was not beneficial to cell culture, if the illumination and nutrition of cell culture were improved, the higher cell density would be gotten.

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氮磷浓度对条斑紫菜细胞培养的影响

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提 要 氮和磷对紫菜的细胞生长和发育有很大的影响，本文讨论了不同氮源、不同氮和磷的浓度和不同细胞密度对条斑紫菜细胞培养的存活率和细胞生长的影响。试验结果显示，对于细胞生长用 NO_3－N 作为氮源优于用 NH_4－N 的。前3天培养的细胞对 N/P 浓度的变化有一些忍耐性，3天后 N/P 的浓度对细胞存活率有影响，当 N/P 浓度为18－20/1.8－2.0 mg l^{-1}时细胞存活率最高，第6天时，可达到77－80%，细胞分裂速度随 N/P 的浓度而增加。细胞密度为1－2×10^5细胞/ml时，其存活率和生长较好，如果密度越高，则存活率和生长就越低。因此，在进行紫菜体细胞育苗生产时，细胞悬浮培养前三天，可加入 N/P 浓度为20/2.0 mgl^{-1}。细胞密度以1－2×10^5细胞/ml为宜。

关键词 细胞培养，氮和磷，存活率，分裂率，条斑紫菜

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