OPTIMIZATION OF THE PRODUCTION OF ASTAXANTHIN BY *PHAFFIA RHODOZYMA*

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ABSTRACT

Of thesis entitled

OPTIMIZATION OF THE PRODUCTION OF ASTAXANTHIN BY *PHAFFIA RHODOZYMA*

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The red pigment astaxanthin is the principal carotenoid pigment responsible for the distinctive orange red pigmentation of marine invertebrates, fish and birds. Astaxanthin can be used as a colorant for the red-pink color of farmed salmon, trout and shrimp. Relatively few species can produce astaxanthin. Although the intracellular astaxanthin content is relatively low when comparing with *H. pluvialis*, which is another promising microorganism for industrial use, the yeast *P. rhodozyma* is a possible candidate for commercial production because of its high biomass yield. In order to increase astaxanthin content of *P. rhodozyma* to meet increasing market needs in the future, one of the fast and efficient approaches seems to be the strain improvement of *P. rhodozyma* by mutagensis. However, the methods for both astaxanthin and biomass production are also of prime importance. As *P. rhodozyma* is a Crabtree positive yeast, its cell yield and pigment production are reduced at high sugar concentrations. In order to minimize

the effect of high sugar concentration, fed-batch fermentation has to be used. D.O.-stat and exponential fed-batch cultures of *P. rhodozyma* have been investigated previously. In this study, pH-stat cultures of *P. rhodozyma* were extensively investigated.

To optimize biomass and carotenoid production by *Phaffia rhodozyma* in pH-stat cultures, two methods of feeding glucose were studied. In the first method, which is comparatively simple to operate, the glucose feeding set point (pH 5.02) was higher than the culture pH (5.00) and *P. rhodozyma* grew at a low specific growth rate (μ = 0.055 h^{-1}). In the second method, the glucose feeding set point (pH 4.98) was lower than the culture pH 5.00 and the yeast grew at a specific growth rate (μ = 0.095 h^{-1}). With the second method of glucose feeding, which is more complex, in order to prevent overfeeding of glucose, a 'time interval" was added to the control strategy of the glucose pump and allowed to expire before the next dose of glucose was added. The length of the "time interval" affected biomass and carotenoid production. A critical time interval (T_c) was defined. In pH-stat cultures of *P. rhodozyma*, it was found that if the "time interval" was set longer than the critical time interval, the yeast did not grow.

Apart from investigating the cultivation method, the effect of adding ethanol to D.O.-stat cultures of *P. rhodozyma* was examined. The time of addition of ethanol to the

system and the concentration of ethanol added are important. When ethanol was added to the system simultaneously with glucose during the log phase, a low concentration of feeding ethanol (0.01% volume of ethanol / volume of fermentation medium) increased the total carotenoid content. On the other hand, a higher concentration of feeding ethanol (0.02% - 0.04% volume of ethanol / volume of fermentation medium) increased the cell biomass. If ethanol concentrations in the culture were too high, the cells were inhibited. When ethanol was added to the system during the stationary phase, provided that the ethanol concentration was not too high (less than 4.4% volume of ethanol / volume of fermentation medium) in the medium, it increased the total carotenoid production of *P. rhodozyma*. Moreover the increase of the total carotenoid content might be inversely proportional to the concentration of ethanol.

GLOSSARY OF SYMBOLS AND ABBREVIATIONS

A	absorbance
°C	degree Celsius
CDW	cell dry weight
d	days
D.O./DO	dissolve oxygen tension
g	gram
h^{-1}	unit of specific growth rate (per hour)
HMGS	β-hydroxy-β-methylglutaryl-CoA synthase
HMGR	β-hydroxy-β-methylglutaryl -CoA reductase
HPLC	high performance liquid chromatography
hr(s)	hour(s)
kg	kilogram
L	litre
mg	milligram
min(s)	minute(s)
ml	millilitre

mm	millimetre
N	normal (concentration)
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced form
nm	wavelength (nanometre)
rpm	rotation per minute
μ	specific growth rate
μg	microgram
UV	ultraviolet
v	volume
w	weight
YM agar	yeast extract and malt extract agar

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CHAPTER 1

INTRODUCTION

In 1972, Phaff and collaborators discovered a yeast referred to as *Rhodozyma montanae*, which contains astaxanthin as the principal carotenoid pigment. Later, Miller described a new genus of this yeast, *Phaffia* with the single species, *Phaffia rhodozyma*. It has two striking characteristics, the intense reddish color associated with mature cultures, coupled with the ability to ferment several sugars.[1] The red pigment astaxanthin is the principal carotenoid pigment responsible for the distinctive orange-red pigmentation of marine invertebrate, fish and birds.[2] Astaxanthin can be used as colorant for the red-pink color of farmed salmon, trout and shrimp.[3] It was forecasted that the farmed salmon production would be 460,000 tonne in year 2000 and possibly more than 100,000 kg of astaxanthin would be required by the year of 2000. This potential demand could open a large market for microbially produced astaxanthin.[3] For this reason and because of the growing importance of astaxanthin in aquaculture and medical field, it was chosen as the target of this study. Relatively few microorganisms can produce astaxanthin. These microorganisms include the yeast *P. rhodozyma*;[1, 2, 4] the algae *H. pluvialis*;[5] the bacteria *A. aurantiacum*.[6] Among them, the *P. rhodozyma* is a possible candidate for commercial production because of its high

astaxanthin content.[3] Since the demand for astaxanthin will be large in the future, scientists have tried to obtain astaxanthin over-producing mutants of *P. rhodozyma*.[7-11] In November 1998, a technical bulletin of Igene Biotechnology Company stated that the astaxanthin content produced by their *P. rhodozyma* mutated strain reached 8000 ppm. Strain improvement of *P. rhodozyma* by mutagenesis seemed to be a fast and efficient way of increasing the yield. Although it is easy to obtain overproducing mutants by the method of mutagenesis, methods for both astaxanthin and biomass production are also a very important issue in industial production. As *P. rhodozyma* is a Crabtree positive yeast, its cell yield and pigment productions are reduced at high sugar concentration.[12] In order to minimize the effect of high sugar concentrations in industrial fermentations, fed-batch fermentation is normally used instead of batch fermentation. Three different kinds of fed-batch fermentation were studied in my undergraduate project [13]. Fermentation parameters for D.O.-stat[14] and exponential fed-batch cultures[15] of *P. rhodozyma* were optimized. In 1991, D. Porro et al developed a pH-controlled fed-batch system for budding yeast[16] and in 1993, H. Yamada et al developed an efficient pH-stat method for fed-batch cultures of *E. coli*.[17] In these two studies, the substrate was fed to the system when the culture pH increased and reached a substrate-feeding set point. Based on this information, a pH-stat fermentation of *P. rhodozyma* was conducted and the set point for glucose feeding was

set a higher value than the culture pH. That is, the substrate-feeding pump will be activated when the pH rises above the pH set point and reaches the feeding set point. However, pH-stat cultures of *P. rhodozyma* using this method of control did not grow very well.[18] In order to optimize the pH-stat method for culturing *P. rhodozyma*, other feeding strategies for the pH-stat will be investigated in this study. In 1977, N. Nishio et al developed a method for fed-batch culture of methanol-utilizing bacteria using the pH-stat. In this method, the substrate was fed to the system when the culture pH decreased and reached a substrate-feeding set point; i.e. the substrate-feeding set point was set at a value lower than the culture pH and ammonia solution was used as base.[19] In 1997, Y. Yamane et al used two-stage fed-batch cultures with pH-stat and D.O.-stat to investigate the effect of glucose and ethanol feeding on astaxanthin production in *P. rhodozyma*. They used pH-stat culture in the first stage for cell growth, the method that was based on the study of Nishio et al.[19] However, details on the pH-stat culture of *P. rhodozyma* were not given in their paper. Therefore, in this study, a detailed investigation of pH-stat cultures of *P. rhodozyma* will be made.

In a study [13] involving D.O.-stat and exponential fed-batch cultures of *P. rhodozyma*, results showed that when there were high residual glucose concentrations in the system, ethanol would be produced by the yeast due to the Crabtree effect. D.O.-stat

fermentation with a large dose size of 2.5 g/L (2.5 g/L glucose was fed each time to the fermenter when the feeding set point was reached) yielded a high carotenoid content. Also, exponential feeding with a high specific growth rate of $0.12h^{-1}$ yielded a high carotenoid content in the cells. In both cases, glucose accumulated and ethanol was detected. These findings together with results from shake-flask studies strongly indicate that ethanol may have a positive effect in the production of carotenoids. Gu et al [20] and Yamane et al [21] also showed the positive effect of ethanol in the production of astaxanthin and they also found that the enhancing effect of ethanol was not restricted only in the stationary phase. However, most of these results were obtained from shake flask experiments. In the current study, the effects of ethanol on growth and carotenoid production of *P. rhodozyma* in the fermenter will be investigated.

In this thesis, since some of the experimental results are presented in the same graph for easy observation and comparison, at the end of the thesis, the results of these experiments with more detailed information are presented independently in the Appendix section.

CHAPTER 2

LITERATURE REVIEW

2.1 Astaxanthin

2.1.1 Properties of astaxanthin

Astaxanthin (3,3'-dihydroxy-ß,ß-carotene-4,4'-dione) (Figure 2.1) is the principal carotenoid pigment responsible for the distinctive orange-red pigmentation of marine invertebrates (lobsters, crabs and shrimps), fish (salmon and trout) and birds (flamingoes).[2, 12] The molecular formula of astaxanthin is $C_{40}H_{52}O_4$ with a molecular weight of 596.86. Crystalline astaxanthin is a fine and dark violet-brown powder. Its melting point is approximately 224°C. It is insoluble in aqueous solutions and most organic solvents but can be dissolved at room temperature in dichloromethane (~30 g/L), chloroform (~10 g/L), acetone (~0.2 g/L), dimethylsulfoxide (DMSO) (~0.5 g/L), and other nonpolar solvents. Astaxanthin has two asymmetric carbon atoms at the 3 and 3' positions and can exists in four configurations, including the identical enantiomers (3S, 3'S; 3R, 3'R) and meso forms (3R, 3'S; 3'R, 3S) (Figure 2.2). Carotenoids contain a long conjugated double bond system. They are less stable than other isoprenoids and precautions must be taken to avoid artifacts and destruction of the pigments. Light, heat, acids, and oxygen are particularly detrimental to carotenoids, and enzymatic

destruction also can occur during extraction from biological samples. Carotenoids are generally extracted with water-miscible polar organic solvents and can be separated conveniently by thin-layer chromatography (TLC) and HPLC.[3] The maximum absorbence of astaxanthin in Petroleum ether is at 475nm. Since animals cannot synthesize carotenoids, these components must be provided in their diets for deposition into the flesh or carapace.[20]

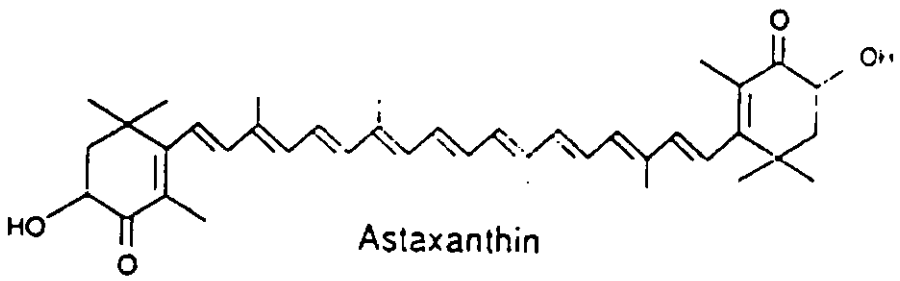

Fig. 2.1. Chemical structure of astaxanthin.

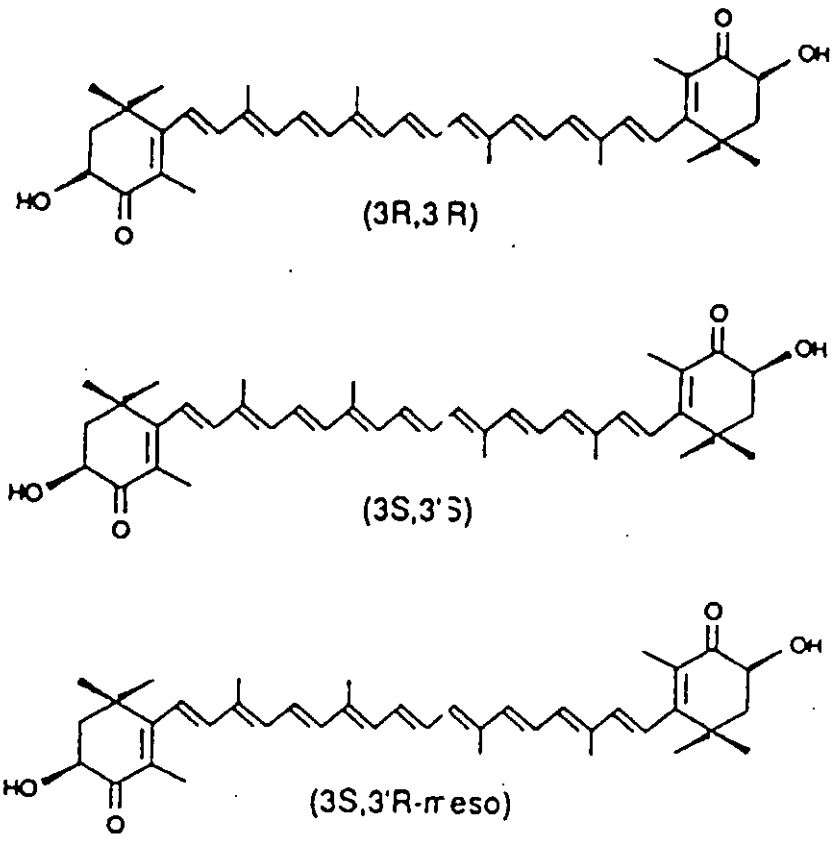

Fig. 2.2. Four configurations of astaxanthin.

2.1.2 Importance of astaxanthin

In the past decade, there was a growing interest in the use of astaxanthin as colorant for the red-pink color of farmed salmon, trout and shrimp. Salmon aquaculture harvest exceeded 400,000 metric tonnes in 1995. It was forecasted that the farmed salmon production would be 460,000 tonnes in 2000 and possibly more than 100,000 kg of astaxanthin would be required by 2000. The corresponding market for astaxanthin as a feed component would be more than $80 million.[3, 20, 22-24] In 1964, canthaxanthin was used as a pigmenter for foods and feeds. Synthetic astaxanthin is presently the principal source used in feeds. Astaxanthin is preferred as a pigmenter over synthetic canthaxanthin for salmonids since it is more efficiently absorbed and also imparts a more natural color to salmon. Chemically synthesized astaxanthin sells for $2000 to $2500/kg in beadlets containing 5% astaxanthin and is stabilized by various ingredients, including gelatin, sucrose, corn starch, modified food starch, ascorbyl palmtate and ethoxyquin. Since the commercial procedures used presently in the chemical synthesis of astaxanthin are not available to the general public and it is not possible to accurately estimate the cost of manufacture, scientists are seeking other methods for the production of astaxanthin.[3] Among all the possible methods, production of astaxanthin by using astaxanthin biosynthetic microorganisms seems to be the most convenient and low-cost method.

Carotenoids have recently received considerable interest because of their potential in delaying or preventing degenerative diseases such as arteriosclerosis, cancer and aging.[22] Experiments showed that the existence of singlet oxygen, oxygen radicals and peroxyl radicals could regulate the carotenoid biosynthesis in microorganism that could produce carotenoids.[25] Several mechanisms have been suggested for carotenoid protection in biological systems, which include the deactivation of electronically activated species such as singlet oxygen ($^1O_2$) and the deactivation of reactive chemical species, such as peroxyl or alkoxyl radicals, that can be generated within cells and might otherwise initiate harmful oxidative reactions. Direct evidence of carotenoids functioning as antioxidants in vivo has been reported in animal models. These studies show that carotenoid protection is related either to a direct antioxidant activity or to a modulation of cellular antioxidant levels by carotenoids.[26]

2.1.3 Source of astaxanthin

Several microorganisms have been shown to produce astaxanthin, including *Agrobacterium aurantiacum*,[6] *Mycobacterium lacticola*, *Phaffia rhodozyma*, [1, 4] *Peniophora*, *Paracoccus carotinifaciens* sp.[27] and *Haematococcus pluvialis*.[3, 5] Apart from the microbial sources of astaxanthin, the non-microbial sources of astaxantin include the crustacea, feathers of birds, *Adonis annua* flowers and synthetic

astaxanthin.[28] Crustacean wastes have been used traditionally as the natural pigment sources for trout and salmon but they have relatively low contents of astaxanthin (0-200 mg/kg dry weight) and high levels of moisture ash and chitin. The red yeast *Phaffia rhodozyma* and unicellular green alga *Haematococcus pluvialis* have become the most promising microorganisms used as sources for the production of astaxanthin.

2.2 *Phaffia rhodozyma*

2.2.1 General characteristics of *Phaffia rhodozyma*

The yeast *Phaffia rhodozyma* is a possible candidate for commercial production of astaxanthin because of its high astaxanthin content.[2] *Phaffia rhodozyma* was first isolated during the 1970s by Herman Phaff, Martin Miller, Minoru Yoneyama, and Masumi Soneda from exudates of deciduous tress in Japan, Alaska, and the U.S.S.R. The yeast had several unusual characteristics and the remarkable property was that the colonies were red to orange due to the presence of carotenoid pigments. It grows from 0 to 27°C.[3] The teleomorphic state of *Phaffia rhodozyma* was described in 1995[29] and a new genus *name Xanthophyllomyces dendrorhous*, a synonym of *Rhodomyces dendrorhous*, in *Filobasidiaceae*, *Ustilaginales* has been introduced. In 1998, sexual activity was induced in the basidiomyceteous *Phaffia rhodozyma* by depletion of nitrogen from the culture medium and its life cycle exhibited homothallic features.[30] Its basidiomycetous relationship was elucidated by the demonstration of a multilayered cell wall and enteroblastic budding. Its basidiomycetous affinity is supported by the carbohydrate composition of the cell wall.[3] A major property that distinguishes *Phaffia* from other genera of related yeast is the composition of carotenoids it produces. Astaxanthin has been identified as the major pigment in *P. rhodozyma*.[3] Wild strains of *P. rhodozyma* so far isolated contain up to 500 μg/g dry yeast of total carotenoid, of

which 40 to 95% is astaxanthin.[3] In 1976, Andrewes & Starrr found that the absolute configuration of astaxanthin produced by *P. rhodozyma* was 3R, 3R', opposite to the normal 3S, 3S' which is found in other organism such as fish.[4, 28] Soon after the discovery of astaxanthin in *Phaffia rhodozyma*, feasibility studies were undertaken to demonstrate the efficiency of this organism as a pigmenter in salmonid and crustacean diets as well as poultry feeds.[28] Analysis of *P. rhodozyma* strains by laser confocal fluorescence microscopy (LCFM)[8] showed that an early step of carotenogenesis in *P. rhodozyma* may be associated with the mitochondria and that lipid globules contain carotenoids that become dispersed to the cell membrane as the cells age.[3] When fed to rainbow trout, the deposition of astaxanthin in the fish flesh was dependent on the proper preparation of yeast cells before their inclusion into the feed. If *P. rhodozyma* was mechanically ruptured, its pigments were transferred to the flesh of rainbow trout, coloring it salmon-pink. The most efficient deposition of astaxanthin in trout occurred when the cell wall of *P. rhodozyma* was partially removed by enzymatic digestion.[31]

2.2.2 Nutrition

Yeasts require carbon, hydrogen, oxygen, nitrogen, phosphorus, sulphur as their major elemental building blocks. Macromolecules such as proteins, polysaccharides, nucleic acids and lipids together with the bulk inorganic ions such as potassium and magnesium

as well as trace elements play a variety of structural and functional roles in the yeast cell. Yeasts acquire essential elements from their growth environment from simple food sources which need to be available at the macronutrient level in the case of C, H, O, N, P, K, Mg and S or at the micronutrient level in the case of trace elements.[32] *Phaffia rhodozyma* grows very well in a simple aqueous medium at pH 5.0 comprising a hexose sugar, ammonium salt, various minerals, trace elements and a few vitamins.

2.2.2.1 Carbon

Phaffia rhodozyma can ferment glucose, maltose, sucrose and raffinose. The yeast was described by Miller and associates as having several unique characteristics, principally its fermentative capabilities, unusual carotenoid composition, and cell wall composition. Other pertinent properties include its ability to assimilate carbon compounds, including D-glucose, maltose, sucrose, cellobiose, trehalose, raffinose, soluble starch (latent or negative), ethanol (latent or negative), α-methylglucoside (latent or negative), D.Mannitol, salicin, 2-ketogluconate, DL-lactate (latent), succinate, and glycerol. The yeast does not grow on lactose, galactose, glucosamine, D-ribose, or D-arabinose.[3] Other carbon sources such as fructose from grape juice,[33-34] xylose,[35] corn wet-milling co-products [36] and raw sugarcane juice [37] as well as depolymerized bagasse [38] can also be utilized *by P. rhodozyma*. As a potential

industrial microbial source for the production of astaxanthin, scientists tried to seek for a lower cost for cultivation as well as for astaxanthin production. Hydrolysates from Peat,[39] *Eucalyptus globulus* Wood [40-41] can serve as a comparatively low-cost medium. In order to maximize assimilation of the carbon energy source by the cells in the microbial conversion process, potassium, phosphorus, magnesium, calcium, sulphur, iron, zinc, copper and manganese are required.[42] *P. rhodozyma* can also grow in commercial complex media such as yeast extract, soy peptone and corn steep liquor.

2.2.2.1.1 Crabtree Effect

This phenomenon relates glucose concentration with the particular catabolic route adpoted by glucose-sensitive yeasts in the presence of oxygen and states that, even under aerobic conditions, fermentation predominates over respiration. Thus, even though oxygen may be present, NADH generated during glycolysis is mainly oxidized by fermentation, rather than by respiration. Two main mechanisms are proposed to cause the Crabtree effect. They are catabolite repression and catabolite inactivation. Catabolite repression occurs when glucose, or an initial product of glucose metabolism, represses the synthesis of various respiratory and gluconeogenic enzyme; whereas catabolite inactivation results in the rapid disappearance of such enzymes on addition

of glucose. In catabolite repression, enzyme activity is lost by dilution with cell growth. That is, although enzymes are still present, they are no longer being synthesized due to gene repression by signals derived from glucose. [32]

Catabolite inactivation, which is more rapid than repression, is thought to be due to glucose-induced deactivation of a limited number of key enzymes like fructose 1,6-bisphosphatase. Enzyme inactivation occurs firstly by enzyme phosphorylation, followed by a slower vacuolar proteolysis of the enzyme. [32]

2.2.2.2 Hydrogen

Elemental hydrogen is present in yeast cellular macromolecules and is available from carbohydrates and other sources. Hydrogen ions (protons) are very important in yeast cell physiology since variations in both extracellular and intracellular pH can have a dramatic influence on growth and metabolism of yeast cells. *Phaffia rhodozyma* can grow in the culture medium with pH between 4-6.[32] The optimum pH for the growth of *P. rhodozyma* is pH 5.00.

