CHAPTER 4
RESULT AND DISCUSSION

Since objective of this study aimed to establish effective medium from pineapple juice concentrate, by product from canned pineapple industries capable of promoting growth and astaxanthin production by *X. dendrorhous*. Therefore, the study started by characterizing pineapple juice concentrate, then, investigating whether pineapple juice is capable of supporting *X. dendrorhous* proliferation. Subsequently, results on optimization of growth and astaxanthin accumulation were provided. According to optimized condition found, batch cultivation was in order to validate the result obtained previously.

4.1 Pineapple Juice Characterization

Chemical composition of pineapple juice concentrate, a primary raw material derived from canned pineapple industry, employed in this study was characterized to determine whether proliferation of *X. dendrorhous* could be sufficiently and satisfactorily promoted.

4.1.1 Sugar Concentration

In this study, the concentrations of sucrose, glucose, and fructose present in pineapple juice concentrate were quantified using High Performance Liquid Chromatography (HPLC). It is evident from Figure 4.1 that there are four distinct peaks apparent at the retention time of 6.19, 8.41, 10.25 and 11.98 minutes which corresponds sucrose, glucose and fructose, after comparing against standard chromatogram obtained using sucrose, glucose and fructose whose retention times were 8.41, 10.25, and 11.98 minutes, respectively, whereas the first peak appearing at 6.19 minutes was unidentified. Results further showed that sucrose, glucose and fructose present in pineapple juice concentrate were at the concentration of 23.58, 39.14, and 36.86 g/L, in other words, at the ratio 1:1.6:1.6, respectively (Figure 4.1). Results found are in good keeping with that reported by Abdullah and Mat (2008) who found that pineapple juice concentrate obtained from pineapple juice cannery contained sucrose, glucose, and fructose at the concentration of 16.75, 19.72, and 20.62 g/L, respectively, which corresponds to the ratio of 1:1.2:1.2. This is not surprising since pineapple juice

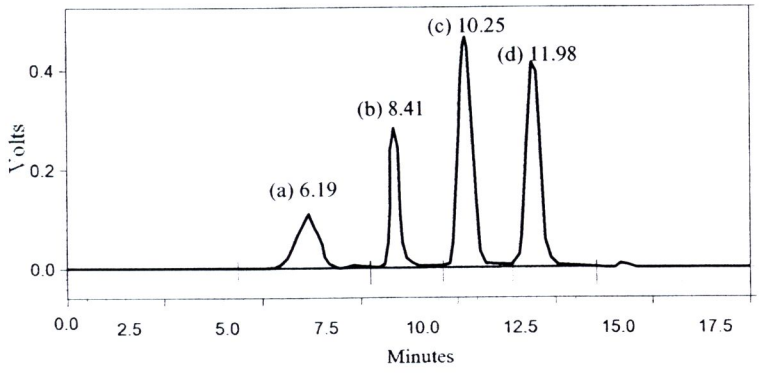


Figure 4.1 Typical sugar profile of diluted pineapple juice concentrate (~100 g/L total sugar) which contained unidentified (a), sucrose (b), glucose (c) and fructose (d) with retention time of 6.19, 8.41, 10.25 and 11.98 minutes, respectively.

Table 4.1 Constituents of pineapple juice stock medium (100 g/L total sugar)

Composition	Amount
Sugar contents	(g/L)
Sucrose	23.58 ± 0.36
Glucose	39.14 ± 0.60
Fructose	36.86 ± 0.57
Acidity of Pineapple Juice	(mM)
Citric acid	21.70 ± 0.00
Acetic acid	2751.30 ± 533.58
Iso-Butyric acid	6.45 ± 1.63
Butyric acid	8.50 ± 0.56
Propionic acid	51.00 ± 10.75
Nitrogen content	(%w/w)
Nitrogen	0.042 ± 0.005
Protein	0.26 ± 0.03
Carbon Content*	(%w/w)
Ash	2.95 ± 0.05
Total carbon	53.92 ± 0.03
Organic matter	97.05 ± 0.05

Note. Pineapple juice base medium was prepared by diluting pineapple juice concentrate with appropriate amount of deionized water to yield the concentration of total sugar 100 g/L. *Carbon Content of undiluted pineapple juice concentrate.

concentrate obtained from fresh pineapple juice through the process of pressing, concentrating, heating and evaporating, with which may possibly destroy the glycosidic bond of sucrose leading to an increase in concentration of both glucose and fructose (Butler, 1913).

