韓國營養學會誌 : 第12卷 第2號
K.J.N., Volumb 12, Number 2, 1979

The Effect of the Different Time Period between the Fermentation and the Freeze-drying on Protein Efficiency Ratios of Candida utilis

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—요 약—

Single Cell Protein(Candida utilis)에 있어서 Fermentation과 Freeze-Drying 과정 사이의 시간 차이가 Protein Efficiency Ratio에 미치는 영향

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인구의 증가와 사료 가격의 상승으로 인한 육류 제품의 가격이 상승하고 있음은 우리나라를 비롯하여 세계적인 현황이다. 이 문제에 대처하는 한 방법으로 새로운 단백질 급원으로써 Single Cell Protein이 개발되었고 이에 대한 여러가지 연구가 되어지고 있다. Single Cell Protein이 인간에게 식용이 될 동물이나 나아가서는 인간에게 직접 식품으로 사용되기 전에 인체나 동물 체내에 끼칠 영향과 그 안전성을 확인하려는 하나의 노력으로 이 연구가 시도되었다.

동물 체내 대사에 미치는 SCP의 악영향의 주 원인은 함유된 독생물질로 보고 그 독성물질이 SCP생산 및 처리 과정중 어느 시기에 생겨나는가를 규명해 보고자 하였다.

Fermentation을 끝낸 SCP의 Supernatant내에 함유된 Inorganic phosphate(I.P.)의 함량을 측정하여 SCP세포의 Viability를 알아보았다. Fermentation을 끝낸 후 10일까지는 I.P.가 차츰 줄다가 40일이 지났을 때에는 I.P.의 증가를 보였으나 직후보다는 낮음을 나타냈다. 또한 냉동 건조시킨 뒤에도 SCP Cell이 살아있음을 보였다.

또한 위와 관련지어서 SCP를 Protein급원으로 써서 사육한 흰쥐에 나타난 Protein Efficiency Ratio(PER)를 통하여 SCP의 질을 평가하였다. 실험결과로 나타난 것을 보면 표준 식이에 사용된 Casein의 PER($\bar{X}$=2.58)이 SCP의 PER(Dict 1; $\bar{X}$=0.888, Dict 2; $\bar{X}$=0.893, Dict 3; $\bar{X}$=0.860)보다 유의적으로 높았다. 그리고 총실험기간의 평균치를 볼 때, Fermentation을 끝내고 3일 후와 6일 후에 냉동 건조시킨 SCP의 PER은 시판되는 SCP의 것과 별다른 차이를 보이지 않았다. 그러나 후반기에 있어서는 Fermentation을 끝내고 3일과 6일 후에 냉동 건조시킨 SCP의 PER이 시판되는 SCP의 것보다 약간 저조함을 보였다. 그리고 Casein의 PER은 총 사육기간을 통하여 별 변동이 없음에 반하여 후반기에 SCP의 PER이 급격한 저하를 보임은 SCP사용상의 문제점을 나타낸 것으로 해석된다.

I. INTRODUCTION

The present and foreseeable shortage of protein for human and animal consumption constitutes a compelling incentive to search for new and reliable sources of that nutrient. Single cell protein may be such a source. Single cell protein (SCP) by definition is a "generic term for crude or refined sources of protein whose origin is unicellular or simple multicellular organisms, for example, bacteria, yeast, fungi and algae"[1].

During World War I, Candida utilis(Torula yeast) was manufactured in Germany to supplement the other sources of protein in short supply. In the United States of America and elsewhere, the production has continued, although interest in this yeast diminished after World War I, probably for economic reasons[2]. However, the search for the new protein sources became very important due to the recent and foreseeable world food shortage.

The single cell protein used in this study is derived from Candida utilis* that was grown through a continuous fermentation with ethanol being the carbohydrate substrate.

There have been several studies on nutritional value of SCP showing that protein from single cell protein compared well with high quality protein sources such as egg, milk, meat, and fish in terms of the amount of crude protein[3]. However, it has been reported that yeast single cell protein as the sole source of protein produced a lower growth rate than casein, possibly due to the low content of sulfur containing amino acids[4]. Furthermore, recent studies have suggested that the weight loss of rats fed the bacterial single cell protein may be caused by certain unknown toxic substance(s) rather than the unbalanced amino acid pattern of single cell protein[5,6]. Therefore, it would be desirable to find out what is (are) the toxic substance(s).