2.2.2.3 Oxygen

Yeasts are unable to grow well in the complete absence of oxygen. This is because, as well as providing a substrate for respiratory enzymes during aerobic growth, oxygen is required for certain growth-maintaining hydroxylations such as those involving the biosynthesis of sterols and unsaturated fatty acids. In the case of *P. rhodozyma*, that is the biosynthesis of carotenoids.[32] Aerobic fermentation in the presence of fully aerobic culture conditions and high levels of readily metabolizable sugars, resulting in a reduced biomass yield and the formation of fermentation products, is known as Crabtree effect.[43] *Phaffia rhodozyma* is a Crabtree positive yeast. Therefore, the cell yield and pigment production of *P. rhodozyma* are reduced at high sugar concentrations.[12]

2.2.2.4 Nitrogen

Yeast cells have a nitrogen content of around 10% of their dry weight. Although yeasts cannot fix molecular nitrogen, simple inorganic nitrogen sources such as ammonium salts are widely utilized. Ammonium sulphate is a commonly used nitrogen source in yeast growth media since it also provides a source of assimilable sulphur. A variety of organic nitrogen compounds: amino acids, peptides, purines, pyrimidiines and amines can also provide the nitrgoenous requirements of the yeast cell.[32] *P. rhodozyma* does

not utilize nitrate, but does hydrolyze urea. Limitation of nitrogen in the presence of ample glucose could increase the proportion of carbon incorporated in lipid.[3]

2.2.2.5 Sulphur and phosphorus

Yeasts require sulphur principally for the biosynthesis of sulphur-containing amino acids. Yeast sulphur content represents around 0.3% of cell dry weight. Inorganic sulphate and the sulphur amino acid methionine are the two compounds central to the sulphur metabolism of yeast.[32]

Phosphorus is present in nucleic acids and in phospholipids and therefore is essential for all yeasts. A significant contribution to the negative charge of the yeast cytoplasm is due to the presence of inorganic phosphates and phosphate groups in organic compounds. The phosphate content of yeast cells accounts for around 3-5% of dry weight.[32]

2.2.2.6 Vitamin and trace element

Yeast requirements for minerals are similar to that of other cells. A supply of potassium, magnesium and several trace elements is necessary for growth. Yeasts have an absolute growth requirement for potassium, which is essential as a cofactor for a wide variety of

enzymes involved in oxidative phosphorylation, protein biosynthesis and carbohydrate metabolism. It is also involved in the uptake of other nutrients like phosphate, as a non-specific charge-balancer and as a stabilizer of macromolecules and ribosomes. Yeast cellular K content varies according to growth conditions but generally represents 1-2% of dry weight.[32]

Magnesium is an absolute requirement for yeast growth and is present in cells at around 0.3 % of dry weight where it plays essential structural and metabolic functions.[32] Vitamins are known as growth factors. They are organic compounds required in very low concentrations for specific catalytic or structural roles in yeast, but are not utilized as energy sources.[32]

For large-scale fermentation *of P. rhodozyma*, a vitamin mixture (**Table 2.1**) and trace element mixture (**Table 2.2**) are also required to achieve maximum cellular yields with maximum cell density in the cultures.[42]

Table 2.1 Vitamin mixture for growth of *Phaffia rhodozyma*

Component	Amount (g/L)
Biotin	0.08
Inositol	5
Thiamine	5
Calcium Pantothenate	1
Pyroxidine HCL	2.25

30 ml/L of vitamin mixture is needed for each fermentation.

Table 2.2 Minerals mixture for growth of *Phaffia rhodozyma*

Component	Amount (g/L)
$FeCl_3 \bullet 6H_2O$	3
$Na_2MoO_4 \bullet 2H_2O$	2
$ZnSO_4 \bullet 7H_2O$	8
$MnSO_4 \bullet 4H_2O$	3
$CuSO_4 \bullet 5H_2O$	6

20 ml/L minerals mixture is needed for each fermentation.

2.2.3 Effects of nutrient and environmental conditions on astaxanthin production

The proposed metabolic pathway for production of astaxanthin in *P. rhodozyma* is originated from the mevalonate pathway. Mevalonate is formed from acetyl-CoA and acetoacetyl CoA synthase and reductase (HMGS and HMGR). The mevalonate pathway and the proposed astaxanthin biosynthetic pathway are shown in Figure 2.3 and Figure 2.4, respectively.[3] The production of astaxanthin by *P. rhodozyma* is affected by the nutrients in the medium and the environmental conditions during cultivation.

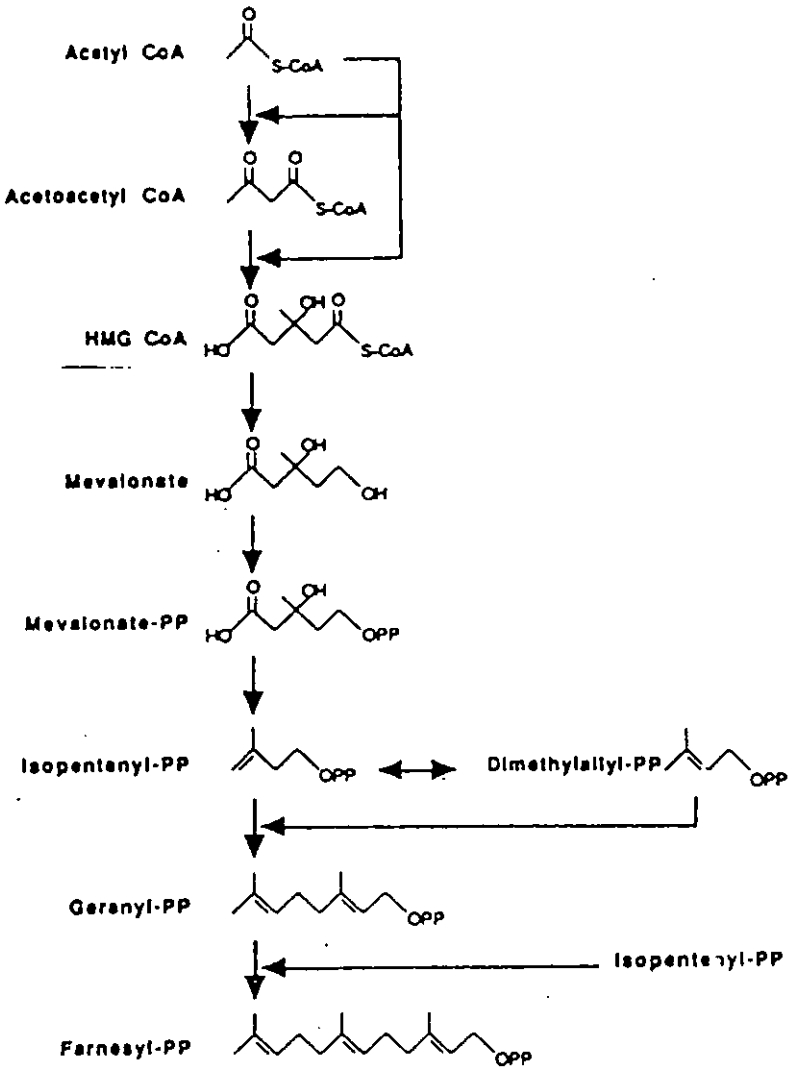

Fig. 2.3. The mevalonate pathway (Proposed)

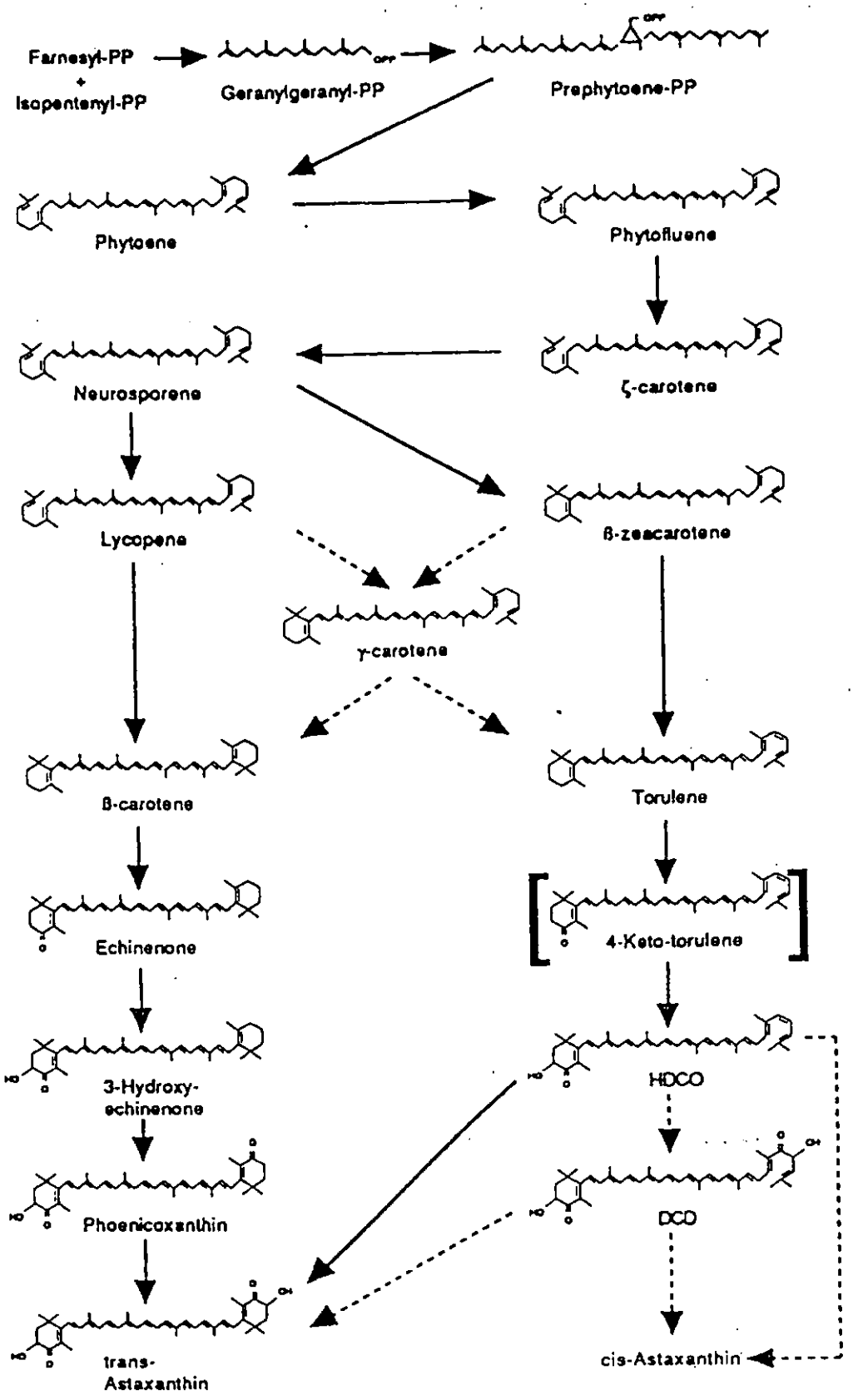

Fig. 2.4. **The astaxanthin biosynthetic pathway (Proposed)**

2.2.3.1 Nutrient Factors

Owing to the long biosynthetic pathway of astaxanthin in *P. rhodozyma*, the intermediate carotenoids in the pathway may have an effect on the production of astaxanthin. Acetic acid acts as direct precursor of acetyl-CoA. Low concentrations of acetic acid decreased the growth rate of and astaxanthin production by *Phaffia rhodozyma* on glucose, with growth completely inhibited by 2 g acetic acid/L. When H_2SO_4 was used for pH control after sugar depletion, it caused a decline in the biomass concentration, whereas when acetic acid was used as a titrant, it resulted in an increase in the biomass with a high astaxanthin content of 1430 μg/g cell.[44] The effect of monoterpenes on astaxanthin production was reported in 1994. The total pigment and astaxanthin content of *Phaffia rhodozyma* increased with an increasing concentration of α-pinene up to 500 μl α-pinene/L.[45] In 1995, mevalonic acid has been tested as enhancer of pigment biosynthesis in wild-type *Phaffia rhodozyma*. The addition of 0.1 % mevalonic acid to the culture media stimulated both trans-astaxanthin and total carotenoids biosynthesis.[46] Apart from the intermediate carotenoids within the pathway, different nutrients in the medium may also account for the productivity of astaxanthin. Studies with antimycin, an antibiotic that affects respiration and leads to accumulation of oxygen radicals, and chemicals that produce activated oxygen species such as superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2),[47] hydroxyl radical (OH)

and singlet oxygen ($^1O_2$) have shown that they play an antioxidant role during growth of *P. rhodozyma* and enhance the productivity of astaxanthin.[48] Glucose, sucrose and xylose were used to investigate the effect of these three carbon sources on the carotenoid profiles of *P. rhodozyma* and the study found that xylose was identified as the carbon source leading to the highest volumetric concentrations of astaxanthin.[49] The influence of oxygen and glucose supply on primary metabolism and astaxanthin production in the yeast *Phaffia rhodozyma* was investigated.[50] When *P. rhodozyma* grew under fermentative conditions with limited oxygen or high concentrations of glucose, the astaxanthin production rate decreased remarkably. The astaxanthin production was enhanced by an initial high carbon/nitrogen ratio (C/N ratio) present in the medium.[50] Glycerol was used as a carbon source for the growth and astaxanthin production by *Phaffia rhodozyma* in a recent study. With yeast extract and peptone in the medium, the maximum specific growth rate was $0.24h^{-1}$. The percentage of astaxanthin in the total pigment produced was constant and its yield from glycerol was almost 0.97 mg/g.[51] The presence of ethanol in the medium also has some effects in the production of astaxanthin. Addition of ethanol (2% v/v) to cultures of *Phaffia rhodozyma* increased the specific rate of carotenoid production and the study provided evidence that biosynthesis of isoprenoids including astaxanthin and other carotenoids in *P. rhodozyma* was enhanced by ethanol.[20] For micronutrients, phosphate has not

been reported to influence carotenogenesis in *P. rhodozyma*. Manganese and iron were demonstrated to affect carotenoid formation in *P. rhodozyma* when it was grown on succinate. Leucine and valine can stimulate carotenogenesis in certain fungi but these amino acids do not seem to be carotenogenic in *P. rhodozyma*.[3]

2.2.3.2 Environmental factors

Astaxanthin production is affected by several environmental factors. Light is important for the regulation of carotenogenesis in a wide variety of organisms. Growth and pigmentation of *P. rhodozyma* were inhibited by high light intensities.[3] Carotenogenesis in *P. rhodozyma* was induced by lower light intensities. Blue light induced higher pigmentation than red, yellow, or green light when the yeast was grown on the surface of YM agar at 7.5°C for 1 month.[3] The light response of the yeast was increased when it was exposed to respiratory chain inhibitor and chemicals that generate activated oxygen species.[3] *P. rhodozyma* grows between the temperature range of 0 to 27°C.[3] The cell yield decreases above 22.5°C, the optimum for growth rate. The carotenoid content of the yeast is fairly constant from 14 to 26°C. The inability of the yeast to grow above 27°C is a serious drawback to its industrial development.[3] When *P. rhodo*zyma is grown at a low temperature (4°C) in the existence of low diphenylamine (inhibitor of β-carotene oxidation) concentration, it can overcome the inhibition of β-carotene oxidation.[52] This study shown that *P. rhodozyma* may have a higher tolerance for inhibitors at a low temperature.

2.2.4 Strain improvement and recent discovery

Currently, industrial astaxanthin production by *P. rhodozyma* is limited by a lengthy low-temperature fermentation and especially by the unavailability of stable astaxanthin high-producing strains.[3] Therefore, until now the development of strain and the fermentation methods of *P. rhodozyma* are still under investigation in order to increase the stability of both the strain and the pigment.

2.2.4.1 Strain improvement

Three approaches can be used for genetic improvement of industrial asexual yeasts such as *P. rhodozyma*: mutagenesis, recombination of mutants and gene cloning and amplification. Each approach has potential benefits and limitations. Mutagenesis is often used because of its simplicity and the fact that it is not necessary to have considerable knowledge of the biosynthesis and genetic regulation of the desired product. The success of this method depends on the efficiency of mutagenesis and proper screening. Fortunately, the color of astaxanthin is orange/red and yeast mutants can be screened by visual examination.[3] By using visual screening after mutagenesis with UV light, ethyl methanesulfonate (EMS), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), ethidium bromide and acriflavin,[7] the study found that EMS and NTG were the most effective mutagens. However, after several consecutive uses of these

mutagens, isolation of carotenoid hyperproducing mutants (CHMs) became very difficult and isolated CHMs were usually unstable. Astaxanthin-overproducing mutants of *P. rhodozyma* can easily be spotted on β-Ionone (an end ring analog of β-carotene)-containing yeast malt agar plates.[10] Isolation of *Phaffia rhodozyma* auxotrophic mutants by enrichment methods was reported in 1993. An enrichment scheme using the polyene antibiotic nystatin for the isolation of auxotrophic mutants was designed. The treatment resulted in auxotrophic isolates at a frequency of 1×10^{-3} [9, 11, 53-54] and the auxotrophic mutants were very useful for further genetic examination of *P. rhodozyma*. Astaxanthin-overproducing mutants of *P. rhodozyma* could also be isolated when the yeast was grown on corn-based fuel ethanol stillage (thin stillage, TS, or fuel ethanol byproducts). The mutants isolated from this method can be grown in a low cost stillage medium and have potential for commercial production of astaxanthin from corn byproducts.[55] Resistance to carotenoid biosynthesis inhibitors can also be employed as a screening method. *P. rhodozyma* mutants, which are resistant to the carotenoid biosynthesis inhibitor diphenylamine, can have a higher astaxanthin production.[56] Apart from using mutagenesis, astaxanthin-overproducing mutants can also be isolated by protoplast fusion. In 1992, scientists from South Korea tried to improve *P. rhodozyma* by using protoplast fusion. They first isolated some auxotrophic mutants from the parental strains that produced

approximately 1600 μg carotenoid /g yeast. They then successfully constructed carotenoid hyperproducing hybrids from these auxotrophic mutants. The hybrids were stable and consistently produced more than 2000 μg carotenoid /g yeast.[57] Gene cloning is an alternative method for strain improvement. In 1998, scientists in Japan cloned the gene clusters that were responsible for the biosynthesis of carotenoids from non-carotenogenic bacteria and yeasts. They showed that the edible yeasts *Candida utilis* as well as *Saccharomyces cerevisiae*, which possessed no carotenoid biosynthetic pathway, acquired the ability to produce carotenoids, when the carotenogenic genes were expressed under the control of yeast-derived promoters and terminators.[58] However, the search for genes responsible for producing astaxanthin from *P. rhodoyzma* and from other carotenogenic microorganisms was very difficult and time consuming.

2.2.4.2 Recent Discoveries

In order to understand the relationship between the astaxanthin biosynthetic enzyme and the astaxanthin biosynthetic genes in *Phaffia rhodozyma* and other carotenogenic microorganisms, scientists have studied this subject intensively in the last 10 years. In 1994, the difference in the genetic information (DNA fragments) between astaxanthin-overproducing mutants and wild-type *P. rhodozyma* was reported by using

randomly amplified polymorphic DNAs (RAPDs). When a single, short oligonucleotide primer, which can bind at many different loci, is used to randomly amplify sequences from a complex DNA template, it is known as RAPDs.[59] From these results, we can find out whether there will be differences in genetic information between astaxanthin-overproducing mutants. More genetic information on *P. rhodozyma* was established. Four double-stranded RNA (dsRNA) molecules were isolated from *Phaffia rhodozyma* UCD 67-385. These dsRNAs can be copurified with isometric virus-like particles and also encode a killer system.[60] The electrophoretic karyotypes of strains from the astaxanthin-producing yeast *Phaffia rhodozyma* were also established. Intact chromosomal DNA molecules released from protoplasts were separated by orthogonal field alternation gel electrophoresis (OFAGE) and contour clamped homogeneous electric field (CHEF)[61] revealing more about chromosomal DNA in *Phaffia rhodozyma* had been known. In 1995, a carotenoid biosynthesis gene cluster for the production of astaxanthin was isolated from the marine bacterium *Agrobacterium aurantiacum* and the astaxanthin biosynthetic pathway was proposed for the first time at the level of the biosynthesis gene.[6] This discovery was very important for the genetic modification of astaxanthin production in non-carotenogenic bacteria and yeasts. Due to the discovery of the astaxanthin biosynthesis gene, the improvement of astaxanthin production in *P. rhodozyma* can be accomplished at the

molecular level. Transformation of *P. rhodozyma* by electroporation was found to have higher transformation efficiency.[62] The gene coding for actin [63] and glyceraldehyde-3-phosphate dehydrogenase [63] from *Phaffia rhodozyma* was found. These findings provided us with more information about the genetic properties of *Phaffia*. An integrative transformation vector for *Phaffia* was also successfully constructed. Based on the above discoveries, scientists in the Netherlands developed an efficient transformation system for *Phaffia rhodozyma*. The system was developed based on electroporation that routinely yielded approximately 1000 transformants per μg of plasmid DNA. The high transformation efficiency depends on vector integration in the ribosomal DNA (rDNA) and the presence of the homologous glycolytic glyceraldehyde-3-phosphate dehydrogenase (gpd) promoter and terminator to drive the expression of the transposon Tn5 encoded kanamycein resistance gene (Km^R) as a selective marker.[64] Recently, there has been a breakthrough in the genetic studies of *P. rhodozyma*. Phytoene desaturase of *P. rhodozyma* have been cloned and characterized, and the study has found that the four desaturase steps are carried out by a single gene product. The feedback regulation of phytoene desaturase may be the rate-limiting in astaxanthin biosynthesis in *P. rhodozyma*, [48] and it may be the best target to start metabolic pathway engineering.[65]

Beside the molecular biology field, new discoveries of *Phaffia rhodozyma* in other fields have also been reported. A novel long chain alkyl phenol, phaffiaol, has been isolated from *Phaffia rhodozyma*. A potent antioxidant, Phaffiaol has antioxidative activity equivalent to α-tocopherol, which is a well-known natural antioxidant.[66][67] *Phaffia rhodozyma* has been found to produce xylitol when it is grown on xylose medium. Xylitol is a pentitol used in the food industry as a non-caloric sweetener with a growing market.[68] All the above discoveries signal that *P. rhodozyma* is getting attention in both the industrial and academic fields.

2.3 Fermentation

The term "fermentation" is derived from the Latin verb *fervere*, to boil, thus describing the appearance of the action of yeast on extracts of fruit or malted grain. Its biochemical meaning relates to the generation of energy by the catabolism of organic compounds, whereas its meaning in industrial microbiology tends to be much broader.

The term fermentation has been used in a strict biochemical sense to mean an energy-generation process in which organic compounds act as both electron donors and terminal electron acceptors. Industrial microbiologists have extended the term fermentation to describe any process of production by the mass culture of a microorganism.[69]

There are five major groups of commercially important fermentations:

1. Those that produce microbial cells (or biomass) as the product;
2. Those that produce microbial enzymes;
3. Those that produce microbial metabolites;
4. Those that produce recombinant products;
5. Those that modify a compound, which is added to the fermentation process – the transformation process.[69]

Fermentation has been used to produce different commercial products for a very long time. In the past decade, production of products, which were modified by molecular

biology methods, through fermentation became more popular. L. Alberghina et al efficiently produced recombinant DNA protein in *Saccharomyces cerevisiae* by using controlled high-cell-density fermentation.[70] Recombinant DNA technology applied to cellular systems which are able to grow in large-scale fermenters, has given the opportunity to obtain large quantities of proteins that would otherwise be difficult or impossible to obtain.[70] Scientists in Japan also used the same method to enhance the production of β-galactosidase.[71] In 1994, continuous beer production was investigated in a high cell-density culture system, which consisted of two stages for the fermentation and sedimentation of yeast cells. The process was found to be suitable for continuous and stable beer brewing.[72] Apart from the traditional fermentation method, efficient production of acetic acid from glucose was investigated in a mixed culture of *Zymomonas mobilis* and *Acetobacter* sp. in 1996. A shift from ethanol fermentation to acetic acid fermentation was achieved by enhancing the aerobic conditions when the glucose dropped below a certain level.[73]

The fermentation of *Phaffia rhodozyma* is mainly for the production of microbial cells and microbial metabolites since it is mainly used as a feeding supplement. On a large scale, *Phaffia rhodozyma* is generally grown in stirred-tank reactors and controlled for sugar feed and pH. Aeration must be vigorous to obtain good cell mass yields and yeast

containing high levels of carotenoid.[3] The fermentations must be provided with adequate aeration and cooling and be carried out for several days as the yeast grows slowly.[3] Lewis and colleagues have studied various production methods *for P. rhodozyma* with the aim of developing of an inexpensive industrial method of culture. They have successfully cultivated *P. rhodozyma* on alfalfa juice [74] and have evaluated autolysis and mixed culture systems for hydrolysis of the cell wall of *P. rhodozyma*.[75-76] The culture of *P. rhodozyma* is costly compared with other yeast fermentations since extended fermentations at low temperatures are required (up to 5days). Considerable astaxanthin is also produced after *P. rhodozyma* growth stops. Good cooling capacity and aeration are important requirements. Reduction of costs for production of *P. rhodozyma* by cultivation on sugars or other waste streams could improve the economics for yeast astaxanthin.[3] The use of other waste streams as a carbon source has been described in the section of **Nutrition (2.2.2)**. Experiments related to the fermentation of *P. rhodozyma* have been done in order to reduce the costs and improve the production of *P. rhodozyma*. A model was presented which described the fed-batch culture of *P. rhodozyma* and enabled the calculation of a feed regime to obtain the maximum yield of cells and pigment.[12] Demonstration of the Crabtree effect during continuous and fed-batch cultivation [43] has been done, and the influence of oxygen and glucose on primary metabolism and astaxanthin production in

batch and fed-batch cultures[50] has been studied to find out the best glucose concentration during fermentation. Ethanol feeding after fed-batch culture with glucose has shown an increment of astaxanthin production.[21] A continuous culture of *Phaffia rhodozyma* has been used to determine the effect of changes in metabolic patterns in the yeast, when there were different levels of glucose in the medium and various changes in dilution rate and specific growth rate.[77]

2.3.1 Batch Cultivation

Batch culture is a closed culture system, which contains an initial, limited amount of nutrient. The inoculated culture will pass through a number of phases. After inoculation there is a period during which it appears that no growth takes place, this period is thus referred to as the lag phase and may be considered as a time of adaptation. In a commercial process the length of the lag phase should be reduced as much as possible and this may be achieved by using a suitable inoculum. Following a period during which the growth rate of the cells gradually increases, the cells grow at a constant and maximum rate and this period is known as the log, or exponential phase. During the exponential phase nutrients are in excess and the organism is growing at its maximum specific growth rate for prevailing conditions.[69] The stationary phase in batch culture is the point where the growth rate has declined to zero. However, as Bull (1974) pointed

out, the stationary phase was a misnomer in terms of the physiology of the organism, as the population was still metabolically active during this phase and might produce products called secondary metabolites, which were not produced during the exponential phase. Bull suggested that this phase be termed the maximum population phase.[69]

Since batch fermentation is an elementary experiment for the investigation of the growth pattern of microorganisms, most of the characteristics of *P. rhodozyma* were found by this method and batch cultivation can serve as control experiment for the studies of different fermentation methods. In 1979, Johnson and Lewis studied several environmental factors that regulated the astaxanthin production in *P. rhodozyma* by batch fermentation.[2] A group of Japanese scientists investigated the influence of the oxygen and glucose supply on primary metabolism (fermentation, respiration and anabolism) and astaxanthin production in *P. rhodozyma* by batch fermentation.[50] As *P. rhodozyma* is a Crabtree positive yeast, its cell yield and pigment production are reduced at high sugar concentration.[12] Batch fermentation is not very suitable for producing astaxanthin industrially.