4.1.2 Acidity of Pineapple Juice

Concentration of citric acid, acetic acid, iso-butyric acid, butyric acid, and propionic acid present in pineapple juice concentrate were 21.70, 2751.30, 6.45, 8.50, and 51.00 mM, respectively (Table 4.1). However, according to Elkins *et al.* (1997), organic acids present in pineapple juice concentrate were citric acid, isocitric acid, malic acid, acetic acid, and fumaric acid which is similar to those found in this study. In addition, Abdullah and Mat (2008) found that citric acid was the main acid in liquid pineapple waste at the concentration of 2.18 g/L while 0.29 g/L malic acid was also detected.

4.1.3 Measurement of Ash and Carbon Content

Ash and carbon content were approximated using the method recommended by Carter (1993). Total carbon found in pineapple juice concentrate was 53.92 % (w/w), while ash content and organic matter were 2.95 % and 97.05 % (w/w), respectively (Table 4.1). Nevertheless, it should be noted that ash content found in this study was higher than that reported in literature, approximately 1.5 % (Elkins *et al.* 1997).

4.1.4 Determination of the Content of Nitrogen

Nitrogen was quantified as total kjeldahl nitrogen (TKN) according to procedure proposes by Association of Official Analytical Chemistry (AOAC) (1998). The nitrogen concentration in pineapple juice concentrate was 0.042 % (w/w) which corresponds to 0.26 % protein (Table 4.1). On the other hand, Abdullah and Mat (2008) reported that soluble protein concentration and TKN in liquid pineapple waste were 1.13 and 0.64 g/L, respectively.

4.1.5 Amino Acid

Amino acids were determined according to standard protocol recommended AOAC (1990). Amino acids present in pineapple juice were provided in Table 4.2. It was observed that total amount of amino acid detected in pineapple juice was 1552.5 mg/ 100 mL. In addition, Elkins *et al.* (1997) found that the highest amount of amino acid found in pineapple juice concentrate was asparagine (551 mg/100 g) which was not

detected in this study. Further, for pineapple juice concentrate employed, the highest amount of amino acid found was aspartate, approximately 369.5 mg/100 mL. Nevertheless, all amino acids detected were also found by Elkins *et al.* (1997) except cysteine which was slightly lower (8.7 mg/100 mL). In addition, eight essential amino acids were found while nine non-essential amino acids were also present in pineapple juice concentrate.

Table 4.2 Amino acid profile of pineapple juice concentrate

Amino acids	**Concentration**	
	This study (mg/100 mL)	**Literature (mg/100 g)****
Aspartate	369.5 ± 2.1	62 ± 16.7
Glutamate	212.3 ± 0.7	28 ± 17.3
Alanine	147.7 ± 0.1	52 ±16.7
Serine	116.6 ± 0.4	92 ± 16.9
Leusine*	92.3 ± 0.2	13 ± 3.4
Glycine	88.0 ± 0.6	17 ± 4.2
Lysine*	86.9 ± 0.4	18 ± 4.9
Valine*	80.7 ± 0.3	14 ± 3.6
Threonine*	74.7 ±0.6	20 ± 3.93
Proline	68.1 ±1.6	14 ± 3.7
Isoleusine*	60.9 ± 0.3	8 ± 2.1
Phenylalanine *	57.1 ± 0.5	13 ± 2.7
Histidine	31.3 ± 0.0	17 ± 7.5
Methionine*	30.5 ± 0.4	45 ± 9.1
Tyrosine*	17.9 ± 0.4	20 ± 7.6
Arginine	9.3 ± 0.1	18 ± 5.4
Cysteine	8.7 ± 0.6	-
Asparagine	-	551 ± 183
Glutamine	-	27 ± 18.5
Total amino acids	**1552.4 ± 5.6**	**1,029.0**

Note: * designates essential amino acid
** Elkins *et al.* (1997)