Miller considered that the most significant toxicological problem of single cell protein is associated with the gastrointestinal syndrome. It appeared that at very high levels in the diet, cramps, diarrhea, and general discomfort were present in men consuming algal single cell protein. It was assumed to be caused by bacterial contaminants of the algae used in those studies or low digestibility of single cell protein[7].

Further toxicological problems may be associated with the feeding of large quantities of nucleic acid. Meiseles and Marshall's study showed that the kidney weight increased significantly when the single cell protein present in the diet increased[8]. Agren et al. suggested that the higher precent body weight of the kidneys implied that perhaps, renal calcification or hyperplasia occurred in the rats fed single cell protein[9].

Despite those studies, it is not clearly known yet what causes the toxic effect on animals or humans fed single cell protein nor when the toxic substances are produced.

The purpose of this study is to observe whether single cell protein harvested at the same time, but freeze-dried at different time periods has any effect on the protein efficiency ratios when fed growing rats. The inogranic phosphate concentration of the supernatant of single cell protein is a possible method for measuring the viability of the cell, as the inogranic phosphate concentration will change as the cell dies due to the absence of adenosine triphosphate(ATP) synthesis and the hydrolysis of ATP in the cell. Protein efficiency ratio was the chosen method to evaluate the quality of the protein.

In addition, the protein efficiency ratios of rats fed those proteins were compared with that of the conventional single cell protein and casein.

Ⅱ. METHOD AND MATERIAL

A) Measurement of the Inorganic Phosphate Concentration in the Supernatant of SCP

Single cell protein coming out from the fermentor was stored in refrigerator till the samples were taken.

Six samples of the supernatant of the SCP were taken. Group 1: Supernatant of the SCP taken immediately after fermetation, Group 2: Supernatant of the SCP taken three days after fermentation, Group 3: Supernatant of SCP taken six days after fermentation, Group 4: Supernatant of SCP taken ten days after fermentation, Group 5: Supernatant of SCP taken forty days after fermentation.

*Special thanks are due to Arthur E. Humphrey, Ph. D., Dean of the College of Engineering and Applied Sciences, at the University of Pennsylvania for contributing the Candida utilis cells.

The inorganic phosphate concentration in each sample was measured by the method developed by Chen et al[10]

B) Measurement of Protein Efficiency Ratios in Rats fed 15% SCP Diet

C. utilis was grown with ethanol through continous fermentation, centrifuged at 3200 RPM for 5 minutes, washed with buffer solution(MOP) and freeze-dried at two different time intervals, such as, 3 days and 6 days after the fermentation. Casein(standard), conventional-SCP* and the experimental-SCP** were used.

Twenty four-20-day old Sprague-Dawley male rats were individually caged upon delivery to the laboratory and fed Purina Rat Chow until the experiment began. At the onset of the actual experiment, the rats were 34-day-old and weighed from 70 to 100 grams.

The rats were divided into four groups and each group consisting six rats.

The content of the basal diet is shown in Table 1. The composition of the experimental diets is: Diet 1: 15% SCP which was freeze-dried 3 days after the fermentation, Diet 2: 15% SCP which was freeze-dried 6 days after the fermentation, Diet 3: 15% conventional SCP, Diet 4: 15% casein.

The Protein Efficiency Ratio(PER) developed by Osborne was the chosen method to evaluate the protein and the degree of toxicity of SCP. The drinking water was given ad libitum and the casein diet was the reference standard[13]. 1.5% agar solution was added to the diet which solidified the mixture, so that rats can handle their food easily and reduce the spillage and also facilitate more accurate measure of food consumption and wastage.

*Conventional SCP is Torutein-10 from Amoco Foods Co., Chicago, Ill. which is C. utilis grown on ethanol having protein content 52% and nucleic acid content 9%.

**Experimental SCP from University of Pennsylvania which is C. utilis grown on ethanol having protein content 50 % and nucleic acid content 8%.