For investigation of newly obtained mutants, batch cultivation is still a good screening

method. Meyer and Preez investigated the effect of culture condition on astaxanthin production by mutant of *P. rhodozyma* in batch fermentation.[78] Fang and Chiou also investigated astaxanthin production of *P. rhodozyma* mutant NCHU-FS501.[53]

2.3.2 Fed-batch fermentation

Yoshida et al. (1973) introduced the term fed-batch culture to describe batch cultures, which were fed continuously, or sequentially, with medium without the removal of culture fluid. A fed-batch culture is established initially in batch mode and is then fed according to one of the following feed strategies:

1. The same medium used to establish the batch culture is added, resulting in an increase in volume.
2. A solution of the limiting substrate at the same concentration as that in the initial medium is added, resulting in an increase in volume.
3. A concentrated solution of the limiting substrate is added at a rate less than in (1) and (2), resulting in an increase in volume.
4. A very concentrated solution of the limiting substrate is added at a rate less than in (1), (2) and (3), resulting in an insignificant increase in volume.

The use of fed-batch culture takes advantage of the fact that the concentration of the limiting substrate may be maintained at a very low level, thus avoiding repressive effect of high substrate concentration. Furthermore, the fed-batch system also gives some control over the organisms' growth rate, which is also related to the specific oxygen uptake rate controlling to some extent the oxygen demand of the fermentation. The quasi-steady state has the advantage of maintaining the concentrations of substrates

other than those which limit growth and can has a significant effect on biomass composition and product formation.[69]

The early fed-batch systems did not incorporate any form of feedback control and relied on the inherent quasi-steady state to maintain process stability. However, the use of concentrated feeds resulting in very sophisticated feeding programmes has necessitated the introduction of feedback control techniques. In such feedback controlled fermentations, a process parameter directly related to the organism's physiological state is monitored continuously by an on-line sensor. The signal generated by the sensor is then used in a control loop to control the medium feed rate. Parameters which have been utilized in this way include dissolved oxygen concentration, pH, effluent gas composition and limiting substrate concentration.[69] The fed-batch fermentation can be divided into two different feeding methods: pre-determined feeding profile and online feedback feeding profile.

2.3.2.1 Pre-determined feeding profiles

There are three pre-determined feeding profiles. They are known as exponential feeding, constant feeding and incremental feeding. In exponential feeding, the growth-limiting substrate is fed exponentially so that a constant specific growth rate, μ, can be obtained. Moreover, it is assumed that a quasi-steady state exists for the substrate concentration, also the biomass yield coefficient, $Y_{x/s}$ is constant. The specific growth rate μ and biomass yield coefficient $Y_{x/s}$ in batch culture can be used to determine the feeding profile by using the following equation to calculate the feed rate,

$F = (\mu X_0 V_0 e^{\mu t}) / (S_0 Y_{x/s})$ [79]

Where F is the feed rate;
μ is the specific growth rate;
X_0 is the initial cell dry weight;
V_0 is the initial working volume of the fermentation;
t is the time;
S_0 is the initial substrate concentration;
$Y_{x/s}$ is the biomass yield coefficient.

In constant feeding profile, a fixed feed rate has been determined before the feeding of growth-limiting substrate. The feed rate would be kept constant through out the feeding period.

2.3.2.2 Online feedback feeding profiles

The physical variables in the fermenter have to be maintained as close as possible to the desired values, the set points. Dissolve oxygen (DO) and pH can be used to control the growth-limiting substrate feeding to the system. These online feedback feeding profiles are known as DO-stat and pH-stat.

During fermentation, when the growth-limiting substrate is depleted, the cell metabolism becomes inactive and uses less oxygen, so the DO in the fermenter increases and this change can be determined by the DO sensor of the fermenter. When growth-limiting substrate is added, it will be used by the cells aerobically and so the DO drops to a certain level until the added substrate is depleted and a control loop is established. It is the working principle of DO-stat.

In the pH-stat method, utilization of growth-limiting substrate by the cells results in production of organic acids and lower pH. After all the limiting substrate in the fermenter has been used up, the cells then use the organic acid as the carbon source and the pH will increase until a new feeding of growth-limiting substrate is added to the system. The pH of the system will drop again and the feeding cycle is established. Very little information is available on pH-stat culture of *P. rhodozyma*.

Fed-batch culture studies of *P. rhodozyma* have appeared in recent literature. Reynders studied the Crabtree effect of *P. rhodozyma* [43]during fermentation and used a mass balance approach and Monod growth kinetic model to describe the fed-batch culture.[12] Other scientists such as Vazquez used a low-cost xylose-containing media from wood hydrolysates to grow *P. rhodozyma* in fed-batch cultures. He obtained 10.3 g cells/L and 8.15 mg total carotenoids/L.[41] Scientists in South America were also interested in fed-batch culture of *P. rhodozyma*.[77] Ho et al [18] have also investigated the growth and carotenoid production of *P. rhodozyma* in fed-batch cultures as well with different feeding methods. The feeding methods investigated include D.O.-stat, pH-stat, constant feeding and exponential feeding.

2.3.3 Online computer control

As computer and computer software are well developed, they can be widely applied in both industrial and commercial areas including fermentation industries. Scientists also use the computer control system in laboratories and fermentation plants for the production of commercially valuable products. Fike et al enhanced hybridoma productivity by using an automated concentrate supplementation method. The concentration of nutrient supplement was monitored and the nutrient supplement was added to the system when its concentration was lower than a perdetermined value.[80] In 1996, Blackmore developed a complete computer monitoring and control system for laboratory and pilot plant *Escherichia coli* fermentations by using commercially available, configurable software.[81] In 1997, Chen used an automated fed-batch fermentation for production of DNA vaccines and the total yield of the plasmid DNA was increased approximately 10-fold.[82] In the past 20 years, it became more common that fermenter manufacturers would supply factory-made software for fermenters. However, the software usually was too rigid and the components were preset and could not be changed easily. Thus, they were not flexible enough for use in different fermentation methods. In this study, the software used is Genesis for Windows (GFW). This program is designed for the control of many industrial processes and it is used in many industries. The program is installed in Windows and provides a user-friendly

workplace for users. The components of the program can be configured in different ways so that it can support different processes and meet the needs of various users. For on-line computer control, however, computers and good software alone are enough since the signal from computer and the signal from the apparatus are different. The signal from the computer is known as digital signal while the signal from the apparatus is known as analog signal. Therefore, a interface must be installed between the computer and the fermenter. The interface contains two components, the analog to digital converter (ADC) and the digital to analog converter (DAC).

CHAPTER 3

GENERAL MATERIALS AND METHODS

This chapter describes the equipment, materials and procedures that were routinely used for the laboratory studies. Details of specific experimental procedures will be described in the relevant chapters.

3.1 Organism

An astaxanthin hyperproducing strain *Phaffia rhodozyma* P 5/6 was used and the yeast was supplied by Prof. E.A. Johnson at the University of Wisconsin, Madison, Wisconsin.

3.2 Culture medium and culture conditions

The culture medium is a slight modification of the medium in a US Patent [42].

The fermentation medium in shake flask cultures contained (per liter):

3 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 1.5 g $MgSO_4 \cdot H_2O$, 1 g yeast extract (Difco), 10 g corn steep liquor (Sigma), 2 g high soy (Quest International), 35 g glucose. The culture was grown in 1-litre Erlenmeyer flasks, on orbital shaker at 250 rpm, with 200 ml culture medium containing 5 % inoculum, at 20 °C and initial pH 5.0 for 96 hours.

3.3 Fermenter

The fermenter cultures were conducted at 20 °C and pH 5.0. A bioengineering 2000 fermenter with a 3.7 L glass tank was used and the working volume was 2.5 L 5% inoculum was used. The pH was controlled with 1.0 N NH_4OH (**Chapter 4**) or 1.0 N NaOH (**Chapter 5**) and 1.0 N HCl. The pH was detected by an Ingold pH electrode. The dissolved oxygen tension was monitored with an Ingold polargraphic electrode. Aerobic condition was maintained by airflow rate of 1.5 L/min and agitation to maintain the D.O. above 40% saturation, a Sigma antifoam 289 was added to minimize foaming. The fermenter was interfaced by a Modicon device interface with an Intel Pentium-75 Hewlett Packard Computer and the fermentation process was controlled by Genesis for Windows 3.0 software. In fed-batch fermentation, 30 g/L glucose was fed intermittently by Tygon® Tubing with calculated volume of 50% glucose solution.

3.4 Sterilization

All medium and solutions were autoclaved in a Hirayama HA-300 autoclave. Solutions that could not be autoclaved were filter sterilized with 25 mm, 0.02 micron Corning® cellular acetate membrane.

3.5 Inoculum

The inoculum medium was the same as the fermentation medium. 0.5 ml of *P. rhodozyma* P 5/6 from a 1 ml frozen vial was inoculated into a flask containing 200 ml of medium and incubated for 48 hours on a rotary shaker at 250 rpm at 20°C. The frozen seed culture was preserved in 1 ml vials containing 0.5 ml cell broth with 15% glycerol stored at –70 °C.

3.6 Sampling

Samples were withdrawn from the sampling tube of the fermenter. By blocking the exhaust line of the fermenter, pressure was created in the fermenter and the culture broth was forced out via the sampling tube. Samples were collected in autoclaved universal bottles.

3.7 Analytical Methods

For fermentation kinetics studies, specific growth rate, biomass yield, biomass yield coefficient, glucose concentration, inorganic nitrogen concentration, alcohol production were determined.

3.7.1 pH measurement

Medium and solution pH were determined by an Orion® digital pH meter Model 520 which was calibrated each time before use by standard pH buffer solutions of pH 4.00 and pH 7.00.

3.7.2 Specific growth rate

Specific growth rate (μ) was determined by measuring the dry weight during log phase. The natural logarithm of the cell dry weight was plotted against time and the specific growth rate was determined by the following equation:

$$\ln X_t - \ln X_0 = \mu t$$

where X_t is the biomass concentration at time t
X_0 is the biomass concentration at the beginning of log phase
μ is the specific growth rate
t is the time

3.7.3 Biomass

Biomass (cell dry weight g/L) was determined by filtering the cells from the culture broth and then drying the filters in a Ohaus® MB 200 moisture balance at 98°C for 20 minutes under the instruction of the user manual or until constant weight.

3.7.4 Biomass yield coefficient

Biomass yield coefficient ($Y_{x/s}$) is the gram cell produced per gram glucose used, gram cell produced will be the dry weight produced and gram glucose used will be determined by measuring the glucose concentration in the culture broth.

3.7.5 Glucose concentration

Glucose concentration was measured by a YSI 2700 food analyzer. It contains a glucose membrane that oxidizes glucose to release H_2O_2 and the amount of H_2O_2 is detected by a probe. The glucose concentration is known when the current generated by the sample is compared with the standard.

3.7.6 Inorganic nitrogen concentration

Inorganic nitrogen concentration was measured by a Corning® ammonium electrode. The inorganic nitrogen in the broth was converted to ammonium gas with NaOH and detected by the probe.

3.7.7 Alcohol concentration

Alcohol produced by the cells was assayed by detecting NADH formation at OD_{300} by the action of alcohol dehydrogenase and NAD^+ or by using the YSI 2700 food analyzer which contained an ethanol membrane that oxidized ethanol to release H_2O_2 and the amount of H_2O_2 was detected by a probe. The ethanol concentration is determined when the current generated by the sample is compared with the standard.

3.7.8 Carotenoid extraction and analysis

For carotenoid extraction and analysis, a 0.5 ml cell sample was transferred into a screwcap tube. The cell sample was mixed with one spoon of 0.4 mm glass beads and vortexed for 2 minutes with 3 ml acetone, then 30 seconds with 2 ml acetone to break up the cell since the pigment was embedded in the cell. After that, 2 ml of petroleum ether was added to the mixture in order to extract the pigment. The petroleum ether extract was removed after the mixture was washed by 2 ml NaCl and 1 ml cold water.

The absorbance of the extract was measured at 475 nm. The total carotenoid yield was calculated by using the 1 % extinction coefficient = 2100 by the formula:

Total carotenoid (μg/ml cell broth) = {(ml of petrol) (A_{475})} / 0.21 ([7])

CHAPTER 4

GROWTH AND CAROTENOID PRODUCTION BY pH-STAT CULTURES OF *Phaffia rhodozyma*

4.1 Introduction

Fed-batch cultures of *P. rhodozyma* [13, 18] have been extensively studied by procedure such as exponential feeding and D.O.-stat. Very little information is available on pH-stat cultures of *P. rhodoyma*. Preliminary results of a study showed that the specific growth rate of *P. rhodozyma* grown in pH-stat culture was the lowest of the fed-batch cultures investigated.[18] pH-stat fermentation is a fed-batch fermentation with a pH feedback control. The substrate is fed to the system in response to the change of pH value. The use of fed-batch culture takes advantage of the fact that the concentration of the limiting substrate may be maintained at a very low level, thus avoiding the repressive effect of high substrate concentration. In pH-stat fermentations, the yeast cells consume the substrate that is fed to the fermenter and produce organic acids. The organic acids produced by the cell will affect the pH of the system. A pH-stat study was conducted so that the substrate feeding set point was higher than the pH control set point. That is, the substrate feeding pump would be activated when the pH rose above the pH set point and reached the feeding set point. However, this control

method did not work consistently. During the growth of the cells at late log phase, the pH would sometimes rise very slowly or not at all even if the glucose in the system had been depleted. The hydrogen ion released from the protein source and nitrogen source might have affected the control of the pH-stat. Other components in the growth media may also have affected the pH-stat. An alternative method to control the pH-stat[19] is to have the pH feeding set point below the pH control set point; however, little information is available on using this method for cultivating *P. rhodozyma*. In this study, an intensive investigation of pH-stat cultures of *P. rhodozyma* was conducted.[83]

4.2 Materials and Methods

4.2.1 pH stat Fermentation

The same medium (containing 5g/L glucose), as described in **Chapter 3.2** was placed in the fermenter and inoculated with 8% (v/v) inoculum. pH-stat was started when the initial glucose was completely consumed. 30 g glucose/L was then fed to the system when pH reaches the feeding set point. Feeding was stopped when 30 g/L glucose was fed.

pH-stat is a form of fed-batch culture with pH feedback control. Glucose is fed to the fermenter when pH reaches the feeding set point. Two methods have been used to operate the pH-stat. In the first method, the glucose feeding set point (5.02) is higher than the culture pH (5.00). Since the medium pH increases as a result of the depletion of glucose, a dose of glucose is added when the pH reaches the set point.[17-18] In this method, HCl is not necessary to control the pH of the medium.

In the second method, the glucose feeding set point is set below the culture pH (5.00). Utilization of glucose by the cells results in production of organic acids and lowers the pH and a dose of glucose would be fed again to the fermenter in response to the pH drop.[19]-[21] To prevent the pH control system from interfering with the glucose

feeding, a "low gap" has been added to the pH set point so that base would not be added until the pH equals the pH set point plus the low gap. Therefore, the pH value will vary between the pH set point and the low gap (**Figure 4.1**). The feeding set point was set between the pH set point and the low gap to make sure that the pH stat fermentation could function properly.

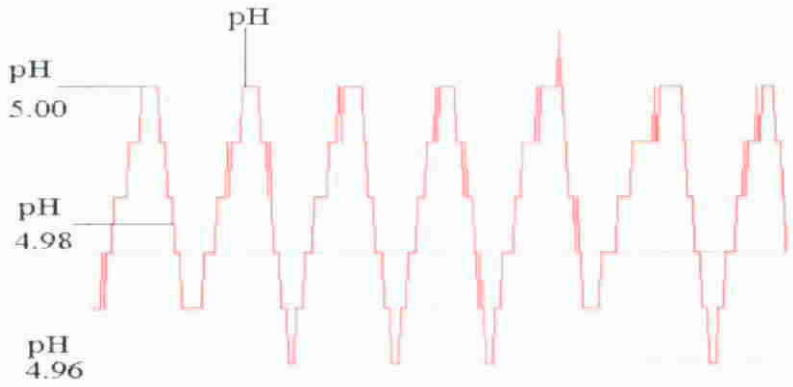

Fig. 4.1 The repetitive pH pattern of a pH-stat culture of *P. rhodozyma*. The glucose feeding set point (4.98) is below the culture pH (5.00)

4.2.2 Verification of pH-stat fermentation with glucose feeding set point below pH of the culture

The glucose concentration in the system was monitored by a YSI 2700 glucose analyzer. The exhaustion of glucose in the system could be monitored by a sudden rise in dissolved oxygen (D.O.) tension. In fed-batch fermentation, the sudden increase of the D.O. indicated that the substrate fed to the system had been consumed. In order to prove that, the pH stat fermentation developed in this study fed glucose only when it was exhausted. Several experiments were designed to investigate if the rise in D.O. and drop in pH would coincide when the glucose was used up. A D.O. control point (Cp) was introduced. In pH stat fermentation with a D.O. control point (Cp), glucose would only be fed to the system when both pH feeding set point and Cp were reached at the same time.

This is equivalent to a combined pH stat-DO stat control. That is the glucose feeding pump will switch on only when pH meets the pH feeding set point and the D.O. meets the Cp. It can be presented by the following logic gate in the computer control strategy:

pH feeding set point = 1 and D.O. control point = 1

Pump switches on, a dose of glucose added to fermenter

The time for the yeast to completely consume an added dose of glucose (T_s) was measured in several experiments. T_s will be different when the dosage of glucose added is different.

4.3 Results and Discussion

Intensive studies of pH-stat fermentation for cultivation of *P. rhodozyma* have been conducted. Different parameters have been investigated in order to find out the most suitable condition in which *P. rhodozyma* could have optimum growth or total pigment content in pH-stat fermentation. The cell dry weight and the cellular carotenoid content in stationary phase stated in this section are calculated by taking the average of the last four samples in the stationary phase.

4.3.1 Batch Fermentation

Fig. 4.2 shows a batch Fermentation of *P. rhodozyma*. 35 g/L glucose was used in this fermentation.

Table 4.1. Cell dry weight and total carotenoid yield of a batch culture of *P. rhodozyma* P 5/6

Time (hrs)	Cell dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.62	0.4824	314	193.8
18	4.42	1.4861	1010	228.5
24	8.98	2.195	1918	213.6
31	11.58	2.4493	4076	352
49	13.02	2.5665	5887	452.2
55.5	13.12	2.5741	5962	454.4
67	14.6	2.681	5863	401.6
79	14.92	2.7027	5686	381.1
96	14.58	2.6797	6560	449.6

Table 4.2. The change of glucose concentration and ethanol production of a batch culture of *P. rhodozyma* P5/6

Time (hrs)	Glucose conc. (g/L)	Ethanol conc. (g/L)
0	31	0
24	28	0.47
48	2.27	11.78
72	0.3	15.35
96	0.26	7.6

The maximum specific growth rate μ was 0.103 h^{-1} and the average biomass yield in the stationary phase was 14.7 g/L. The total carotenoid yield and cellular carotenoid content in the stationary phase was 6036.3 μg/L and 410.767 μg/g. Glucose was depleted after 50 hrs and ethanol was produced to about 70 hours and then decreased rapidly after 72 hours. A large amount of ethanol was produced since the Crabtree effect was significant when the glucose concentration was high. The biomass yield coefficient was 0.374 g cell/ g glucose. The pH was controlled at pH 5 throughout the process.

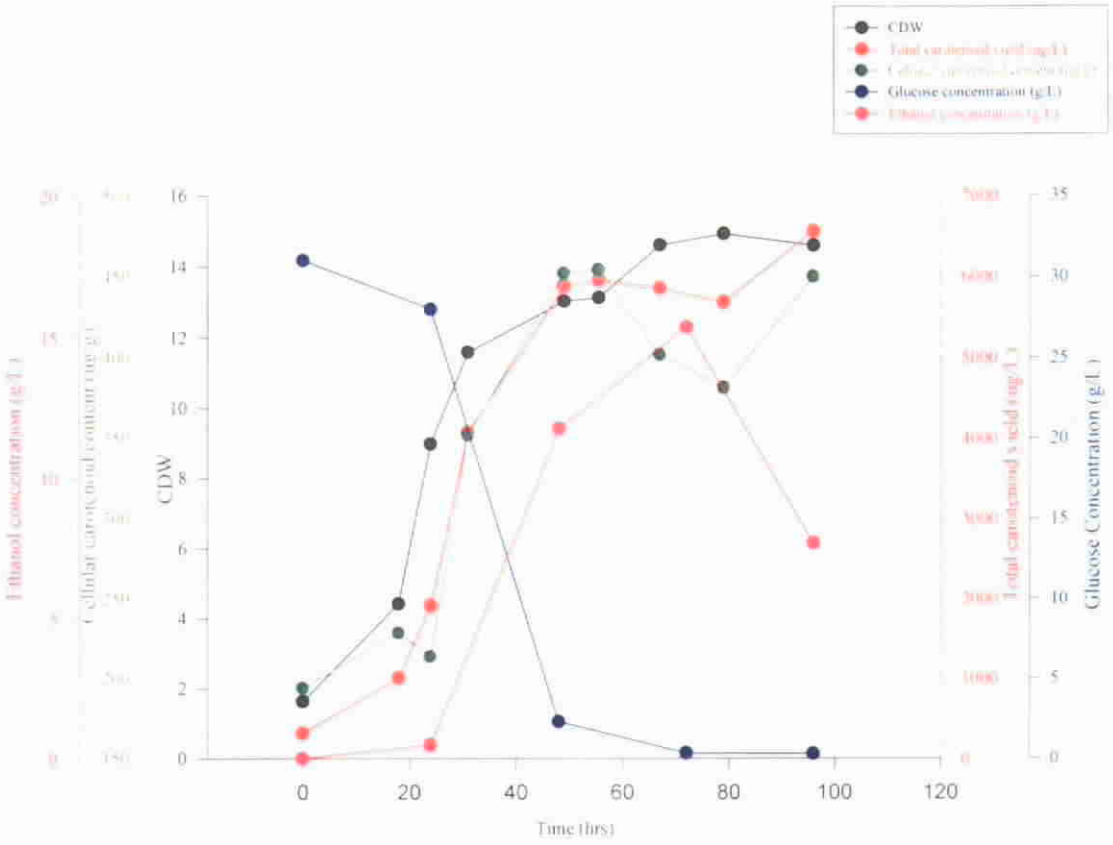

Fig. 4.2 Batch Fermentation of *Phaffia rhodozyma* P 5/6

4.3.2 Two methods of substrate addition in pH-stat fermentation

Two different methods of adding glucose in pH-stat fermentation of *P. rhodozyma* were investigated in this section. The first method was conducted as such that the glucose feeding set point was higher than the pH of the culture. The second method was conducted such that the glucose feeding set point was lower than the pH of the culture.