4.2 Cultivation of *X. dendrorhous* in Traditional YM Medium and Pineapple Juice Base Medium

The results on *X. dendrorhous* cultivation in YM medium are shown in Figure 4.2A. It was found that the biomass production in the YM medium showed a rapid growth during the period of 12-48 hours, with the highest cell concentration of 4.2 g/L at 48 hours of cultivation and then entered the stationary phase from 48 until 144 hours of cultivation. Results found are in good keeping with that of Kongtragool (2006) and Johnson and Lewis (1979) who obtained the highest biomass production of 4.21 g/L after 48 hour post inoculation, in other words, volumetric yield 0.44 g/L. It could also be observed that no lag phase was evident in this cultivation. The astaxanthin accumulation by *X. dendrorhous* cultivated in the YM medium increased rapidly at initial time of cultivation until 72 hours and increased slightly until 144 hours, providing that the highest astaxanthin production was 1.04 mg/L or 278.13 $\mu g/g_{yeast}$. This indicates that astaxanthin formation in *X. dendrorhous* was growth-associated which is of the same pattern reported by Johnson and Lewis (1979). However, astaxanthin accumulation by both *Rhodotorula glutinis* and *Sporoblomyces reseus* (Bobkova, 1965; Vecher and Kulikova, 1968) has been reported to be non growth-associated. Moreover, it was observed that total sugar in YM medium was quickly consumed from initially 10.4 g/L to 1.9 g/L within 24 hours and stabilized until 144 hours. These time courses showed that a higher sugar concentration in the culture medium is favorable for rapid cell growth. In addition, total nitrogen (TKN) slightly decreased at initial time of cultivation and then remained steady during 24-144 hours of cultivation and eventually increased towards the end of cultivation because of cell lysis which corresponded to the dead phase of growth.

Moreover, since this study aimed at selecting the effective low cost medium; therefore, the by product from canned pineapple industries, namely, pineapple juice concentrate, was chosen as base medium for cultivating *X. dendrorhous*. This experiment compares growth and astaxanthin production of *X. dendrorhous* cultivated in pineapple juice concentrate diluted to contain glucose at the final concentration of 10 g/L and the traditional YM medium. Growth and astaxanthin production by *X. dendrorhous* cultivated in pineapple juice base growth medium was presented in Figure 4.2B. It could be observed that the cell growth in the pineapple juice concentrate medium is of a similar trend to that of YM medium. Cells grew rapidly during 12-48 hours, without lag