Ⅲ. RESULT

The protein content of the Candida utilis was found to be 50% with total nucleic acid content of 8%.

The inorganic phosphate concentration of the superenatant of C. utilis changed when the time period increased after the fermentation. There was no significant difference between the inorganic phosphate concentration of the supernatant of SCP measured three days after the fermenation($\bar{X}$=1.861 mg, SE=±0.018) and the inorganic phosphate concentration of the supernatant of the SCP measured six days after the fermenation ($\bar{X}$=1.830 mg, SE=±0.045) ($p<0.05$), However, the inorganic phosphate concentration of the supernatant of the SCP measured ten days after the fermenation($\bar{X}$=1.742 mg, SE = ± 0.08) showed significant difference from the inorganic phosphate concentration of the supernatant of the SCP measured three days($\bar{X}$=1.861 mg, SE=±0.018) or six days after the fermentation($\bar{X}$=1.830 mg, SE=±0.045, $p<0.05$) (Table1). The most interesting observation was that the inogranic phosphate concentration of the supernatant of the SCP measured forty days after fermentation($\bar{X}$=1.873 mg, SE=±0.018) was not only significantly higher than that of SCP measured six days and ten days after fermentation($\bar{X}$=1.80 mg, SE=±0.045, $\bar{X}$=1.742 mg, SE=±0.018 respectively) but also close to that of the single cell protein measured three days after the fermentation($\bar{X}$=1.861 mg, SE=±0.018) (Table 1 and Fig. 1).

The mean protein efficiency ratio of the diets is given in Table 5. It appears that the PER of casein($\bar{X}$=2.58, SE=±0.24) is significantly higher than the PER of the single cell protein, Diet 1($\bar{X}$=0.888, SE=±0.180), Diet2 ($\bar{X}$=0.893, SE=±0.140) or Diet 3 ($\bar{X}$=0.860, SE=±0.11) ($p<0.05$). The mean PER of the SCP freeze-dried three days after the fermentation($\bar{X}$=0.888, SE=0.180) and that of the single cell protein freeze-dried six days after the fermentation($\bar{X}$=0.893, SE=±0.40) are insignificantly different from the

Table 1: The Inorganic Phosphate Concentrations of Supernatant of *C. utilis* which have different Time Period between the Fermentation and the Freeze-drying

Group 1	$\bar{X}$[1]	1.970 mg P/ml
(supernatant taken immediately	SD[2]	±0.045
after fermentation)	SE[3]	±0.018
Group 2	$\bar{X}$	1.861 mg P/ml
(supernatant taken 3 days after	SD	±0.045
fermentation)	SE	±0.018
Group 3	$\bar{X}$	1.830 mg P/ml
(Supernatant taken 6 days after	SD	±0.077
fermentation)	SE	±0.045
Group 4	$\bar{X}$	1.742 mg P/ml
(supernatant taken 10 days after	SD	±0.045
fermentation)	SE	±0.018
Group 5	$\bar{X}$	1.873 mg P/ml
(supernatant taken 40 days after	SD	±0.045
fermentation)	SE	±0.018

1) Mean, 2) Standard Deviation, 3) Standard Error.

PER of the conventional SCP($\bar{X}$=0.86, SE=± 0.11).

It was also observed that the PER of the conventional SCP($\bar{X}$=0.56, SE=±0.20) is slightly higher than experimental SCP, Diet 1($\bar{X}$=0.44, SE=±0.32) and Diet 2($\bar{X}$=0.40, SE=±0.32) for the second period of feeding (from the sixth day to the eleventh day). The paired 't' test shows that the mean PER of the Diet 3, conventional SCP is significantly different from the mean PER of the experimental SCP, Diet 1($0.001<p<0.002$) and diet 2($0.01<p<0.02$).

It is interesting that there is a drastic decrease in PER in the second period(from the sixth day to the eleventh day) of feeding, compared to the first period(from the first day to the fifth day) of feeding except the PER of the casein diet. The PER of the casein diet was consistent throughout the feeding period, compared to the PER of the single cell protein diets.