4.3.2.1 pH-stat culture of *P. rhodozyma* with a glucose feeding set point (5.02) higher than the pH of culture (5.00)

The glucose dose size used in this experiment was 0.5g/L. 30g/L glucose was dissolved in double amount of water to make a 50% glucose solution.

Table 4.3. Cell dry weight and total carotenoid yield of pH-stat culture of *P. rhodozyma* with a glucose feeding set point higher than the pH of culture

Time (Hours)	Cell dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.3	0.2624	222	171.5
5	1.72	0.5423	334	194.4
16	3.08	1.125	838	272.1
25	4.36	1.472	1085	249
29	5.4	1.686	1465	271.4
40	7.66	2.036	2150	280.7
48	8.54	2.145	2541	297.6
52	9.04	2.202	2797	309.4
64.5	10.58	2.359	3561	336.6
73	11.36	2.43	3802	334.8
86.5	12.8	2.549	4354	340.2
96	13.46	2.6	5211	387.2

Table 4.4. The residual glucose concentration of pH-stat culture of *P. rhodozyma* with a glucose feeding set point higher than the pH of culture

Time (Hours)	Glucose conc. (g/L)
0	5.06
5	4.47
16	0.473
25	0.014
29	0.027
40	0.007
48	0.005
52	0.145
64.5	0.088
73	0.193
86.5	0.173
96	0.219

The maximum specific growth rate of the cells was 0.055 h^{-1} and the culture reached its maximum biomass and total carotenoid yield at approximately 100 hours. As the log phase could be observed, the maximum specific growth rate of the cells was calculated by using the cell dry weight at the beginning of log phase and at the end of the fermentation. The result is consistent with results from an earlier work[18] which showed that *P. rhodozyma* cultured by this type of pH-stat had a low specific growth rate.

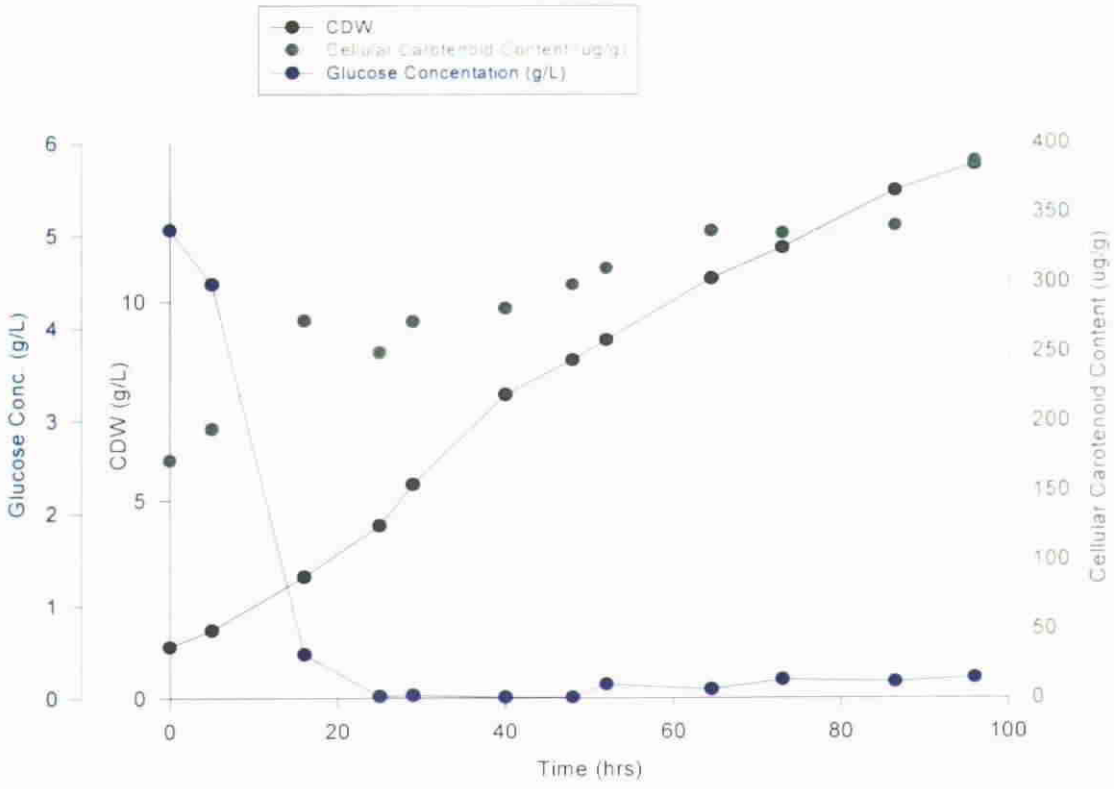

Fig. 4.3 pH-stat culture of *P. rhodozyma* with a glucose feeding set point (5.02) higher than pH of the culture (5.00)

4.3.2.2 pH-stat culture of *P. rhodozyma* with a glucose feeding set point (4.98) lower than the pH of culture (5.00)

The glucose dose size used in this experiment was 0.5g/L. 30g/L glucose was dissolved in double amount of water to make a 50% glucose solution.

Table 4.5. Cell dry weight and total carotenoid yield of pH-stat culture of *P. rhodozyma* with a glucose feeding set point lower than the pH of culture

Time (Hours)	Cell dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.54	0.4318	389	252.6
6.5	1.88	0.6313	90.5	48.1
18	2.66	0.9783	371	139.5
24	4.12	1.4159	789	191
30.5	6.9	1.9315	1636	237.1
42	14.18	2.6518	4629	326.4
49	14.56	2.6783	5431	373
53.5	14.78	2.6933	5107	345.5
73	15.04	2.7107	4845	322.1
77.5	15	2.7081	5537	369.1
88	15.9	2.7663	5303	333.5
96	14.62	2.6824	5757	393.8

The result shows that this is a better method of culturing *P. rhodozyma* by using pH-stat cultivation. pH-stat cultures with glucose feeding set point below the culture pH took approximately 70 hours to attain maximum biomass.

The cell dry weight in the stationary phase is 15.07 g/L and the cellular carotenoid content at stationary phase is 352.8 μg/g. The specific growth rate is 0.095h^{-1} and the biomass yield coefficient is 0.43 g cell/g glucose.

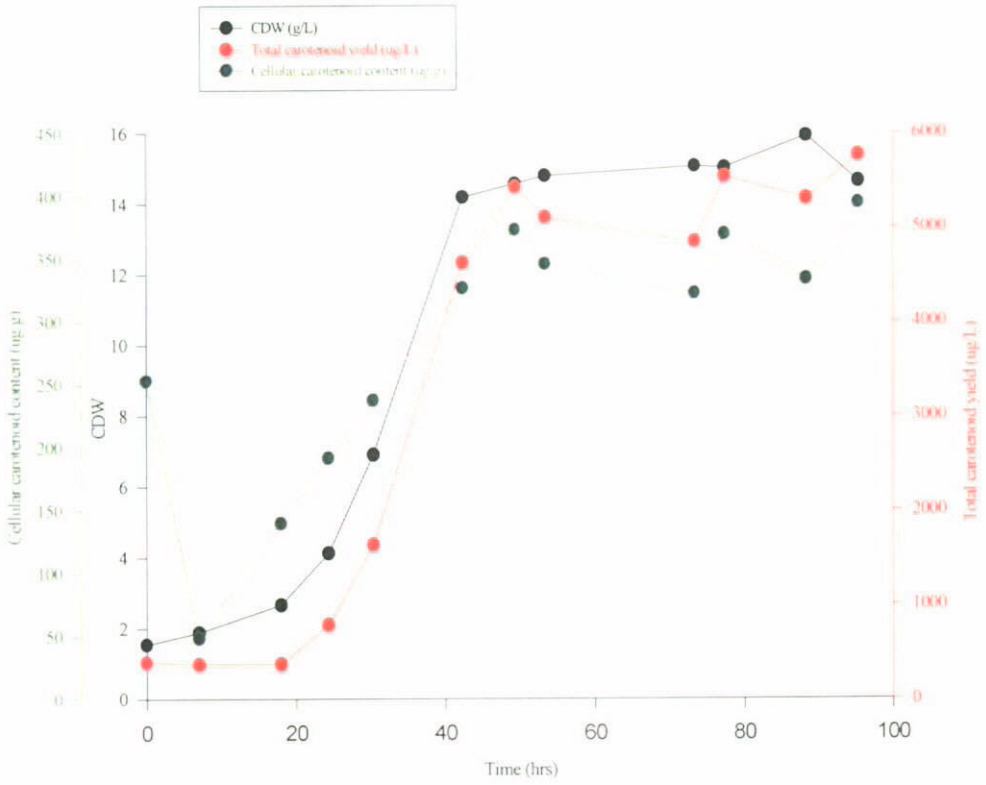

Fig. 4.4 pH-stat culture of *P. rhodozyma* with a glucose feeding set point (4.98) lower than pH of the culture (5.00)

With the glucose feeding set point below the pH of the culture, the specific growth rate of the cells was higher and the yeast cells reached the stationary phase at about 60 hours into the fermentation process. With a glucose feeding set point higher than the pH of the culture, the specific growth rate of the cells was lower and the maximum cell dry weight was not reached until about 100 hours into the fermentation process.

Although the cellular carotenoid content of the cells produced by these two methods were about the same, the cell dry weight produced by the second method was higher than the first one. However, the control of the second method was more complicated.

4.3.3 The effect of the length of the "time interval" between glucose doses on pH-stat cultures

To prevent overfeeding of glucose and to allow sufficient time for the yeast to metabolize the added dose of glucose, a "time interval" was added to the control strategy of the glucose pump and allowed to expire before the next dose of glucose added.[83]

4.3.3.1 The investigation of the length of the "time interval" with 0.5 g/L glucose dose size

0.5 g/L glucose dose size was used because the time for the cells to consume 0.5 g/L glucose was long enough tha the change in dissolved oxygen tension and pH could be observed and the consumption of the glucose by the cells can be measured. However, if the dose size was too large, the Crabtree effect would be significant and the growth of cells would be inhibited. That would alter the result of pH-stat culture.

In this experiment, the feeding set point was set at pH 4.98 and the value of the low gap for pH control was set at 0.04; i.e. the base was added to the culture when the system pH is equal to pH 4.96.

4.3.3.1.1 "Time interval" equal to T_s

Table 4.6. Cell dry weight and total carotenoid yield of *P. rhodozyma* with "time interval" equal to T_s (0.5 g/L glucose dose size) in pH-stat culture

Time (Hours)	Cell dry weight (g/L)	ln CDW	Total content yield(μg/L)	Cellular carotenoid content (μg/g)
0	1.42	0.3507	97	64.4
18.5	2.74	1.008	495	180.7
22.5	3.14	1.144	723	230.5
26.5	3.94	1.371	941	238.8
30.5	4.96	1.6014	1371	276.5
41.5	12.06	2.4899	3657	303.2
46	13.98	2.6376	4754	340.1
54	15.3	2.728	4650	304
67	15.46	2.7383	4827	312.3
71	16.16	2.7825	4933	305.3
79	16.46	2.801	4774	290.1
96	15.5	2.7408	4483	289.2

Please refer to Fig. A.1

With continuous monitoring, the time interval between each dose of glucose feeding was determined to be 13 mins. Repeated measurements of the residual glucose concentrations in the fermenter during the 13-minute interval showed that the dose of glucose added at the 1st minute was always exhausted at the 13th minute.

Therefore, in this experiment, the "time interval" between glucose dose or the time it took for the culture to completely utilize each dose of glucose (T_s) is 13 minutes. The cell dry weight and the cellular carotenoid content at the stationary phase are 15.78 g/L and 300.2 μg/g, respectively. The specific growth rate is 0.0708 h^{-1} and the biomass yield coefficient is 0.451 g cell/g glucose.

Table 4.7. The residual glucose concentration and ethanol production of *P. rhodozyma* with "time interval" equal to T_s (0.5 g/L glucose dose size) in pH-stat culture

Time (Hours)	Glucose conc. (g/L)	Ethanol (g/L)
0	6.63	0.3726
6	4.37	0.6969
18.5	1.82	1.986
22	0.289	-
25	0.635	-
26.5	0.084	2.788
30.5	0.3763	-
41.5	0.017	1.359
46	0.0133	-

4.3.3.1.2 "Time interval" set longer than T_s

After the T_s of the pH-stat fermentation of *P. rhodozyma* with a glucose dose size of 0.5 g/L was determined, I further attempted to investigate whether setting the "time interval" longer than T_s would affect the dry weight and carotenoid production of pH-stat cultures. Experiments were done in which the "time interval" was progressively increased from T_s and the growth of the cells monitored. It was found that the "time interval" between each dose of glucose could not be infinitely increased. When the "time interval" increased from T_s to above a certain value, the pH-stat fermentation of *P. rhodozyma* could not be maintained and cells growth ceased when glucose dose size was 0.5 g/L; this critical time interval was 18 minutes.

Table 4.8. Cell dry weight and total carotenoid yield of *P. rhodozyma* with "time interval" equal to T_c (0.5 g/L glucose dose size) in pH-stat culture

Time (Hours)	Cell dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	2.96	1.522	371	125.3
18	3.94	1.085	1000	253.8
25	4.58	1.371	859	187.6
29	5.56	1.716	1035	186.2
40	10.2	2.322	2336	229
44.5	9.88	2.291	4480	453.4
48	11.4	2.434	4777	419
52.5	11.84	2.471	5154	435.3
68	12.38	2.516	4781	386.2
76.5	12.92	2.559	5133	397.3
89	12.66	2.538	4966	392.3
96	12.5	2.526	5082	406.6

Please refer to Fig. A.2

With the "time interval" set at 18 minutes, the cell dry weight and the cellular carotenoid content at the stationary phase are 12.46 g/L and 403.5 μg/g, respectively. The specific growth rate is 0.0472 h^{-1} and the biomass yield coefficient is 0.356 g cell/g glucose.

The "time interval" beyond which the cells will not grow can be known as the "critical time interval" (T_c) for pH-stat cultures of *P. rhodozyma* with glucose feeding set point below the pH of the culture. The time interval between feeding in this experiment was 18 mins and it was the T_c for feeding glucose. T_c includes the time for the yeast cells to consume the added dose of glucose in the fermenter and a period of glucose starvation. Cells cannot recover from the starvation if the time interval is longer than T_c.

Table 4.9. The residual glucose concentration of *P. rhodozyma* with "time interval" equal to T_c (0.5 g/L glucose dose size) in pH-stat culture

Time (Hours)	Glucose Conc. (g/L)
0	4.84
18	0.899
22	0.142
25	0.3677
29	0.296
40	0.013
44.5	0
89	0
96	0

These results (Fig. 4.5) showed that pH-stat culture of *P. rhodozyma* grown with "time interval" set as T_s yielded a higher cell dry weight than cells grown with "time interval" set as T_c. For carotenoid production, when the "time interval" was set as T_c, the cellular carotenoid content of the cell was higher than cells grown with "time interval" set as T_s. There was a period of glucose starvation that happened during each "time interval". *P. rhodozyma* may produce more cellular carotenoid during these short time periods. Therefore the cellular carotenoid content of the cells grown with the "time interval" set as T_c was higher.

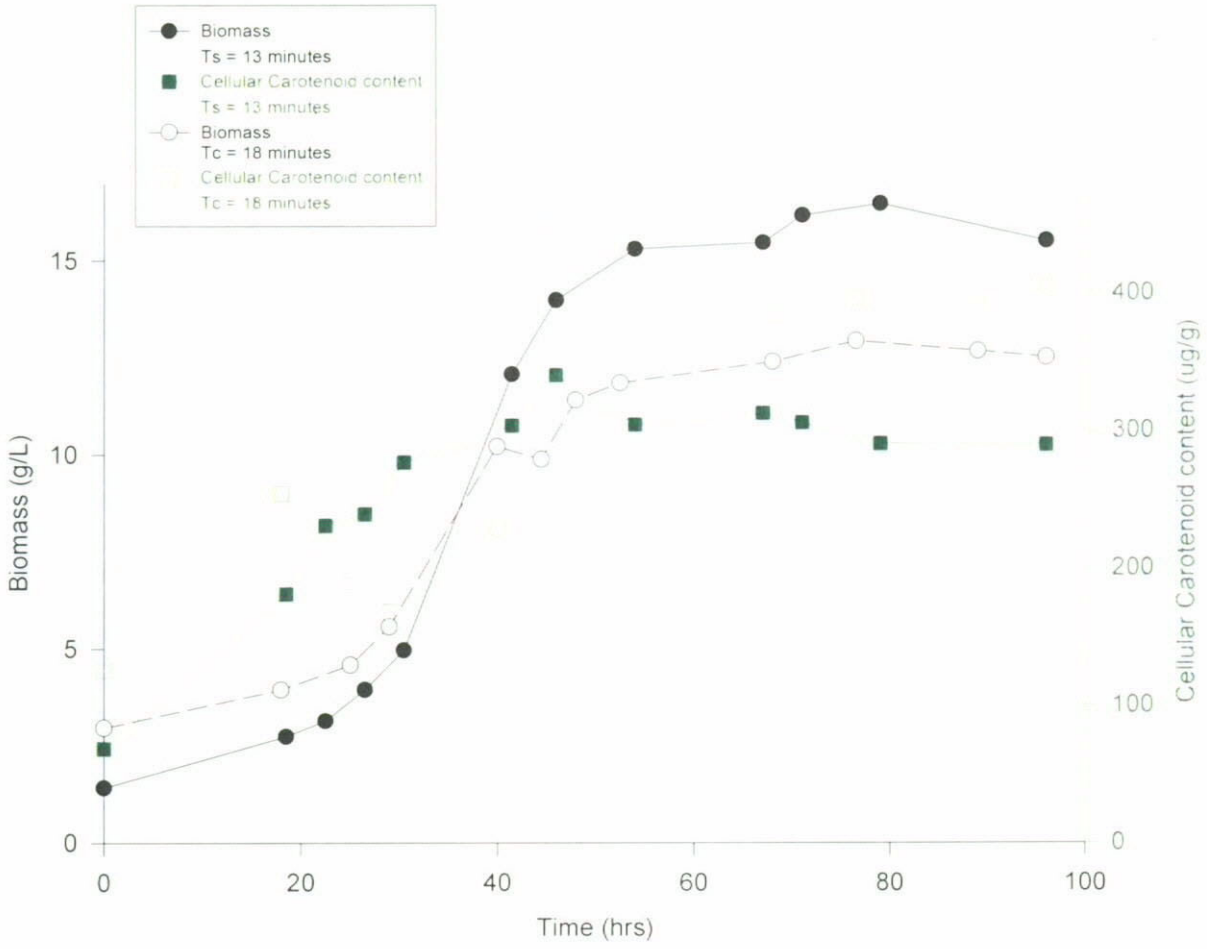

Fig. 4.5. Biomass and cellular carotenoid content of pH-stat culture of *P. rhodozyma*. A comparison of "time interval" settings of T_s and T_c with 0.5 g/L glucose dose size.

4.3.3.2 The investigation of T_s and T_c with a smaller glucose dose size (0.1 g/L)

As the effect of the "time interval" was so significant in 0.5 g/L glucose dose size, a smaller glucose dose size was investigated. 0.1 g/L glucose dose size was 5 times smaller than the 0.5 g/L glucose dose size. If 2.5 g/L glucose dose size was chosen, i.e. 5 times larger than 0.5 g/L, the glucose dose size would be too large and Crabtree effect in each dosage would significantly affect the growth of *P. rhodozyma*.

The glucose feeding set point in these experiments was set at pH 4.98 and the value of low gap was 0.04; i.e. the base was added to the culture when the system pH was equal to pH 4.96.

4.3.3.2.1 "Time interval" equal to T_s

Table 4.10. Cell dry weight and total carotenoid yield of *P. rhodozyma* with "time interval" equal to T_s (0.1 g/L glucose dose size) in pH-stat culture

Time (Hours)	Cell dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Celllular carotenoid content (μg/g)
0	1.5	0.4055	120	80
5	2.78	1.0225	240	86.3
16	3.82	1.3403	438.1	114.7
23	4.14	1.4207	780	188.4
29	6.08	1.805	1611	264.9
41.5	11.16	2.4123	3909	350.2
45.5	11.6	2.451	3601	305.2
52	12.24	2.5047	3540	289.2
69.5	13.46	2.6	3630	269.8
77.5	13.98	2.6376	4080	291.8
89	14.46	2.6714	4387	303.4
96	14.68	2.6865	3806	259.2

Please refer to Fig. A.3

Table 4.11. The residual glucose concentration, ethanol concentration and inorganic nitrogen concentration of *P. rhodozyma* with "time interval" equal to T_s (0.1 g/L glucose dose size) in pH-stat culture

Time (Hours)	Glucose Conc. (g/L)	Ethanol Conc. (g/L)	Inorganic Nitrogen Conc. (g/L)
0	4.99	0.8	4.81
5	-	-	4.82
16	0.179	0.23	-
19	0.048	-	6.76
23	0.022	1.99	-
29	0.074	-	-
41.5	0.0015	1.01	7.55
52	0	0	-
64.5	0	1.46	-
68.5	0	-	6.24
77.5	0	0.2162	7.03
88	0	0	-

The average cell dry weight and the average cellular carotenoid content at the stationary phase are 14.15 g/L and 281.1 μg/g, respectively. The specific growth rate is 0.0536 h^{-1} and the biomass yield coefficient is 0.404 g cell/g glucose.

4.3.3.2.2 "Time interval" equal to T_c

Table 4.12. Cell dry weight and total carotenoid yield of *P. rhodozyma* with "time interval" equal to T_c (0.1 g/L glucose dose size) in pH-stat culture

Times (hours)	Cell dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.26	0.231	191	151.2
5	1.46	0.378	315	215.3
16.5	2.32	0.842	664	286.1
20	2.74	1.008	985	359.4
29.5	4.7	1.548	1731	368.4
41.75	6.4	1.856	3181	497
49.5	7.84	2.059	4120	525.4
54	8.16	2.099	4307	527.8
67.5	10.48	2.349	4546	433.8
71	10.52	2.353	4491	426.9
89.5	10.86	2.385	3753	345.6
96	12.06	2.49	5139	426.1

Please refer to Fig. A.4

The cell dry weight and the cellular carotenoid content at the stationary phase are 10.98 g/L and 408.1 μg/g, respectively. The specific growth rate is 0.0356 h^{-1} and the biomass yield coefficient is 0.314 g cell/g glucose.

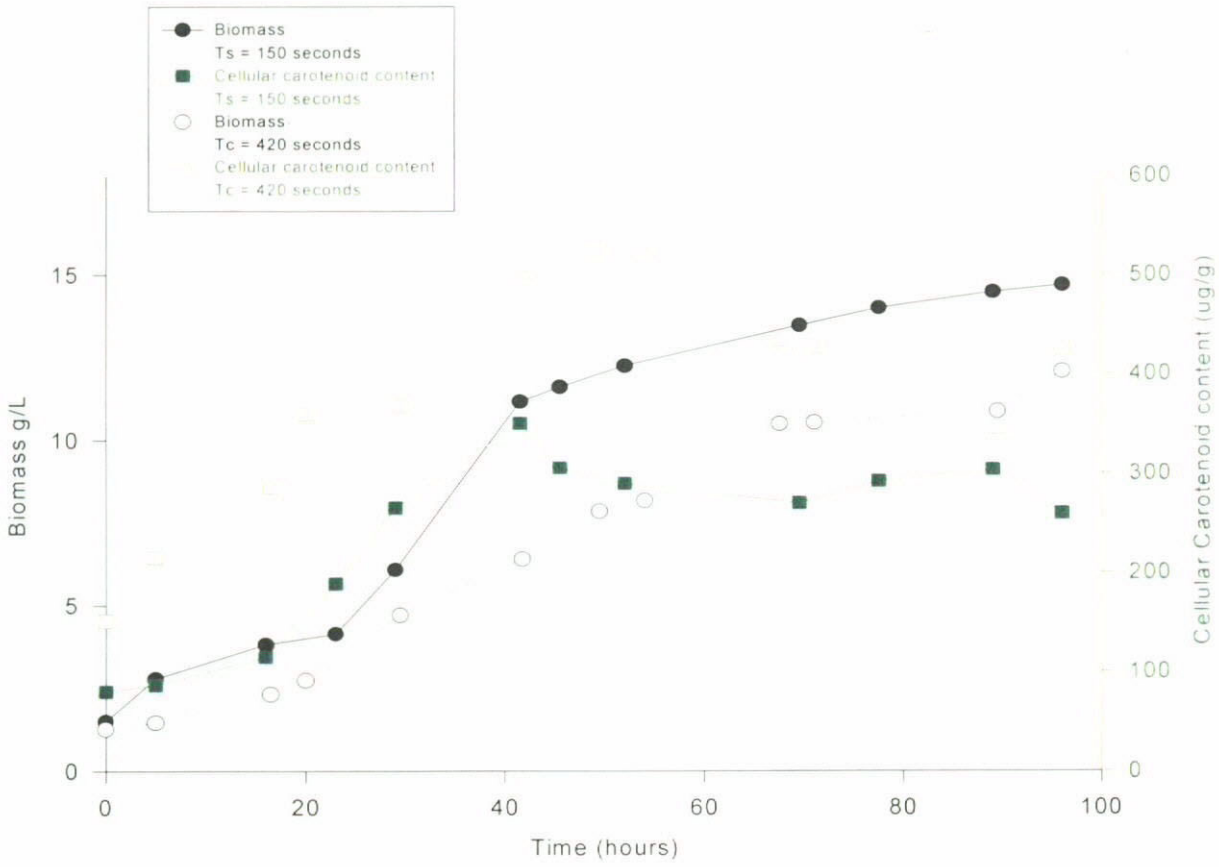

Fig. 4.6 Biomass and cellular carotenoid content of pH-stat culture of *P. rhodozyma*. A comparison of "time interval" settings of T_s and T_c with 0.1 g/L glucose dose size.

The patterns of pH-stat fermentation of *P. rhodozyma* with "time interval" of T_s and T_c are shown in Fig. A.5 and Fig. A.6. "Time interval" settings of T_s and T_c with 0.1 g/L glucose dose size affected the growth and carotenoid production of *P. rhodozyma* similarly to when 0.5 g/L glucose dose size was used.

In pH-stat cultures, when the "time interval" was set shorter than T_s, glucose accumulated in the culture. When the "time interval" was set longer than T_s, the cells became subjected to a period of starvation in between the addition of each dose of glucose. When the "time interval" was increased from T_s, the cells in the pH-stat grew normally until a critical time interval (T_c) was reached. When the "time interval" between each glucose dose was set longer than T_c, the pH-stat culture stopped growing. T_s and T_c for 0.5 g/L glucose dose size pH-stat fermentation was 13 minutes and 18 minutes respectively. "Time interval" of T_s produced more biomass whereas "time interval" of T_c produced cells with a higher carotenoid content. T_s and T_c for 0.1 g/L glucose dose size pH-stat fermentation was 300 seconds and 420 seconds, respectively. With a time interval of T_c, the cells were periodically starved, causing the specific growth rate of the cells to decrease. Since maintenance energy increased, less biomass was produced. At the same time, the cells would produce carotenoid in this short glucose starvation period as they were in the stationary phase. These factors may have

contributed to the increased carotenoid content of the cells.

In these experiments, two different glucose dose sizes were used. pH-stat with 0.5 g/L glucose dose size could yield a higher biomass than that with 0.1 g/L glucose dose size but the total carotenoid content of these two different glucose sizes were very similar. A larger glucose dose size may be more favorable for higher biomass production.

4.3.4 Verification of the pH-stat fermentation of *P. rhodozyma* (pH stat – DO stat) with glucose dose size of 0.5 g/L

A pH-stat culture has a repetitive pH pattern (**Fig. 4.1**). Unlike the D.O. pattern of a D.O.-stat culture, there are no indications in the pH pattern of a pH-stat culture that the glucose that is fed into the culture is completely consumed before the next dose of glucose is added. To verify this in a pH-stat culture of *P. rhodozyma*, controls from the pH-stat and D.O.-stat were combined so that a dose of glucose was added only when the pH and the D.O. reached the glucose feeding set point simultaneously, indicating an exhaustion of glucose.

The feeding set point was set at pH 4.98 and the value of low gap was 0.04; i.e. the base was added to the culture when the system pH equal to pH 4.96. D.O. was maintained at 40 % saturation and D.O. feeding was set at 48% saturation.

Table 4.13. Cell dry weight and total carotenoid yield of *P. rhodozyma* in verification of pH-stat culture with dose size of 0.5 g/L

Time (Hours)	Cell dry weight (g/L)	ln CDW	Glucose (g/L)	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.42	0.3507	4.92	240	169
7	1.76	0.5653	-	307	174.3
20	3.4	1.224	0.041	729	214.3
24	4.4	1.4816	0.332	1086	246.8
27	4.7	1.5476	-	1858	395.2
32	7.22	1.9769	-	2606	360.9
48	13.46	2.5997	0.294	4945	367.4
55.5	14.48	2.6728	0	4940	341.2
66	13.62	2.6115	0	5336	391.8
76	14.88	2.7	0	5271	354.3
80	15.58	2.746	0	5594	359.1
96	16.5	2.8034	0	5464	331.2

The cell dry weight and the cellular carotenoid content in the stationary phase are 15.01 g/L and 355.5 μg/g, respectively. The specific growth rate is 0.0627 h^{-1} and the biomass yield coefficient is 0.429 g cell/g glucose.

The D.O. and pH pattern shown in Fig 4.7 demonstrates that the pH-stat culture protocol developed in this study feeds glucose in a repetitive cycle and only when the glucose is exhausted. The result shows that glucose feeding was really controlled by the drop in pH and the coincident rise of D.O. that indicated exhaustion of glucose.

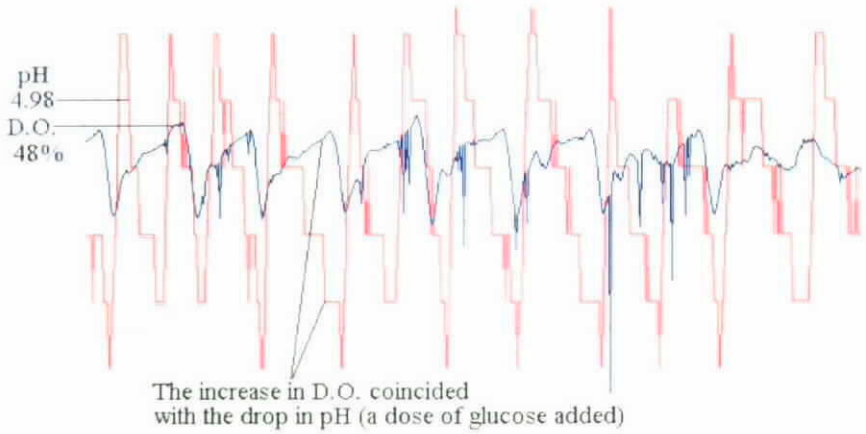

Fig. 4.7 The pH and D.O. profile of a pH-stat culture of *P. rhodozyma* showing the cycles of simultaneous decrease in pH and increase in D.O. when a dose of glucose was added

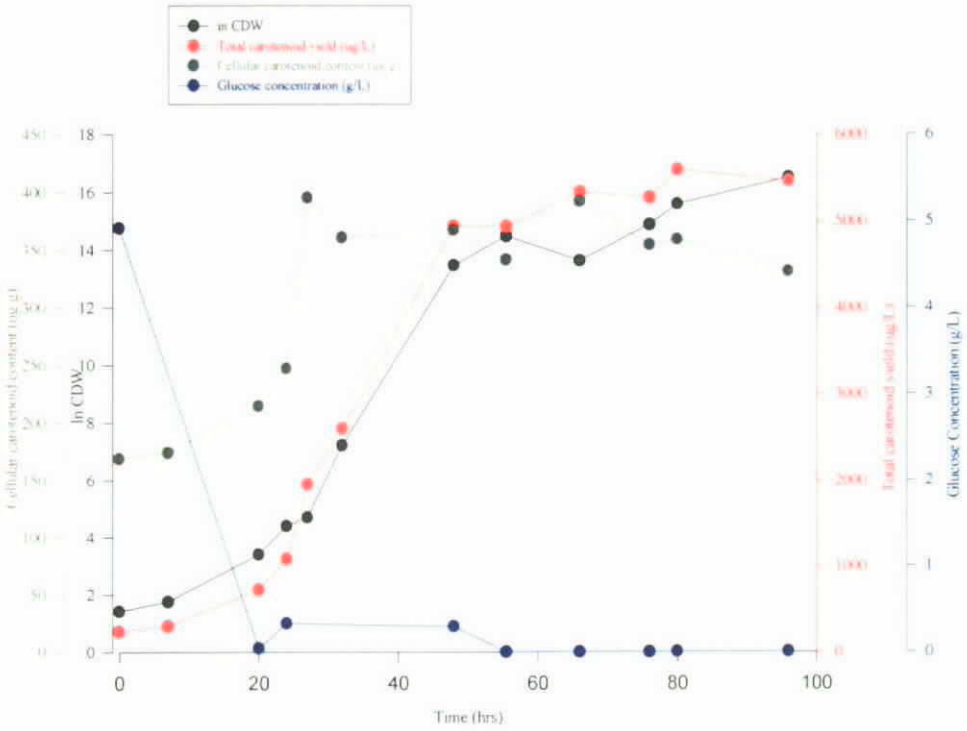

Fig. 4.8 Biomass and carotenoid content of a pH-stat culture of *P. rhodozyma*. Dose of glucose added only when the pH and DO reached the glucose feeding set point simultaneously. Glucose dose size = 0.5 g/L

4.3.5 The effect of potassium dihydrogen phosphorus in a pH-stat

Since the specific growth rate of *P. rhodozyma* is low compared with bacteria such as *E. coli*, the organic acids are not produced in large amounts and may only have a small and gradual effect on the pH of the culture. The nutrient and mineral in the medium may affect the pH-stat fermentation. Therefore, the effect of potassium dihydrogen phosphate in the medium was examined. Two fermentations were compared. One had 1.5 g/L of KH_2PO_4 batched in the medium in the fermenter before inoculation. The other had 0.5 g/L batched in the fermenter before inoculation, the remaining 1 g/L of KH_2PO_4 was fed to the fermenter with the glucose solution.

Table 4.14. Cell dry weight and total carotenoid yield of *P. rhodozyma* with KH_2PO_4 batched in the fermenter before inoculation.

Time (Hours)	Cell dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.32	0.2776	266	201.7
13	2.16	0.7701	520	240.7
17.5	2.96	1.0852	914	308.9
22.5	3.46	1.2413	1425	411.9
27	3.86	1.3507	1276	330.6
37.5	6.18	1.8213	2248	363.7
45	8.48	2.1377	3076	362.8
50.5	9.22	2.2214	3620	392.6
62	10.28	2.3302	3239	315.1
70	11.8	2.4681	3529	299
85.5	10.06	2.3086	3584	356.3
96	11.04	2.4015	3621	328

Please refer to Fig. A.7

Table 4.15. The residual glucose concentration throughout the fermentation process with KH_2PO_4 batched in the fermenter before inoculation

Time (Hours)	Glucose Conc. (g/L)
14.5	3.68
15	3.45
17.5	1.825
22.5	0.035
23	0.0195
24	0.06
26	0.045
27	0.0325
41.5	0.051
68	0
96	0

The cell dry weight and the cellular carotenoid content in the stationary phase are 10.8 g/L and 324.6 μg/g, respectively. The specific growth rate is 0.0398 h^{-1} and the biomass yield coefficient is 0.308 g cell/g glucose.

Table 4.16. Cell dry weight and total carotenoid yield of *P. rhodozyma* with KH_2PO_4 feeding to the fermenter

Time (Hours)	Cell dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)	Glucose Conc. (g/L)
0	1.34	0.2927	451	336.2	4.97
5.5	1.7	0.5306	720	423.5	-
17.5	3.6	1.2809	781	216.9	0.198
21.5	3.9	1.361	1024	262.3	0.02
29.5	5.48	1.7011	1752	319.8	0.1425
41.5	9.08	2.2061	2920	321.6	0.0165
49.5	10.36	2.338	3771	364	-
54	11.48	2.4406	3432	299	0
65.5	13.44	2.5982	5217	388.1	0
73.5	14.2	2.6532	4850	341.5	0
89.5	13.94	2.6348	4723	338.8	0
96	14.1	2.6462	4680	331.9	0

Please refer to Fig. A.8

The cell dry weight and the cellular carotenoid content in the stationary phase are 13.92 g/L and 350.1 μg/g, respectively. The specific growth rate is 0.0423 h^{-1} and the biomass yield coefficient is 0.398 g cell/g glucose.

The result (Fig. 4.9) shows that when feeding 2/3 of KH_2PO_4 with glucose to the system, the final biomass was approximately 20% higher. KH_2PO_4 was a weak acid that might affect the pH value by releasing the proton when it was fed to the medium with glucose. In the medium containing the 1.5 g/L KH_2PO_4 at the beginning of the fermentation, the weak acid might have been neutralized by the base in the initial 20 hours of non-feeding period and the change of pH might have been slower during the feeding period. This might have affected the pH-stat fermentation.

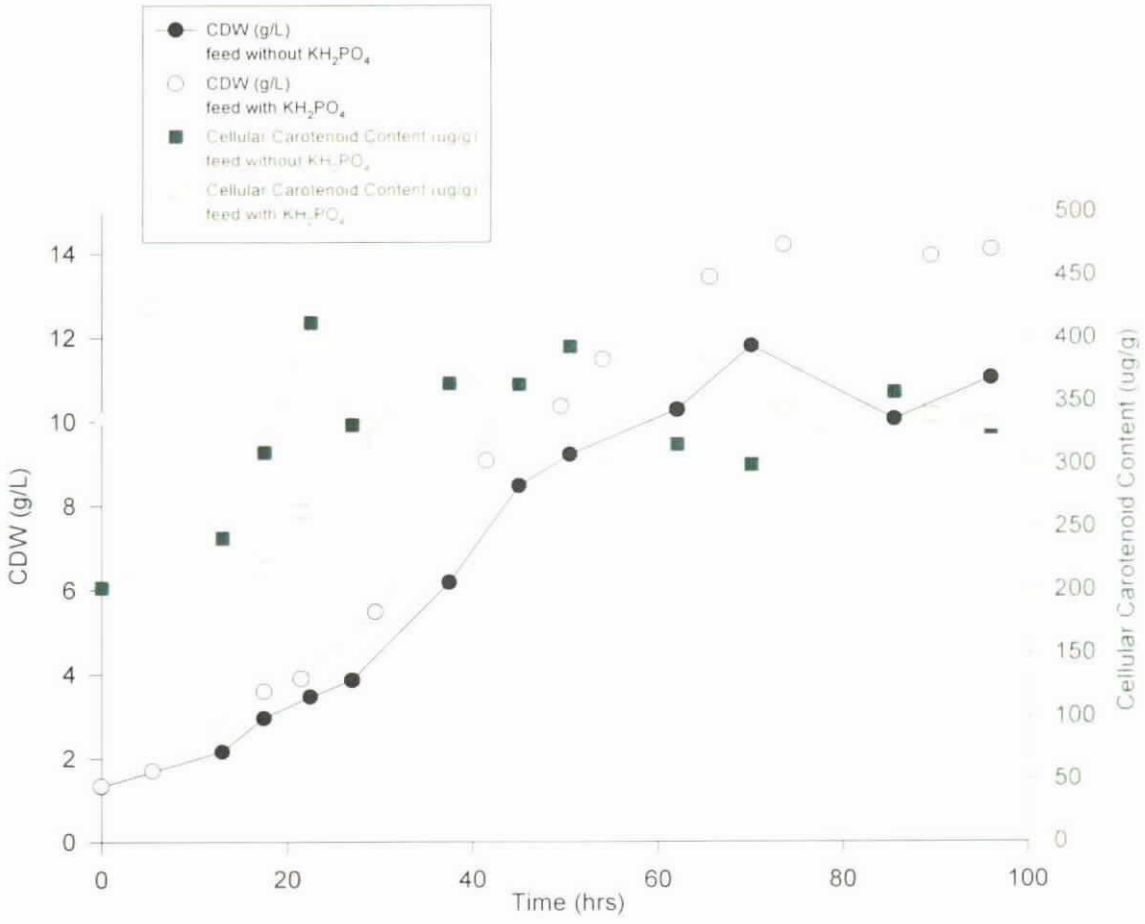

Fig. 4.9 Biomass and carotenoid content of pH-stat cultures of *P. rhodozyma* with and without KH_2PO_4 in the glucose feed.

4.4 Conclusion

Two methods of feeding glucose in pH-stat cultures of *P. rhodozyma* were studied. In the first method, the glucose feeding set point (5.02) was higher than the culture pH (5.00). This method was comparatively simple to operate, though *P. rhodozyma* grew at a low specific growth rate (0.055 h^{-1}). In the second method, the glucose feeding set point (4.98) was lower than the culture pH (5.00). The yeast grew at a specific growth rate of 0.095 h^{-1}, which was similar to the specific growth rates of *P. rhodozyma* in fed-batch cultures as reported by others. The second method of glucose feeding was more complex, and in order to safe guard against overfeeding of glucose, a "time interval" was added to the control strategy of the glucose pump and allowed to expire before the next dose of glucose was added. A critical time interval was defined. In pH-stat cultures of *P. rhodozyma*, it was found that if the "time interval" was set longer than the critical time interval (i.e. 18 minutes in 0.5 g/L gluose dose size), the yeast did not grow. A large glucose feeding dose size was more favorable for the biomass yield in pH-stat fermentation. Feeding 2/3 of the KH_2PO_4 with glucose in the pH-stat culture increased the final cell biomass by about 20% and this indicated that the nutrient with acidic property could alter the pH-stat culture.

CHAPTER 5

THE EFFECT OF ETHANOL ON THE GROWTH AND CAROTENOID PRODUCTION OF *P. RHODOZYMA*

5.1 Introduction

As *Phaffia rhodozyma* is an industrially important microorganism, improvements of the fermentation process of *Phaffia rhodozyma* have been investigated for several years. These improvements included the modification of fermentation strategies, such as using fed-batch culture instead of batch culture to prevent the adverse effects of high concentration of glucose in the culture system,[12, 18, 43, 50, 77] and the modification of the fermentation nutrients used for the fermentation process.[34, 44-46] Scientists have tried to put together the most inexpensive combination of nutrients for the cultivation of *P. rhodozyma* in order to reduce the cost for the production of astaxanthin. They used hydrolysates of *Eucalyptus* wood [41] and peat [84] as nutrients for the fermentation process. A variety of nutrients and compounds were screened for improvement of astaxanthin production by *P. rhodozyma*; the use of ethanol as a supplement in the medium showed consistently that it enhances carotenoid production. As stated in **Chapter 1,** previous studies[13] with D.O.-stat and exponential fed-batch

cultures of *P. rhodozyma* have shown that ethanol produced by the cells in the log phase caused a higher total carotenoid production. The ethanol produced by the cells accumulated in the system until the stationary phase was reached. The cells then started to consume the ethanol in the stationary phase after they had completely consumed the fed glucose. Adding ethanol to *P. rhodozyma* at the beginning of stationary phase in shake flask experiments also showed that it enhanced carotenoid production (**Figure 5.1**). To check whether there is any preference in the consumption of ethanol and glucose by *P. rhodozyma*, simultaneous feeding of ethanol and glucose in these experiments will be investigated.

Different kinds of fed-batch fermentation can be used as a tool to investigate the effect of ethanol on growth and carotenoid production by *P. rhodozyma*. pH-stat fermentation of *P. rhodozyma* developed in this study (**Chapter 4**) can also be used but the control of pH-stat fermentation was too complicated to be suitable for these experiments. Thus, D.O.-stat fermentation was be used. D.O.-stat is a type of fed-batch culture with dissolved oxygen saturation (D.O.) control. Glucose is fed to the fermenter when the D.O. reaches the feeding set point.

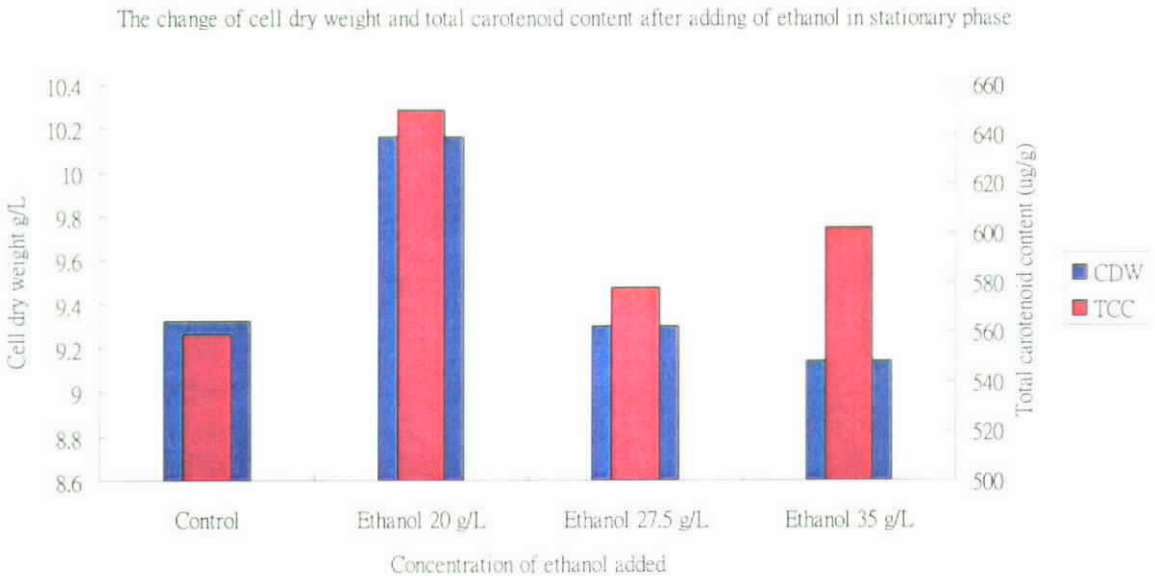

Fig. 5.1 The effect of ethanol (added at stationary phase) on cell dry weight and total carotenoid content.

5.2 Materials and methods

5.2.1 D.O.-stat culture of *P. rhodozyma*

The medium with 5 g/L glucose in the fermenter was inoculated with 8% (v/v) inoculum. D.O.-stat was started when the initial glucose was completely consumed. 30 g/L glucose was then fed to the system when D.O. reached the feeding set point. Feeding was stopped after 30 g glucose/L was added.

D.O.-stat is a form of fed-batch culture with dissolved oxygen (D.O.) control. Glucose is fed to the fermenter when the D.O. reaches the feeding set point. The glucose feeding set point of all the experiments is set at 48% D.O. saturation while the dissolved oxygen of the system is controlled at 40% D.O. saturation. When the glucose is completely consumed in the system, the metabolic rate of the cells will slow down and the cells will use less D.O. in the system. D.O. will increase from the D.O. set point to the feeding set point. Glucose will be fed to the system by the computer controlled pump. When a dose of glucose is added to the system, the cells will consume the carbon source and the metabolic rate of the cells will increase. The D.O. of the system will decrease and remain below the feeding set point at the set point of the system. When the added glucose is consumed, the cycle repeats itself.

5.2.2 D.O.-stat culture of *P. rhodozyma* with simultaneous feeding of glucose and ethanol

In order to investigate the effect of ethanol on growth and carotenoid production during the log phase, experiments were designed to feed glucose and ethanol during the feeding period. Ethanol and glucose were added when the D.O. of the system reached the feeding set point, i.e. 48 % D.O. saturation. Three different ethanol concentrations were used: 5 %, 10 % and 20 % v/v (volume of ethanol/ volume of feeding solution) of ethanol in 150 ml glucose (30g/L) solution. When a dose of the mixture was fed into the fermenter, the concentrations of these two compounds in the medium were 0.08 g/L, 0.16 g/L and 0.33 g/L ethanol respectively with 1 g/L glucose. The ethanol concentrations of samples in each experiment were analyzed as described in **Section 3.8.7.**

5.2.3 D.O.-stat culture of *P. rhodozyma* with ethanol feeding in the stationary phase

In order to investigate the effect of ethanol feeding to a D.O.-stat *P. rhodozyma* culture in the stationary phase, experiments were designed to feed ethanol immediately after the feeding of glucose. A total of 5 g/L of ethanol was added to the system, the addition of each dose of ethanol increased the ethanol concentration of the medium by 0.5 g/L in

the stationary phase. The ethanol was added when the D.O. of the system reached the feeding set point, i.e. 48% D.O. saturation. The ethanol concentrations of samples in each experiment were analyzed as described in **Section 3.8.7**.