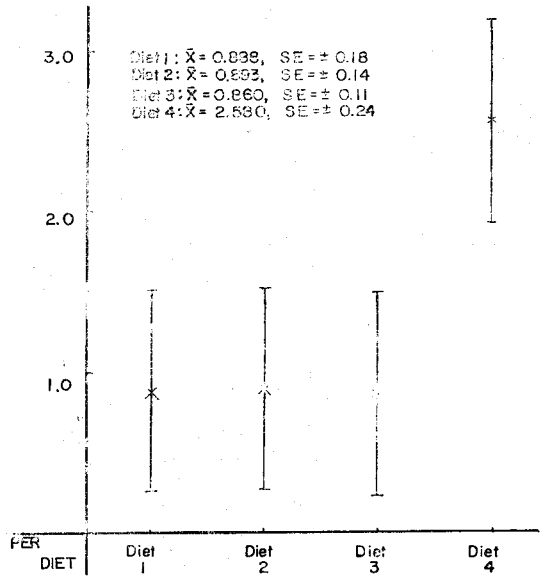

Fig. 1. The LSD test Score(1.278) and the Mean of PERs of Each Diet for Entire Eleven Days.

VI. DISCUSSION

It was shown that the inorganic phosphate concentration of the supernatant of the single cell protein decreased after the fermentation and then started to increase, when stored in refrigerator. However, the freeze-dried yeast cells were shown to be alive when the cells were plated on both Sabouraud and Myco-cell.

In this experiment, the inorganic phosphate concentration of the supernatant of the SCP was used as a indicator of the degree of the cell viability. Since the adenosine triphosphate(ATP) formation is essential for living cell[15], when cells die, the inorganic phosphate concentration of the supernatant of the SCP will increase because of the lack of ATP synthesis and continue hydroly-

sis of ATP in the cells. Therefore, it is assumed that, at the beginning of the storage period, the viability of the yeast cell increased, then starts to decrease ten days after coming off the fermentor, but the yeast cell did not die even after freeze-dried maintaining its minimal viability. It is assumed that the decreased cell viability after ten days is due to the deficiency of nutrients.

From the result of this experiment, it is only possible to discuss the relationship between the length of the storage period of single cell protein after harvesting and the protein efficiency ratio of single cell protein since the inorganic phosphate concentration of the supernatant of the single cell protein did not show a linear relationship with the storage period.

In the entire eleven days of the feeding period, the PER of single cell protein freeze-dried three days and six days after harvesting($\bar{X}=0.888$, SE$=\pm 0.18$, $\bar{X}=0.893$, SE$=\pm 0.14$ respectively) were similar. However, in a comparison of the second period of the feeding(from the sixth day to the eleventh day), the protein efficiency ratio of the single cell protein freeze-dried three days after harvesting, Diet 1 ($\bar{X}=0.04$, SE$=\pm 0.32$) is slightly higher than the protein efficiency ratio of the single cell protein freeze-dried six days after harvesting, Diet 2 ($\bar{X}=0.40$, SE$=\pm 0.32$).

It is not clear yet what caused the lower PER in rats fed the single cell protein freeze-dried six days after harvesting than the PER of single cell protein freeze-dried three days after harvesting. There might be some changes in the cell during the storage period before freeze-drying. For example, certain changes of the cell component may occur during the storage period effecting the amino acid pattern and causing the decreased protein efficiency ratio of SCP. There are several studies done concerning the effect of storage of C. utilis. When they were stored for three to four years under uncontrolled condition, three was decrease in the biological value of C. utilis. In amino acid analysis, the level of methionine was abnormal[16]. However, in the present study, the single cell protein was stored for forty days only for the measurement of the inorganic phosphate concentration of the supernatant of the single cell protein and for six days only for the measurement of the protein efficiency ratio of the experimental single cell protein.

However, Yanet et al.'s study showed that the low biological value of the long-stored cell was not corrected by the simultaneous addition of casein and DL-methionine. Therefore, they assumed that there might be some toxic substances which caused the biological value of C. utilis. However, their histo-pathological data did not suggest the presence of toxic compounds but intestinal disturbances[17]

In addition to the difference of the storage condition between those studies and the present study, the difference of the protein efficiency ratio among the single cell protein diets shown in the present study does not appear to be significant. ($p<0.05$) The effect of the time period between the fermentation and the freeze-drying may not be a major factor on the toxic effect of the single cell protein shown by low protein efficiency ratio of rats. The storage period between the fermentation and the freeze-drying may increase the effect of certain toxic substances already existing.