5.3 Result and Discussion

A batch fermentation (**Section 4.3.1**) and a D.O.-stat culture with 1 g/L glucose dose size will be used as controls. The cell dry weight and the cellular carotenoid content at stationary phase discussed in the following sections are calculated by taking the average of the last four samples at the stationary phase.

5.3.1 D.O.-stat culture of *P. rhodoyzma* with 1 g/L glucose dose size

Table 5.1. Cell dry weight and total carotenoid yield of *P. rhodozyma* in D.O.-stat culture with 1 g/L glucose dose size.

Time (hrs)	Cell Dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.52	0.4187	143	94
6	2.1	0.7419	229	108.8
19	4.34	1.4678	1190	274.3
23.5	5.26	1.6601	1470	279.5
29.5	8.92	2.1882	2234	250.5
42	16.98	2.832	4534	267
46	16.92	2.8284	4149	245.2
50	17.02	2.8344	3920	230.3
67	17.5	2.8622	3581	204.6
71	17.64	2.8702	4307	244.2
78	17.6	2.8679	4043	229.8
96	17.98	2.88926	4198	233.4

The maximum specific growth rate μ was 0.0633 h^{-1} and biomass yield in the stationary phase was 17.68 g/L. The total carotenoid yield and cellular carotenoid content in stationary phase was 4032.4 μg/L and 228.1 μg/g, respectively. Glucose was fed to the system at 22 hours and the feeding was completed at the 42 hours. During the fermentation, a small amount of ethanol was produced. The maximum concentration of

ethanol produced was about 1 g/L. It indicated that the Crabtree effect was not so significant when compared with the batch culture. The biomass yield coefficient was 0.505 g cell/ g glucose.

Table 5.2. The residual glucose concentration and ethanol production of *P. rhodozyma* in D.O.-stat culture with 1 g/L glucose dose size

Time (hrs)	Glucose conc. (g/L)	Ethanol conc. (g/L)
0	4.56	0.29
6	3.98	0.184
19	0.039	0.4
23.5	0.186	0.276
29.5	0.17	0.261
42	0	0.46
46	0.002	0.414
50	0.002	-
54	0.562	0.353
67	0.098	1.18
71	0.407	0.552
75	0.005	-
78	0.494	0.475
96	0	0.23

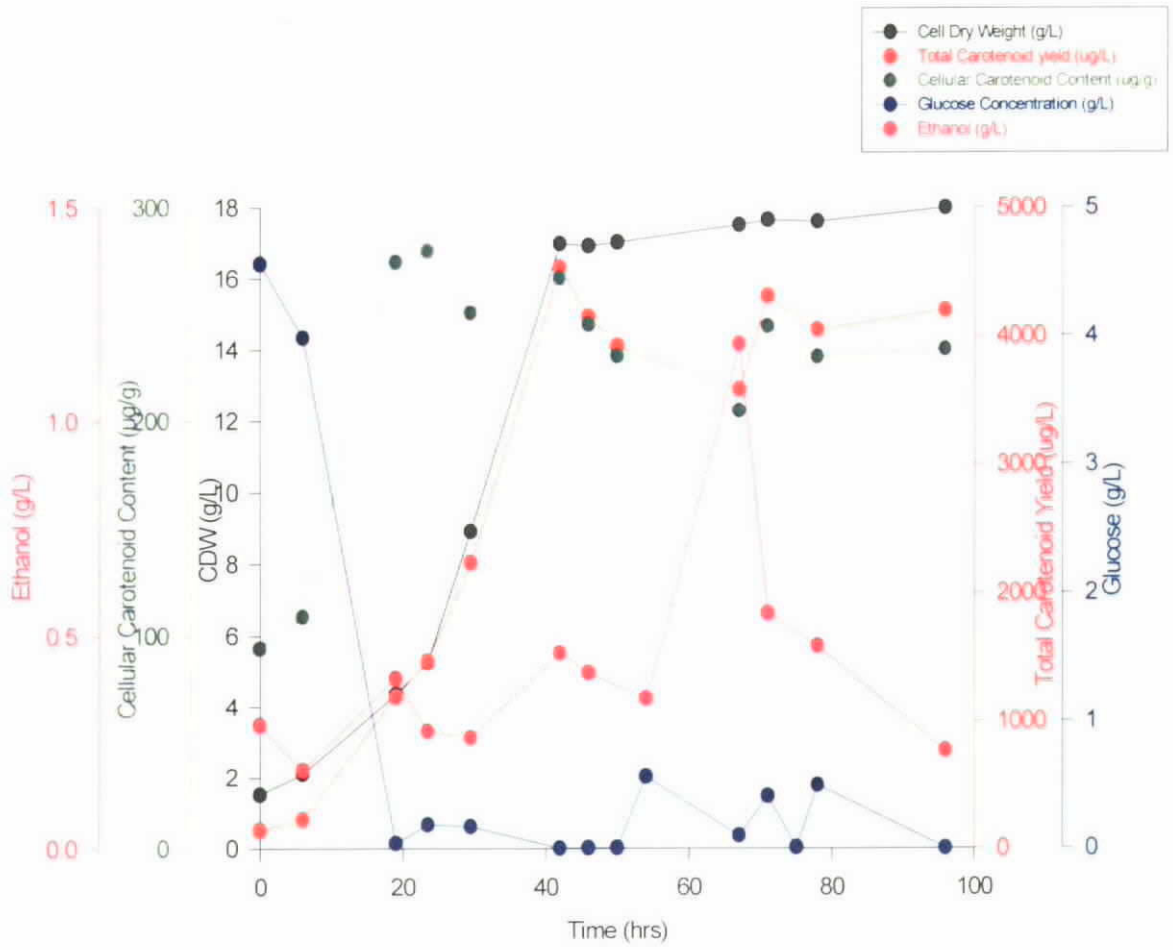

Fig 5.2 D.O.-stat fermentation with 1 g/L glucose dose size

5.3.2 D.O.-stat cultures of *P. rhodozyma* with simultaneous feeding of glucose and ethanol

In order to investigate the effect of ethanol on growth and carotenoid production during the log phase, experiments had been designed to feed glucose and ethanol simultaneously during the glucose feeding period.

5.3.2.1 D.O.-stat culture of *P. rhodozyma* with 1g/L glucose and 0.08 g/L ethanol dose size (0.01 % volume of ethanol / volume of fermentation medium)

Table 5.3. Cell dry weight and total carotenoid content of *P. rhodozyma* in D.O.-stat culture with 1 g/L glucose dose size and 0.08 g/L ethanol dose size

Time (hrs)	Cell Dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	2.22	0.7975	548	246.7
5.5	2.1	0.7419	377	179.6
16	3.36	1.2119	1152	343
26	5.46	1.6974	1550	284
29.5	6.3	1.8406	2095	332.6
40	9.62	2.2638	3338	347
48	14.3	2.6603	4526	316.5
52	13.22	2.5817	4689	354.7
63.5	15.66	2.7511	4669	298.1
72.5	16.08	2.7776	4370	271.7
88	15.98	2.7713	4819	301.6
96	15.78	2.7587	4580	290.2

Please refer to Fig B.1

Table 5.4 Residual glucose and ethanol concentrations of *P. rhodozyma* in D.O.-stat culture with 1 g/L glucose dose size and 0.08 g/L ethanol dose size

Time (hrs)	Glucose conc. (g/L)	Ethanol conc. (g/L)
0	4.38	-
5.5	3.81	0.184
16	0.506	-
22	0.08	0.46
26	0.058	-
29.5	0.362	0.123
40	0.438	0.138
44.5	0.128	0.733
48	0.202	0.414
52	0	0
67.5	0	0.169
77	0	0.169
96	0	0.169

The maximum specific growth rate μ was 0.044 h^{-1} and biomass yield in the stationary phase was 15.88 g/L. The total carotenoid yield and cellular carotenoid content in the stationary phase was 4609.3 μg/L and 290.4 μg/g, respectively. Glucose was fed to the system at the 23rd hour and the feeding was stopped at the 46th hour. During the fermentation, a small amount of ethanol might have been produced since the concentration of ethanol accumulated exceeded the single dose of ethanol added. The maximum concentration of ethanol detected was about 0.8 g/L. The biomass yield coefficient was 0.387 g cell / g carbon source.

5.3.2.2 D.O.-stat culture of *P. rhodozyma* with 1g/L glucose and 0.16 g/L ethanol dose size (0.02 % volume of ethanol / volume of fermentation medium)

Table 5.5 Cell dry weight and total carotenoid yield of *P. rhodozyma* in D.O.-stat culture with 1 g/L glucose and 0.16 g/L ethanol dose size

Time (hrs)	Cell Dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.46	0.3784	160	109.6
6	2.48	0.9083	488	196.9
21	3.82	1.3402	1074	281.2
24.5	4.2	1.4351	1029	244.9
30	6.92	1.9344	1618	233.8
41.5	14.26	2.6575	3771	264.5
49.5	17.5	2.8622	4610	263.4
53.5	16.7	2.8154	4760	285
66.5	18.42	2.9134	5754	312.4
73.5	19.02	2.9455	5173	272
88.5	19.66	2.9786	4260	216.7
96	19.62	2.9765	5012	255.5

Please refer to Fig. B.2

Table 5.6 Residual glucose and ethanol concentrations of *P. rhodozyma* in D.O.-stat culture with 1 g/L glucose dose size and 0.16 g/L ethanol dose size

Time (hrs)	Glucose conc. (g/L)	Ethanol conc. (g/L)
0	3.82	-
6	3.21	3.027
16.5	0.064	-
21	0.03	2.153
24.5	-	1.017
30	0.676	1.279
41.5	0.41	2.562
45.5	0.203	0.598
49.5	0.002	0.0897
53.5	0.001	0.022
66.5	0.004	0.02603
70.5	0	0.0288
73.5	0.971	0.02327
88	0	0.03169
96	0.714	-

The maximum specific growth rate μ was 0.057 h^{-1} and biomass yield in the stationary phase was 19.18 g/L. The total carotenoid yield and cellular carotenoid content in the stationary phase was 5050 μg/L and 263.3 μg/g, respectively. Glucose was fed to the system at the 24th hour and the feeding was stopped at the 49th hour. During the fermentation, a small amount of ethanol might have been produced. The maximum concentration of ethanol detected was about 2.5 g/L. The biomass yield coefficient was 0.408 g cell / g carbon source.

5.3.2.3 D.O.-stat culture of *P. rhodozyma* with 1g/L glucose and 0.32 g/L ethanol dose size (0.04 % volume of ethanol / volume of fermentation medium)

Table 5.7 Cell dry weight and total carotenoid yield of *P. rhodozyma* in D.O-stat culture with 1 g/L glucose dose size and 0.32 g/L ethanol dose size

Time (hrs)	Cell Dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.22	0.1989	110	89.8
7	2.06	0.7227	307	148.9
18	3.92	1.3661	789	201.2
23.5	4.96	1.6014	964	194.4
28	5.62	1.7263	1257	223.7
42	14.32	2.6617	3358	234.5
50	20.42	3.0165	4479	219.3
55.5	22.08	3.0947	5909	267.6
66	22.04	3.0929	5860	265.9
74	21.64	3.0745	5219	241.2
90	22.12	3.0964	5662	256
96	23.36	3.151	5992	256.5

Please refer to Fig. B.3

The maximum specific growth rate μ was 0.0487 h^{-1} and biomass yield in the stationary phase was 22.29 g/L. The total carotenoid yield and cellular carotenoid content in the stationary phase was 5683 μg/L and 255 μg/g, respectively. Glucose was fed to the system at the 23.5 hour and the feeding was stopped at the 50th hour. During the fermentation, a small amount of ethanol might have been produced. The maximum concentration of ethanol detected was about 0.6 g/L. The biomass yield coefficient was 0.378 g cell / g carbon source.

Table 5.8 Residual glucose and ethanol concentrations of *P. rhodozyma* in D.O.-stat culture with 1 g/L glucose dose size and 0.32 g/L ethanol dose size

Time (hrs)	Glucose conc. (g/L)	Ethanol conc. (g/L)
0	3.33	0.52
7	2.73	0.184
18	0.046	0.368
23.5	-	0.184
28	0.728	0.29
31.5	0.117	0.046
42	0.123	-
46	0.648	0.35
50	0.151	0.35
55.5	0.233	0.322
66	0.571	0.474
70	0.523	-
74	0.48	0.368
79.5	0.556	0.368
90	0.464	0.414
96	0.786	0.382

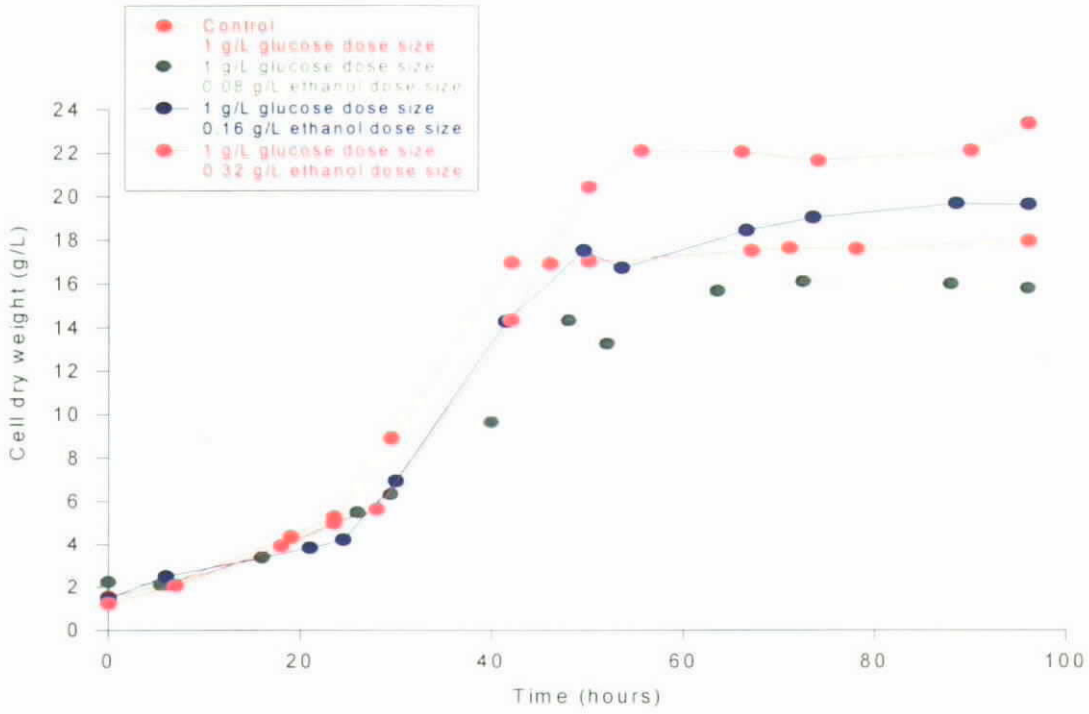

Fig 5.3 Comparison of cell dry weight of D.O.-stat cultures of *P. rhodozyma* with simultaneous feeding of glucose and ethanol.

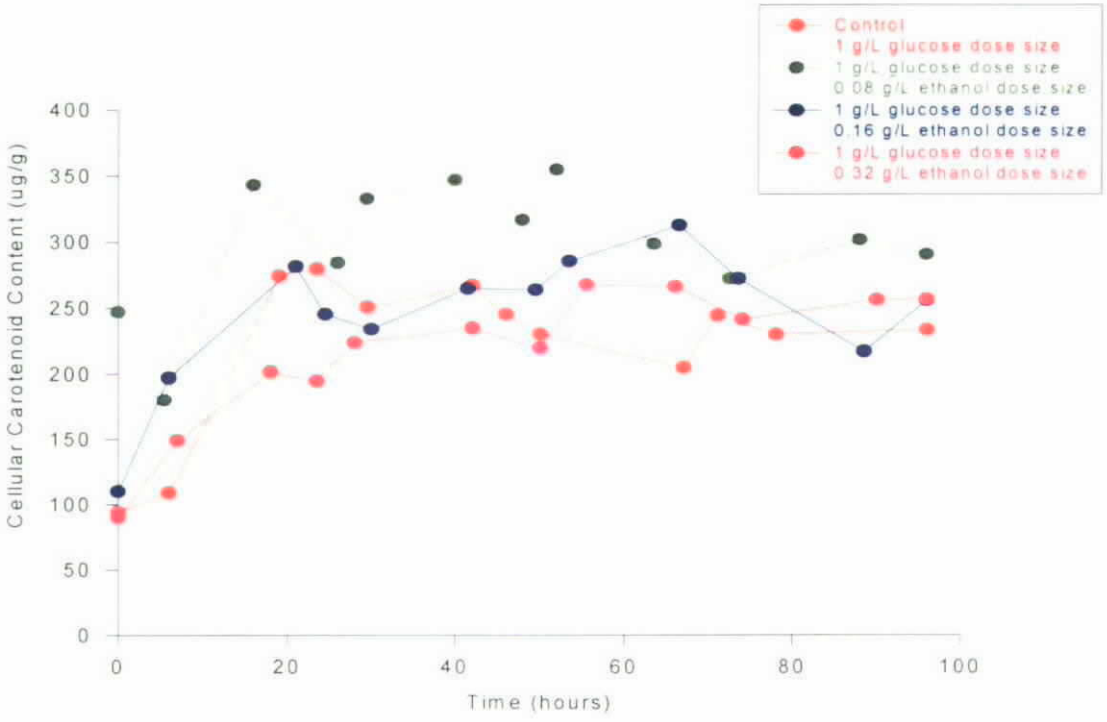

Fig 5.4 Comparison of cellular carotenoid content of D.O.-stat cultures of *P. rhodozyma* with simultaneous feeding of glucose and ethanol

D.O.-stat fermentations, which were fed with higher concentrations of ethanol, yielded higher cell dry weight. D.O.-stat fermentations fed with 0.16 g/L and 0.32 g/L ethanol dose sizes had final cell dry weights higher than the control (no ethanol feedings), whilst the D.O.-stat fermentation fed with 0.08 g/L ethanol dose size had a final cell dry weight lower than the control. Normally, the maximum biomass yield coefficient of *P. rhodozyma* is about 0.5 g cell / g glucose. That is, 35 g/L glucose usually supports about 17.5 g/L cell dry weight and this is shown in the results of the control experiment. However, the cell dry weight for 0.16 g/L ethanol dose size and 0.32 g/L ethanol dose size is 19.18 g/L and 22.29 g/L, respectively. These cell dry weights might be too high for the glucose to support. Total ethanol concentration added in these two experiments was 4 g/L and 8 g/L, respectively. Since the biomass yield coefficient of ethanol is different from the biomass yield coefficient of glucose, the extra increase of cell dry weight may be due to the presence of ethanol, which may act as a carbon source to support the growth of the cells. Gu et al [20] suggested that ethanol was oxidized in cells to acetaldehyde by alcohol dehydrogenase or by a cytochrome P-450 enzyme system [20]. Acetaldehyde is known to be catabolized by at least three routes:

(a) acetadehyde + H_2O + O_2 $\longrightarrow$ acetate + superoxide by aldehyde oxidase;

(b) acetaldehyde + CO_2 $\longrightarrow$ pyruvate by pyruvate decarboxylase;

(c) acetaldehyde + H_2O + NAD(P) $\longrightarrow$ NAD(P)H + acetate + H^+ by aldehyde dehydrogenase.[20]

Experiments with higher cell dry weight may follow the reaction (b) to produce pyruvate in the presence of ethanol, and the pyruvate will be used to support the cell dry weight.

For the experiment with less cell dry weight produced, a higher total carotenoid content was obtained. Furthermore, during the feeding period, the total cartenoid content reached a maximum of about 350 μg/g. In this case, the presence of ethanol have enhanced the total carotenoid content. Ethanol might be oxidized by aldehyde oxidase and aldehyde dehydrogenase and formed acetate as a product as described in reaction (a).[20] The acetate produced increased the total carotenoid formation in the cultivation of *P. rhodozyma*.[20, 44] Acetate has been postulated to inhibit enzymes of the glyoxalate bypass resulting in increased levels of acetyl-CoA. Acetyl-CoA is the precursor for the total carotenoid production. Whether the ethanol enhances the cell dry weight or the amount of total carotenoid content depends on the concentration of ethanol in the cell broth. In this study, it was discovered that concentration of ethanol higher than 0.01 % v/v would increase the yield of cell dry weight. Concentration of ethanol lower than 0.01 % v/v would increase the production of total carotenoid content.

The change of ethanol and glucose concentrations in the fermenter over a period of time was closely monitored using the YSI 2700 biochemical analyzer and the result is shown in Fig. 5.5 and Fig. 5.6. The figures indicate that the consumptions of ethanol and glucose were simultaneous. However, the ethanol in the system, in some cases, could not be completely consumed before the next dose of glucose and ethanol was added. This result is different from that of Yamane et al [21] which stated that ethanol consumption did not take place until glucose was less than 0.3 g/L.[21] The difference might be due to the different experimental design and different strains involved. In this study, ethanol was fed simultaneously with glucose in the log phase and the strain is P 5/6. However, in the study of Yamane et al,[21] the experiments were conducted in shake flasks with strain ATCC 24202 was used. Different strains may respond differently to the ethanol in the system, and the condition of shake flask cultures differs from the fermenter culture. Plus, the yeast cell may react diversely when the ethanol was added in various stages of the fermentation process.

D.O.-stat culture of *P. rhodozyma* with 1 g/L glucose and 0.08 g/L ethanol dose size have increased total carotenoid production compared with the control. In this experiment, the utilization of glucose and ethanol over time was not measured. The results if measured might be different from those obtained in Fig. 5.5 and Fig 5.6. A

small amount of ethanol may accumulate in the system until the cells reach the stationary phase and the ethanol fed to the system may not be consumed simultaneously with glucose as in Fig. 5.5 and Fig. 5.6.

The ethanol concentrations shown in Fig. 5.5 and Fig. 5.6 were a little bit higher than those added in each dose. The excess ethanol existing in the system may be due to the production of ethanol by the yeast cells. Although the Crabtree effect of 1 g/L glucose dose size was not very significant in *P. rhodozyma* strain P 5/6, it was still possible for ethanol production to take place.

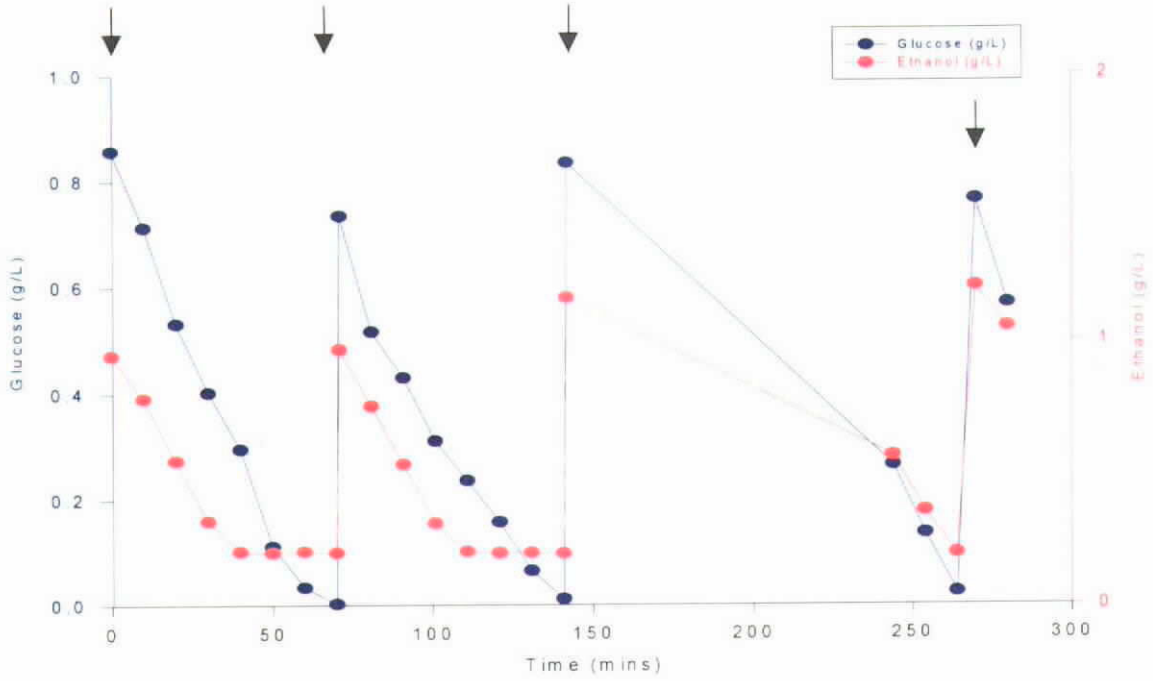

Fig. 5.5 Change of glucose and ethanol concentrations during the first 300 minutes of the feeding period in 0.32 g/L ethanol dose size.

↓-- Dose added

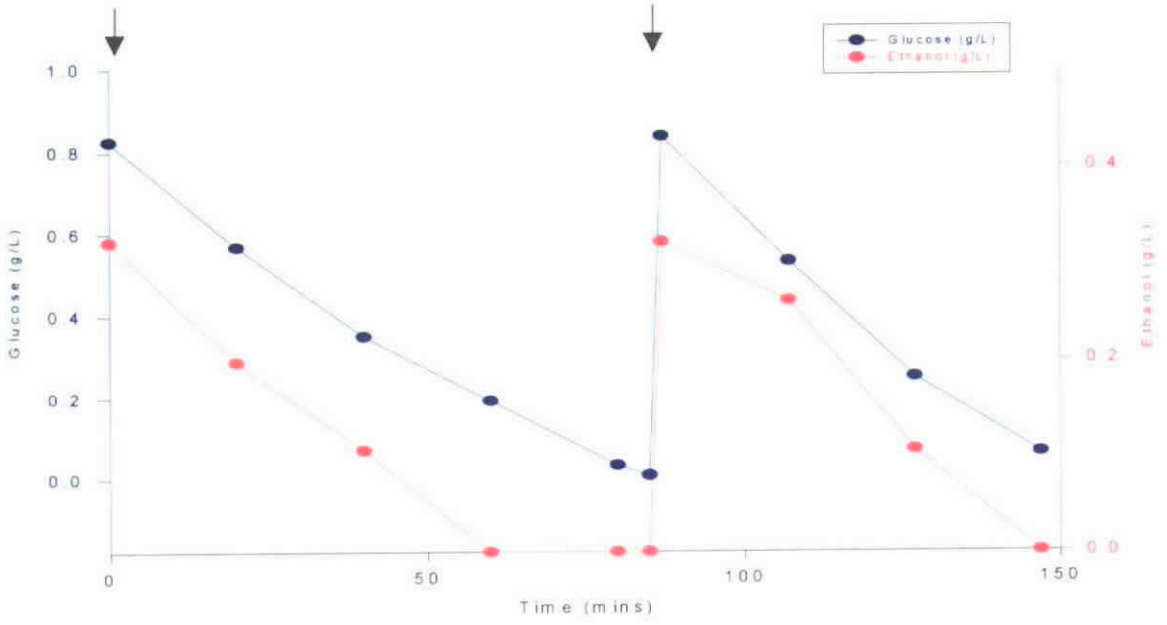

Fig. 5.6 Change of glucose concentration and ethanol concentrations during the first 150 mins of the feeding period in 0.16 g/L ethanol dose size

↓-- Dose added

5.3.3 The effect of ethanol feeding in the stationary phase

D.O.-stat culture of *P. rhodozyma* with ethanol feeding in the stationary phase (ethanol dose size = 0.5 g/L)

Table 5.9 . Cell dry weight and total carotenoid yield of *P. rhodozyma* in D.O.-stat culture with ethanol feeding in the stationary phase (0.5 g/L ethanol dose size)

Time (hrs)	Cell Dry weight (g/L)	ln CDW	Total carotenoid yield (μg/L)	Cellular carotenoid content (μg/g)
0	1.68	0.5188	492	293.1
7	1.9	0.6419	570	299.7
20.5	3.64	1.292	1030	282.8
25.5	4.94	1.5974	1270	257.2
31	5.98	1.7884	1864	311.8
43	9.74	2.2762	3373	346.3
51	12.4	2.5177	4211	339.6
55	13.76	2.6218	4543	330.1
66	13.52	2.6042	5893	435.9
74	14.32	2.6617	6090	425.2
78	15.06	2.7120	6307	418.8
96	15.82	2.7613	5098	322.3

Please refer to Fig. B.4

The maximum specific growth rate μ was 0.0388 h^{-1} and biomass yield in the stationary phase was 14.68 g/L. The total carotenoid yield and cellular carotenoid content in the stationary phase was 5847 μg/L and 398.3 μg/g, respectively. Glucose was fed to the system at the 25.5th hour and the feeding was stopped at the 50th hour. Ethanol was fed to the system after the glucose feeding period. During the fermentation, a small amount of ethanol was produced. The maximum concentration of ethanol detected was about 0.6 g/L. The biomass yield coefficient was 0.419 g cell / g glucose. However, comparing total carotenoid content of this experiment increased by about 74.6%

compared with that of the control.

Table 5.10. The residual glucose and ethanol concentrations of *P. rhodozyma* in D.O.-stat culture with ethanol feeding in the stationary phase (0.5 g/L ethanol dose size)

Time (hrs)	Glucose conc. (g/L)	Ethanol conc. (g/L)
0	4.62	0.184
7	4.11	0.184
20.5	0.052	0.353
25.5	0.796	0.29
31	0.542	0.414
43	0.002	0.552
47	0.012	-
51	0.009	-
55	0.006	-
70.5	0.002	0.46
78	0	0.336
96	0	0.244

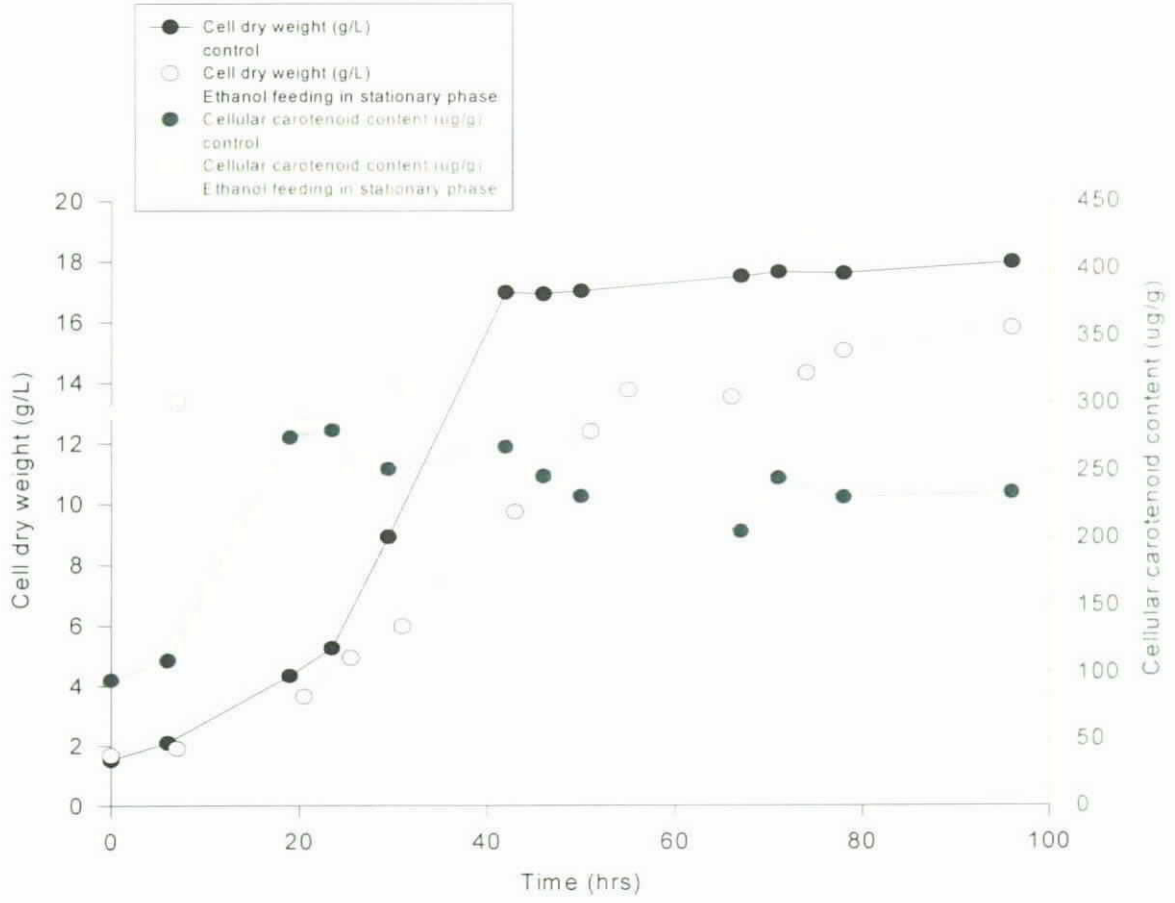

Fig. 5.7 Comparisons of cell dry weight and cellular carotenoid content of feeding and not feeding ethanol in the stationary phase.

When ethanol is added to the culture in the stationary phase, the ethanol will act as a supplement for the carotenoid production. Although the production of carotenoid in *P. rhodozyma* is usually growth-related, the addition of ethanol in the stationary phase could further increase the total carotenoid content. Fig. 5.8, shows that the added ethanol was not completely consumed before a new dose of ethanol was added. During the experiment, it could be observed that, when the cells were consuming ethanol, they used less dissolve oxygen in the system than when they were consuming glucose. That means the cells could consume ethanol at a lower metabolic rate. The consumption of ethanol in the stationary phase was slow and the ethanol thus accumulated in the system. This was totally different from the experiments when glucose and ethanol were added to the system simultaneously. In this case, ethanol may be oxidized again by aldehyde oxidase and aldehyde dehydrogenase and form acetate as a product. The acetate produced may increase total carotenoid formation in the cultivation of *P. rhodozyma*. Acetate has been postulated to inhibit enzymes of the glyoxalate bypass resulting in increased levels of acetyl-CoA. However, if the concentration of ethanol added to the system was too high, the increase of total carotenoid content would not be significant since the high concentration of ethanol would kill the cells. Also, the enzymes in the metabolic pathway for the production of acetyl-CoA might be limited. Although more substrate was added to the system, the production of acetyl-CoA would be restricted.

Therefore, the concentration of ethanol added and the time of adding ethanol would have an effect on the production of carotenoid in *P. rhodozyma*.

For investigating the effect of ethanol in the stationary phase, the result was consistent with the study of Gu et al. In their study, 0.2% v/v of ethanol was added and the carotenoid content increased 27.4% compared with the control.[20] In this study, the concentrations of ethanol used was much lower. The total ethanol added was 0.06 % v/v and the carotenoid content increased to 74.6% compared with the control. Moreover, in my earlier shake flask studies (**Section 5.1**), ethanol concentrations ranged from 2.5% - 4.4% v/v were added and the carotenoid content increased 16.1% compared with the control. Lower concentrations of the ethanol added in the stationary phase may increase the carotenoid content more.

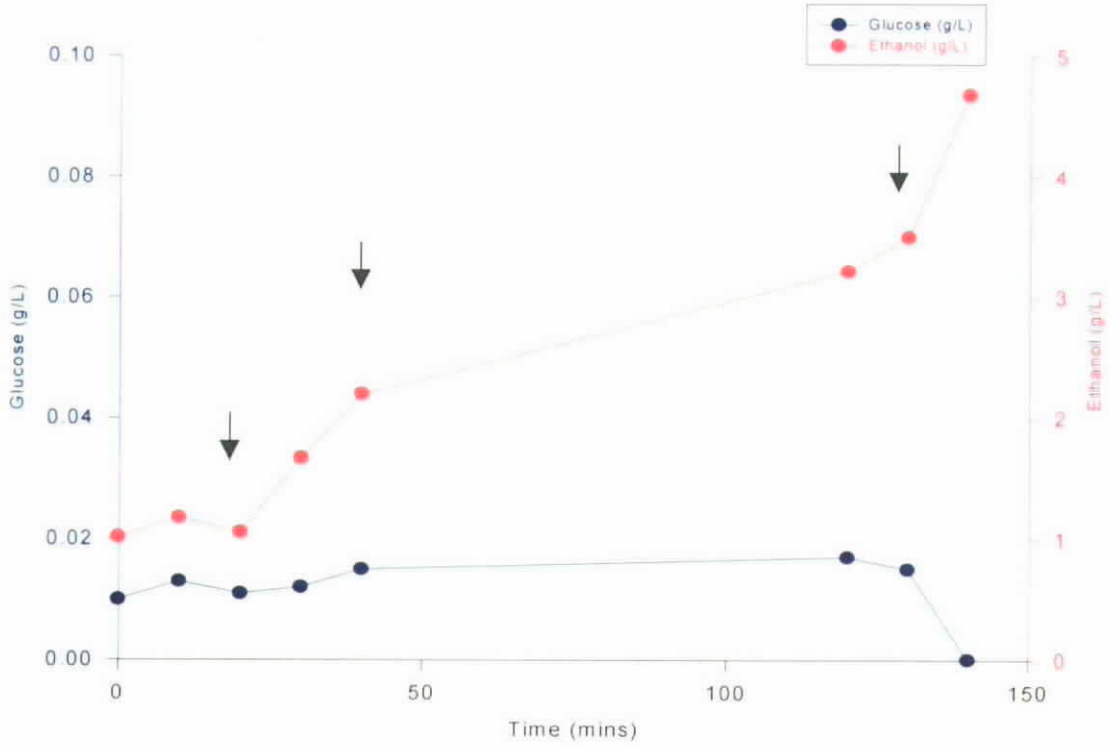

Fig 5.8 Change of glucose concentration and ethanol concentration during the initial 150 minutes of ethanol feeding in the stationary phase.

↓ -- **Dose added**

5.4 Conclusion

Different supplements can be used to increase the total carotenoid production in *P. rhodozyma*. Among them, ethanol is an inexpensive and effective supplement for the enhancement of total carotenoid production. However, the time of ethanol addition to the system and the concentration of ethanol added are important. On one hand, when ethanol was added to the system simultaneously with glucose during the log phase, a low concentration of ethanol (0.01% v/v) increased the total carotenoid content. On the other hand, a higher concentration of ethanol (0.02% - 0.04% v/v) increased the cell biomass. If ethanol concentration was too high, the cells were inhibited. When ethanol was added to the system during the stationary phase, provided that the ethanol concentration was not too high (less than 4.4% v/v), it increased the total carotenoid production of *P. rhodozyma*. Moreover, the increase of the total carotenoid content was inversely proportional to the concentration of ethanol. Addition of a low concentration of ethanol at the stationary phase could yield a higher total carotenoid content in *P. rhodozyma* P 5/6.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1 General Discussion

pH-stat culture and D.O.-stat culture with ethanol feeding of *P. rhodozyma* P5/6 have been investigated in this study. Both pH-stat and D.O.-stat cultures are fed-batch fermentations. Fed-batch fermentation is used for cultivation of *P. rhodozyma* since it is a Crabtree positive yeast and its growth can be effected by high glucose concentration. pH-stat culture with substrate feeding set point lower than the culture pH was shown to be more promising for the cultivation of *P. rhodozyma* than pH-stat culture with substrate feeding set point higher than the culture pH. In the study of pH-stat culture of *P. rhodozyma*, the time interval between the addition of each dose of glucose was found to have an effect on the growth and total carotenoid content of the cells. The time for the cells to completely consume each dose of fed glucose was defined as T_s. In pH-stat cultures, when the "time interval" was set shorter than T_s, glucose accumulated in the culture. When the "time interval" was set longer than T_s, the cells became subjected to a period of starvation in between the addition of each dose of glucose. When the "time interval" was increased from T_s, the cells in the pH-stat grew normally until a critical time interval (T_c) was reached. When the "time interval" between each glucose dose

was set longer than T_c, the pH-stat culture stopped growing. T_s and T_c for 0.5 g/L glucose dose size pH-stat fermentation was 13 minutes and 18 minutes, respectively. "Time interval" of T_s produced more biomass whereas "time interval" of T_c produced cells with a higher carotenoid content. T_s and T_c for 0.1 g/L glucose dose size pH-stat fermentation was 300 seconds and 420 seconds, respectively. With a time interval of T_c, the cells were periodically starved, causing the specific growth rate of the cells to decrease. Since maintenance energy increased, less biomass was produced. At the same time, the cells would produce carotenoid in this short glucose starvation period as they were in the stationary phase. These factors may have contributed to the increased carotenoid content of the cells.

Nutrients that could affect the pH of the system could also affect the pH-stat cultures. Potassium dihydrogen phosphorus (KH_2PO_4) was used to investigate the effect of acidic nutrient in pH-stat culture. When KH_2PO_4 was fed with glucose to the system, the biomass was approximately 20% higher than when the same amount of KH_2PO_4 was batched initially in the system. KH_2PO_4 was a weak acid that may affect the pH value by releasing protons when it was fed to the medium. However, the KH_2PO_4 that was batched initially may have been neutralized by the base in the initial 20 hours of non-feeding period, and the change of pH may have been slower during the feeding

period. Thus the pH-stat fermentation is affected by the medium components and how the components are added to the medium.

In my previous undergraduate study,[13] D.O.-stat and exponential fed-batch fermentation of *P. rhodozyma* were investigated. D.O.-stat and pH-stat fermentations are fed-batch fermentations with online feedback control. One of the purposes of studying pH-stat fermentation was to compare the optimum condition for cultivation of *P. rhodozyma* in D.O.-stat with pH-stat fermentation.

Table 6.1. The comparison of D.O. stat and pH stat with shot size of 0.5 g/L and 0.1 g/L glucose dose size

	Glucose dose size (g/L)	Specific growth rate (μ)	Biomass yield coefficient ($Y_{x/s}$)	CDW (g/L)	Total carotenoid content (μg/g)
D.O. stat [13]	0.5	0.068	0.445	15.73	302.9
pH stat with T_s	0.5	0.069	0.43	15.07	352.8
pH stat with T_c	0.5	0.071	0.451	15.78	300.2
D.O. stat [13]	0.1	0.067	0.439	15.8	385.1
pH stat with T_s	0.1	0.054	0.404	14.15	281.1
pH stat with T_c	0.1	0.036	0.314	10.98	408.1

A comparison in Table 6.1 shows that, for 0.5 g/L dose size, results from the D.O.-stat fermentation is similar to the pH-stat fermentation with T_c, but there is an increase in total carotenoid content of the pH-stat fermentation with T_s. Generally speaking, the results of the pH-stat and D.O.-stat cultivation of *P. rhodozyma* with 0.5 g/L glucose dose size yielded similar biomass and total carotenoid content. That is, for the 0.5 g/L

glucose dose size, pH-stat can serve as an alternative for D.O.-stat fermentation. For 0.1 g/L glucose dose size, the cell dry weight is significantly less in the pH-stat fermentation. The total carotenoid content of cells from pH-stat with T_c as controlling method is about 6% higher than that in D.O.-stat fermentation. Since the total cell dry weight produced is an important factor to consider in industrial cultivation of *P. rhodozyma*, pH-stat cultivation of *P. rhodozyma* with small dose size may not be suitable for industrial use.

The pH-stat culture developed in this study would have been used for investigating the effect of ethanol on growth and carotenoid production of *P. rhodozyma*. However, D.O.-stat fermentation was chosen for such investigation because the control of D.O.-stat fermentation was convenient and easy to operate. In comparison, the control of pH-stat fermentation developed was very complicated. Although it may not be suitable for industrial use, it can serve as an alternative or backup method to D.O.-stat fermentation, especially when the D.O. probe malfunctions during a fermentation, since it is another form of fed-batch fermentation with online feedback control.

Many studies have proved that ethanol has a positive influence on carotenoid production during the stationary phase. [13, 20-21] An inexpensive nutrient, ethanol,

can exert a positive effect when its concentration is below 4.4% v/v. A higher total carotenoid content can be obtained when a lower ethanol concentration was added. If concentration of ethanol fed to the system is higher than 0.01% v/v and the ethanol is fed to the system simultaneously with glucose, the consumption of ethanol and glucose would take place at the same time. The effect on growth and total carotenoid content of the cell will depend on the concentration of ethanol feed. If the ethanol concentration fed to the system is higher than 0.01% v/v, the biomass could be enhanced. Conversely, if the added ethanol concentration is lower than 0.01% v/v, the total carotenoid content of the cells would be increased. When the concentration of the ethanol feed is lower than 0.01%, the consumptions of ethanol and glucose may not be simultaneous. In this case, ethanol may accumulate in the system while glucose may be consumed first.

6.2 Conclusion

Two methods of glucose feeding in pH-stat culture have been studied. The first method is comparatively simple to perform. The glucose feeding set point (5.02) was higher than the culture pH but the yeast grew at a low specific growth rate. In the second method, the glucose feeding set point (4.98) was lower than the culture pH (5.00) and the yeast grew at a specific growth rate of 0.095 h^{-1} which is close to the maximum specific rate of the yeast. However, the second method of glucose feeding is too complicated for operation. *P. rhodozyma* grew better in fed-batch fermentation than in batch fermentation. This study shows that *P. rhodozyma* P 5/6 can be cultured in a pH-stat. *P. rhodozyma* is a yeast. When compared with other microorganisms such as bacteria, the production of organic acid is lower during the consumption of glucose. It is an interesting finding by successfully using pH-stat fermentation for cultivating *P. rhodozyma*. pH-stat cultivation of *P. rhodozyma* can be used as other fed-batch cultivation in the production of astaxanthin industrially but it is too complicated and it is suitable for using as an alternative method when other easy-to-operate methods are not in function.

In order to prevent overfeeding of glucose, a "time interval" was introduced to the control strategy of the glucose pump and allowed to expire before the next dose of

glucose was added. The length of the "time interval" affected biomass and carotenoid production. A critical time interval (T_c) was defined. In pH-stat culture of *P. rhodozyma*, it was found that if the "time interval" was set longer than the critical time interval, the yeast did not grow. As the mechanism of the cells reacted in the "time interval" is still not known, they can serve as the targets for further study.

D.O.-stat has been used to investigate the effect of ethanol on the growth and carotenoid production of *P. rhodozyma*. To study the priority of glucose and ethanol consumption by *P. rhodozyma*, ethanol and glucose were fed simultaneously to a culture during the log phase. Results showed that the consumption of ethanol and glucose was simultaneous and when the ethanol feed concentration was larger than 0.01% v/v, the biomass yield of the system was enhanced. When the ethanol feed concentration was lower than 0.01% v/v, carotenoid content of the cells increased. However, the consumption pattern of ethanol and glucose at this concentration is not known. The consumption pattern may vary in experiments with ethanol concentrations higher than 0.01% v/v. Addition of ethanol in the stationary phase increased the carotenoid content of the cells; the results are consistent with results from other studies.[20-21] Total carotenoid content of *P. rhodozyma* could be increased when ethanol concentrations up to 4.4% v/v was added. These results can be surely that the addition of ethanol can have

effect either on the production of biomass or production of carotenoid but the effect depends on the concentration of ethanol. However, if the ethanol concentration added was too high, the cell growth was inhibited. The increase in the cellular carotenoid content of the cells depended on the ethanol concentration added. A lower ethanol concentration (ranged 0.01% - 2.5% v/v) could yield a higher cellular carotenoid content.

Since the mechanism and physiological explanation are still not completely clear, experiments, such as those in the field of biochemistry and at the molecular level, can be conducted for further study. For the experiment in the field of biochemistry, purification and identification of enzymes in different times during the fermentation process can be done to find out which enzymes are involved in the pathway of ethanol utilization. Then, a much clearer picture can be obtained.