The protein efficiency ratios of all the single cell protein show much lower values than that of casein as indicated in other studies. Several possible causes have been suggested. Agren reported that yeast single cell protein as the sole protein source produced a lower growth rate than he casein, probably due to the lack of sulfur containing amino acid[18]. However, the amino acid pattern of the conventional single cell protein used in the present experiment compares well with that of casein. Therefore, it is difficult to explain that the lower protein efficiency ratio was caused by unbalanced amino acid pattern of the single cell protein.

It has been assumed that the high content of nucleic acid of single cell protein is related with

the toxicological problems in single cell protein feeding. Meiseles and Marshall reported that kidney weight, in relation to body weight, was significantly higher in the single cell protein fed rats when compared to the other groups[19]. These results may indicate that the high nucleic acid: protein ratio found in single cell protein may give rise to high blood levels of urea and uric acid and possibly the accummulation of uric acid in kidney[20]. Agren's study showed that renal calcification and hyperplasia had occurred in the rats fed single cell protein. However it seems unlikely that the nucleic acid content of the diet caused the increased kidney weight. Rats have the enzyme, urease which catalizes the oxidation of uric acid to the more soluble allantoin[21].

It was suggested by Agren et al. that the presence of cretain unknown toxic substances in bacterial single cell protein might be responsible for the arrested growth and weight losses[22].

Meiseles and Marshall's study also showed some toxic effects of bacterial single cell protein to rats. One rat was fed a fifteen percent single cell protein diet for thirteen days, then was placed on a sixty percent single cell protein diet, and within twenty-four hours of this diet change, the rat died. Another rat whose body weight was 206 gram, was fed rat chow until he was five weeks old, then was placed on a 60% single cell protein diet, and within three days he lost 13% of his body weight. These results suggest that a toxic substance was possibly the cause of the rats' weight loss in SCP feeding[23].

In the present study, the drastic decrease of the protein efficiency ratios for the second period of single cell protein feeding(from the sixth day to the eleventh day), compared with the first period of SCP feeding (from the first day to the fifth day) may be considered to be the result of certain unknown toxic substance. However, it is still not answered when the toxic substance is produced nor what is the toxic substance(s) since there was no linear relationship between the cell viability and the storage period of SCP in this experiment.

It is also uncertain yet why the protein efficiency ratios of the conventional SCP show lower values than that of the SCP freeze-dried three days after the fermentation and also show similar value to the single cell protein freeze-dried six days after fermentation. The amino acid content of the conventional single cell protein compares well with that of casein[24,25,26]. The conventional SCP was derived from C. utilis that was grown through a continuous fermentation with ethanol like the experimental single cell protein. The conventional single cell protein was dried by spray-drying, and the experimental single cell protein was dried by freeze-drying. However, it is difficult to conclude that the difference in the protein efficiency ratios is only due to the difference of the method of drying of the single cell protein. It is assumed that there might be some effects of certain different conditions of the production of those single cell proteins. Brown and Rose reported that several cell components of C. utilis differed under various conditions. The greatest difference was in the ribonucleic acid and protein contents of cells grown under NH_4 limitation, which increased as the temperature was decreased[27].

From the present results, it can be concluded that much work must still be done for the complete understanding of the toxicity of SCP before single cell protein can be used as a food source by man. It would be desirable to study more time period between the fermentation and the freeze-drying relating the changes of the inorganic phosphate concentration of the supernatant of the single cell protein. Longer feeding trials for the rats would yield more data. The other factors of the cell production which would effect the quality of SCP should also be carefully studied. The amino acid and the nucleic acid content must be clearly defined and studied. The digestibility of single cell protein must be carefully studied.

Special thanks are due to Carol Marshall, Ph. D., Professor of Home Economics at Rutgers-The

State Unversity of New Jersey, U.S.A. for advising this research.

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