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APPENDIX A

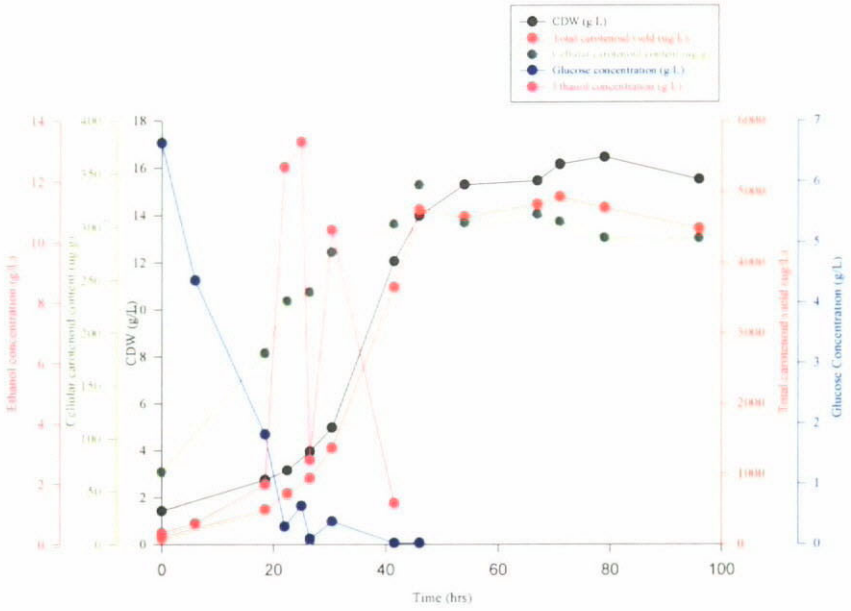

Fig. A.1 **pH-stat culture of *P. rhodozyma*. The "time interval" was set as T_s and the glucose dose size was 0.5 g/L.**

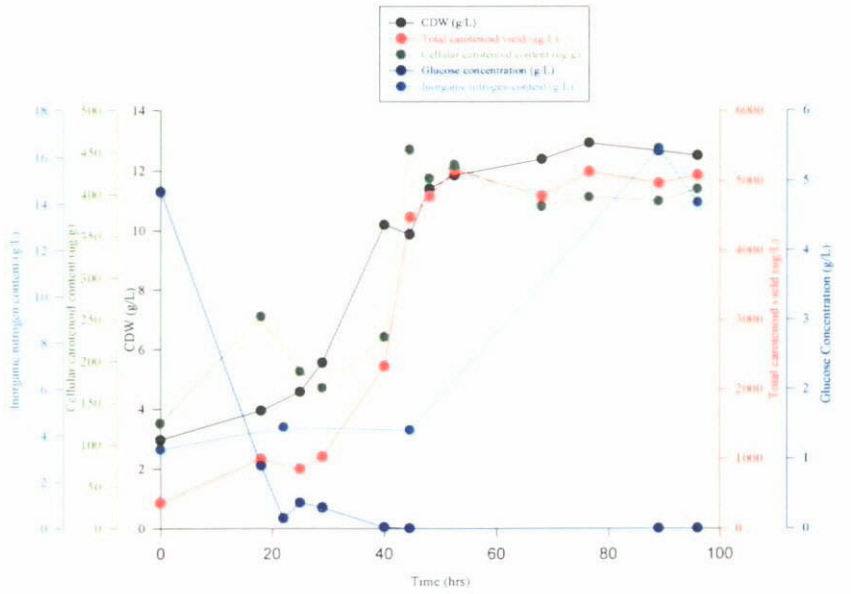

Fig. A.2 **pH-stat culture of *P. rhodozyma*. The "time interval" was set as T_c and the glucose dose size was 0.5 g/L.**

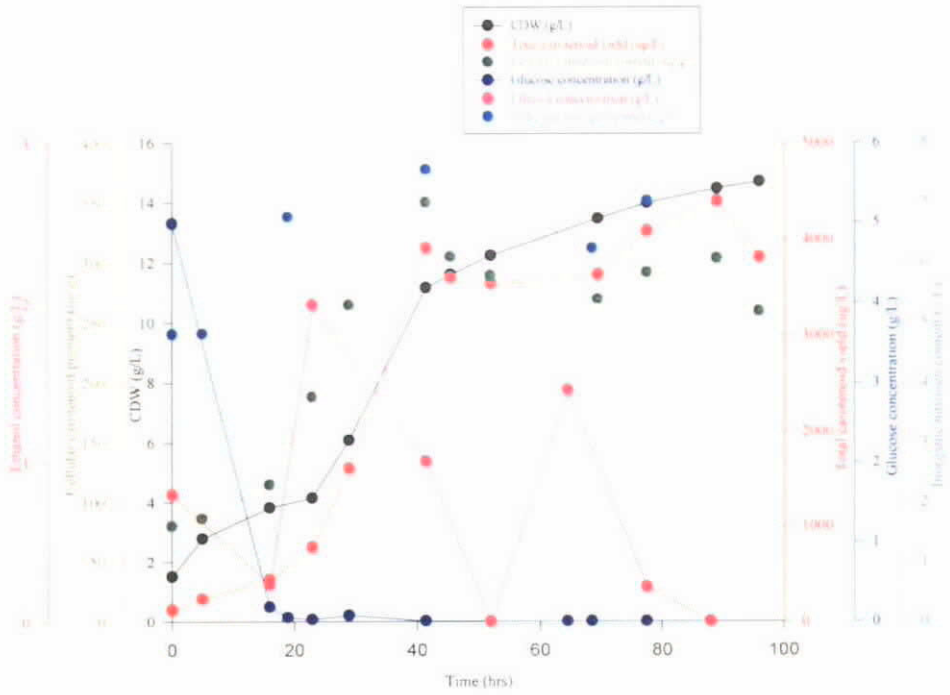

Fig. A.3 pH-stat culture of *P. rhodozyma*. The "time interval" was set as T_s and the glucose dose size was 0.1 g/L.

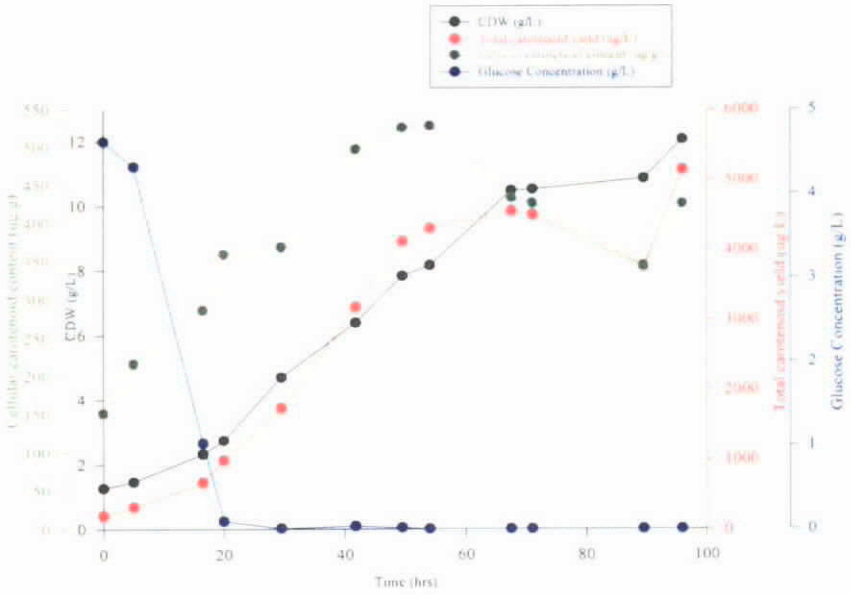

Fig. A.4 pH-stat culture of *P. rhodozyma*. The "time interval" was set as T_c and the glucose dose size was 0.1 g/L

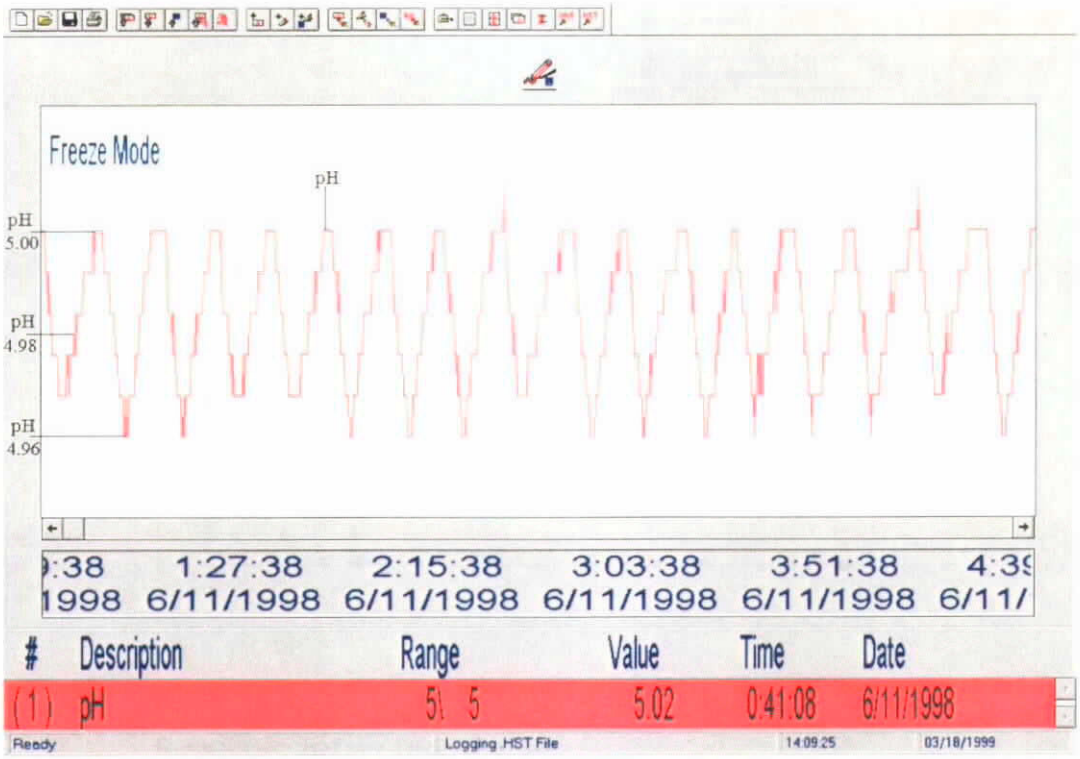

Fig. A.5. pH pattern of pH-stat culture of *P. rhodozyma* with "time interval" setting as Ts.

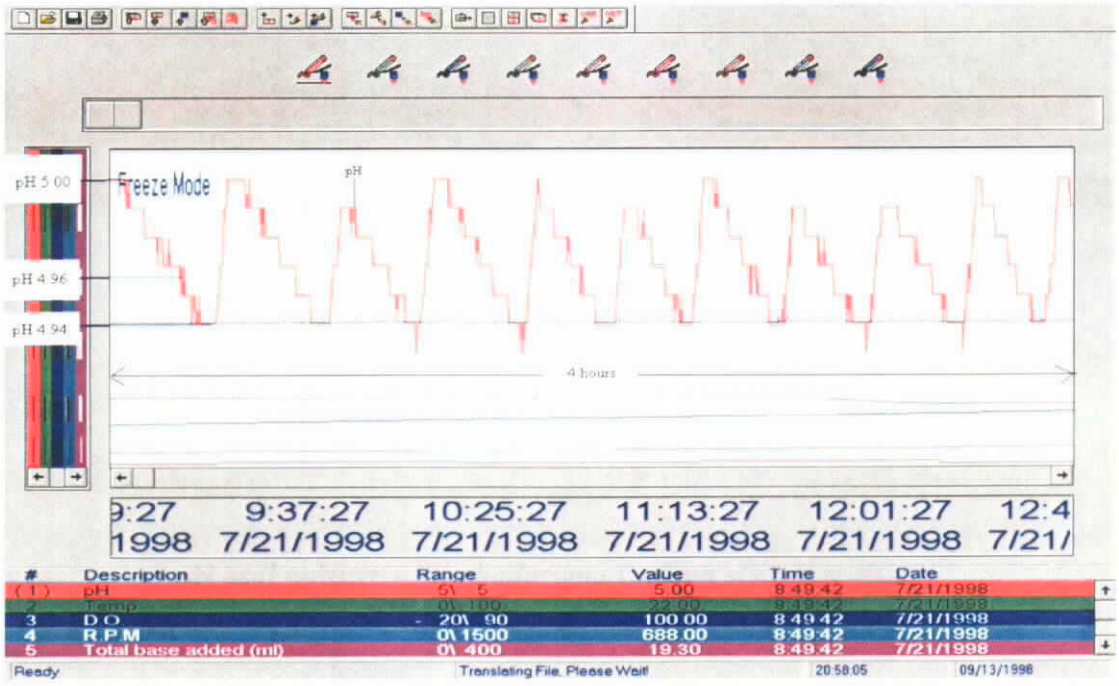

Fig. A.6. pH pattern of pH-stat culture of *P. rhodozyma* with "time interval" setting as T_c

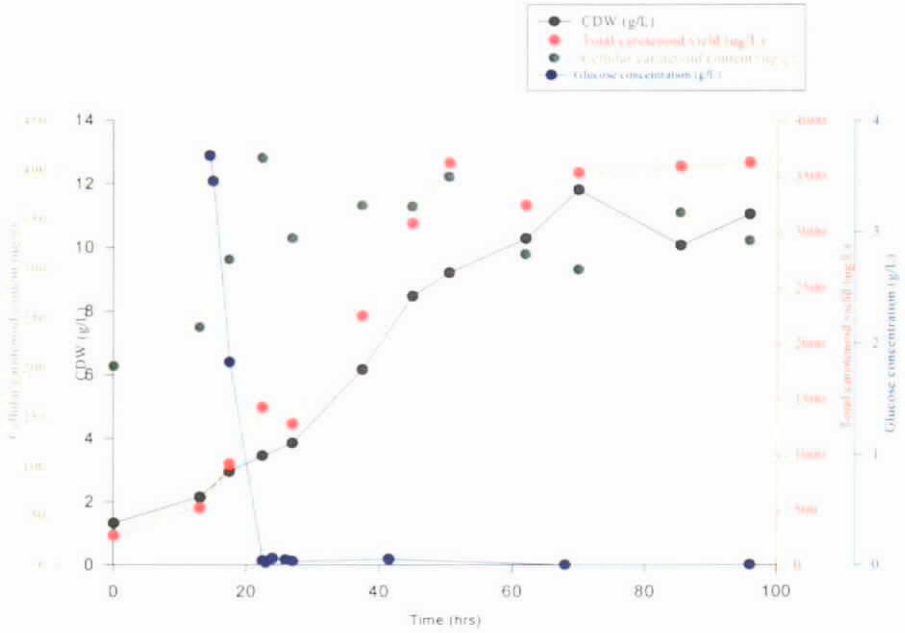

Fig. A.7. pH-stat culture of *P. rhodozyma* with KH_2PO_4 batched in the fermenter before inoculation

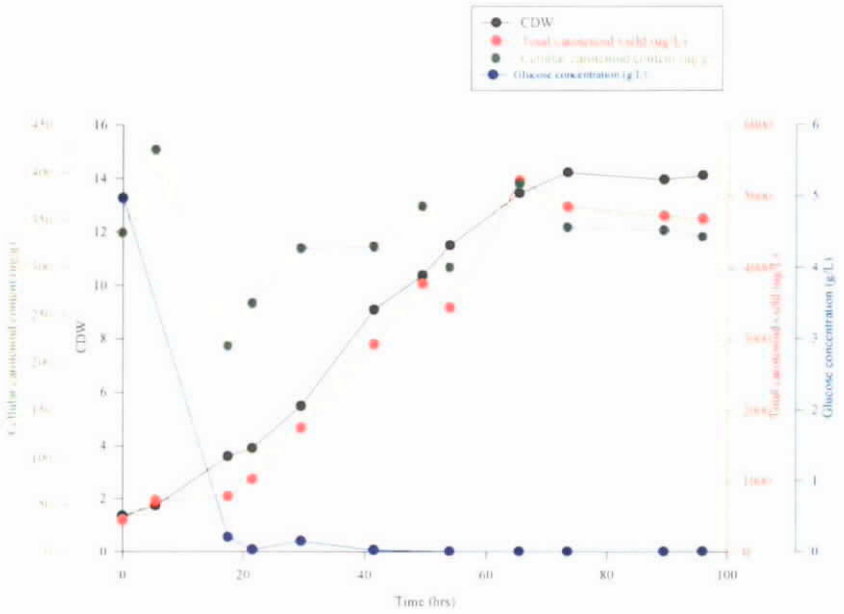

Fig. A.8. pH-stat culture of *P. rhodozyma* feeding with KH_2PO_4.

APPENDIX B

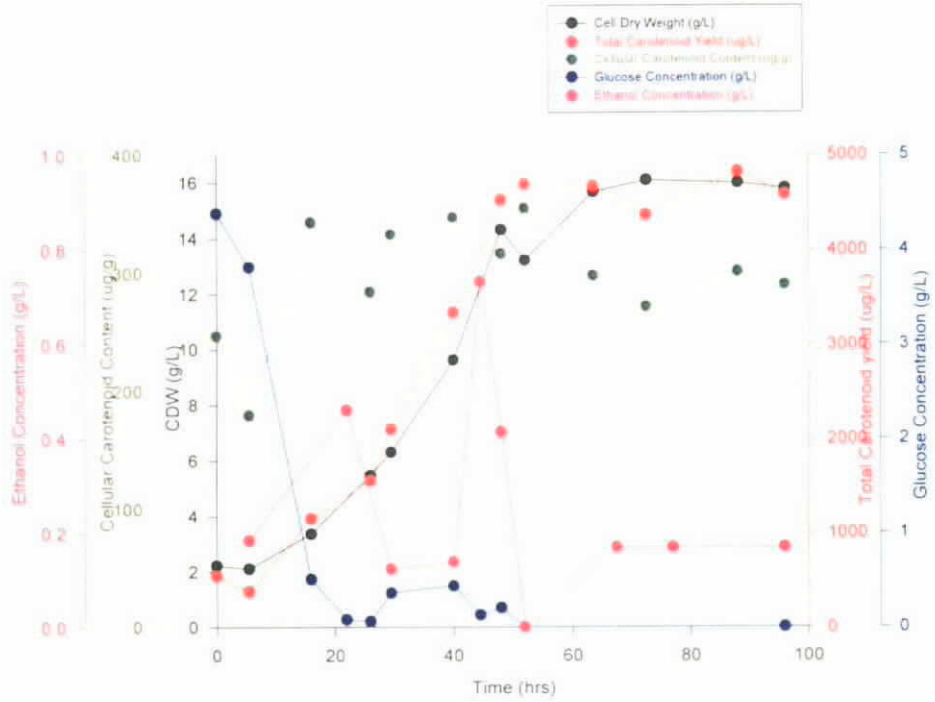

Fig. B.1. **D.O.-stat culture of *P. rhodozyma* with 1 g/L glucose and 0.08 g/L ethanol dose size.**

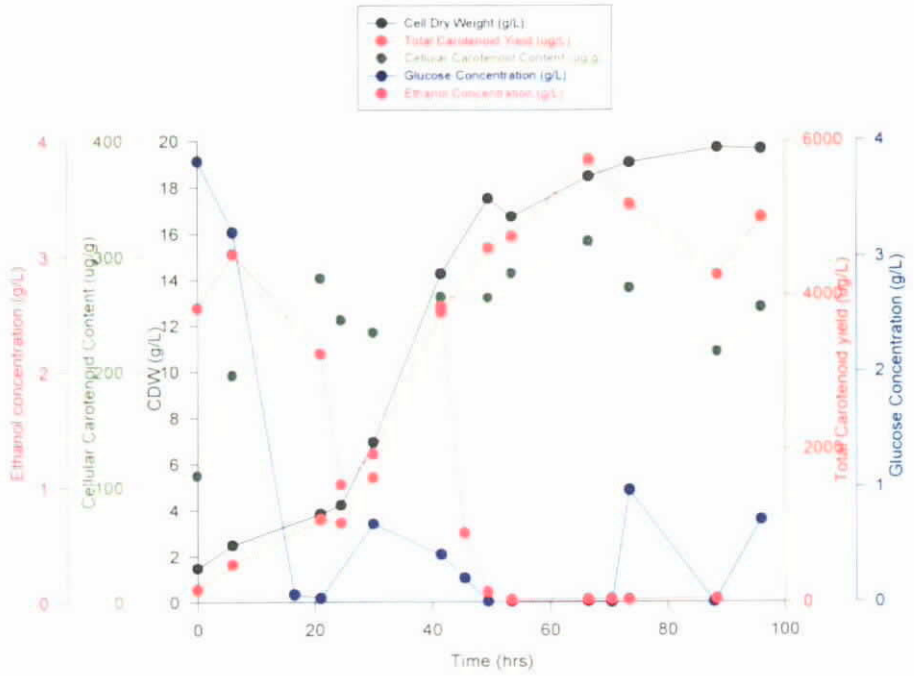

Fig. B.2. **D.O.-stat culture of *P. rhodozyma* with 1 g/L glucose and 0.16 g/L ethanol dose size.**

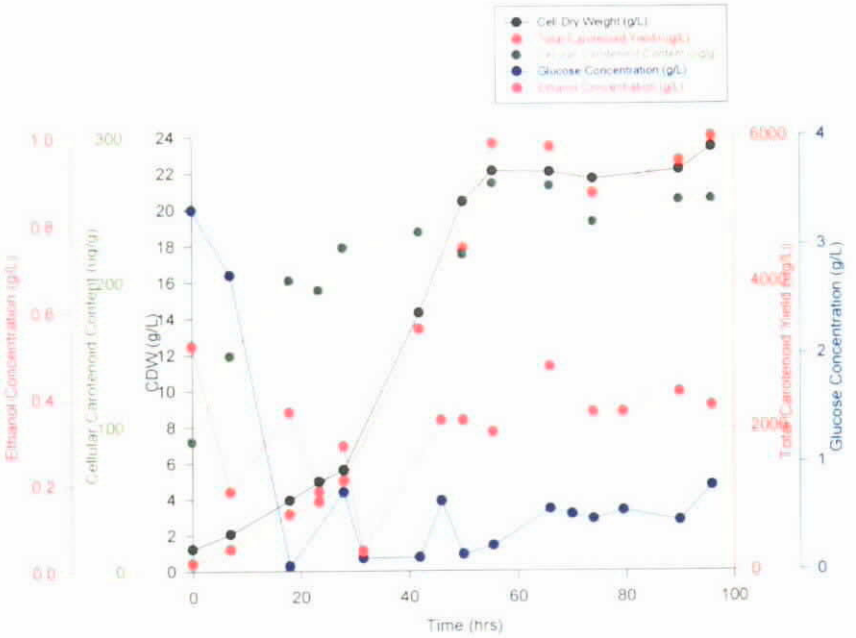

Fig. B.3. D.O.-stat culture of *P. rhodozyma* with 1 g/L glucose and 0.32 g/L ethanol dose size.

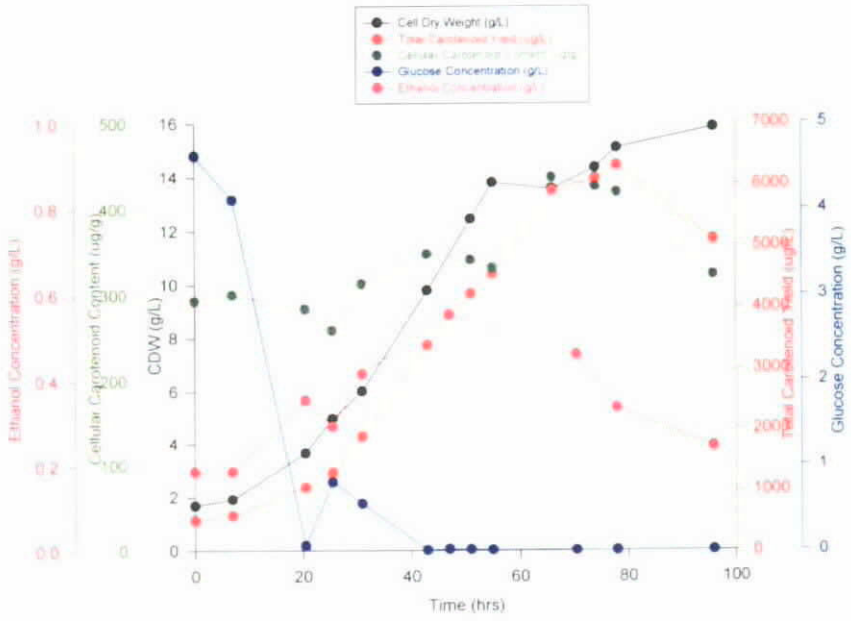

Fig. B.4. D.O.-stat culture of *P. rhodozyma* with ethanol feeding in the stationary phase (0.5 g/L dose size).