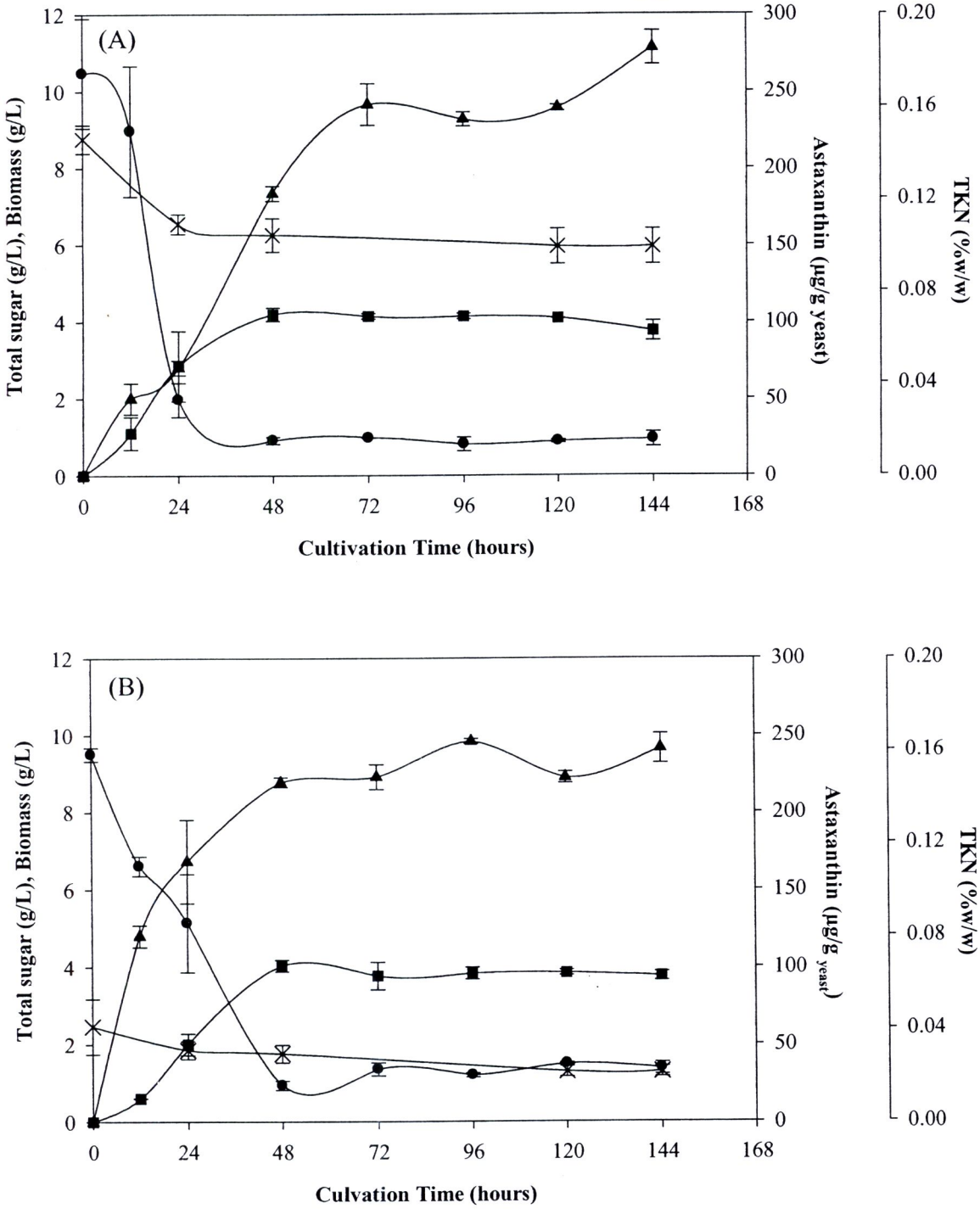


Figure 4.2 Cultivation of *X. dendrorhous* in YM (A) and pineapple juice base medium (B). (●) Total sugar, (■) Cell dry weight, (×) TKN, and (▲) Astaxanthin production

phase and then entered the stationary phase at 48 until 144 hours of cultivation. The highest cell concentration in pineapple juice base growth medium reached was 4.25 g/L at 72 hours of cultivation. However, at initial time of cultivation, growth of *X. dendrorhous* in pineapple juice base growth medium was slower than that observed with YM medium, which may due to the fact that at initial time of cultivation, YM medium contained more readily available nitrogen than pineapple juice base medium. In addition, the astaxanthin production by *X. dendrorhous* increased rapidly until 96 hours and increased slightly until 144 hours, which is almost of similar trend to that of YM medium. Moreover, the highest astaxanthin production was 0.91 mg/L or 241.47 $\mu g/g_{yeast}$ which is slightly different from that obtained with YM medium of which the highest astaxanthin was 278.13 $\mu g/g_{yeast}$, in other words, astaxanthin produced using YM medium was comparable to that of pineapple juice base medium (Figure 4.2). At the beginning of *X. dendrorhous* cultivation, result showed that production of astaxanthin in YM medium is slower than that of pineapple juice medium since at that time nitrogen concentration in YM medium is higher than that of pineapple juice medium (Figure 4.2). According to Vustin *et al.* (2004), abundant nitrogen present in the medium may enhance cell growth but suppress the enzymes involved in converting β-carotene to astaxanthin. Then, *X. dendrorhous* cultivated in pineapple juice produced more astaxanthin initially than that cultivated in YM medium. However, at the end of cultivation, astaxanthin concentration obtained using the two growth media was approximately the same. Furthermore, it was observed that total sugar in pineapple juice was slowly consumed from initially 9.50 g/L to 5.13 g/L within 24 hours and rapidly depleted from 5.13 to 0.93 g/L from 24 to 48 hours post inoculation and remained stable until 144 hours. These time courses showed that sugar present in the culture medium is favorable for rapid cell growth. At the beginning of the cultivation *X. dendrorhous* uptook sugar more slowly when cultivated in pineapple juice base medium leading to low cell concentration in comparison with that obtained with YM medium. This may be due to the fact that YM medium contained solely glucose while many types of sugar, i.e., glucose, sucrose, and fructose, were present in pineapple juice base growth medium. According to An *et al.* (2001), monosaccharide was easier to metabolize by *X. dendrorhous* than that of disaccharide because no hydrolysis was necessary. In addition, total nitrogen (TKN) was slightly decreased at the beginning of cultivation and then reached steady value during 24-144 hours of cultivation which was similar to that observed with YM medium.

4.3 Screening of Factors Affecting Growth of *X. dendrorhous* by The Fractional Factorial Design

4.3.1 Factors Influencing Growth of *X. dendrorhous*

Since several factors, e.g., growth medium compositions, temperature and pH, were reported to greatly influence growth of *X. dendrorhous* (Johnson and Lewis, 1979; Fang and Cheng, 1993; Ramírez *et al.*, 2001), therefore, objective of the present study was to identify parameters significantly affecting growth of *X. dendrorhous* cultivated in pineapple juice concentrate base growth medium.

Table 4.3 provides experimental runs required for 2_{IV}^{6-2} fractional factorial design providing that 3 center points were also included to determine whether curvature is present within the tested range of variables selected. Responses employed in this study were biomass concentration and astaxanthin content attained at day 8 post inoculation.

As can be seen from Table 4.3, the highest cell concentration of 12.35 g/L was obtained (Run 14) where sucrose, $(NH_4)_2SO_4$, and KNO_3 were set at high levels while glucose, *n*-hexadecane, and pH were provided at low levels. Furthermore, for experimental runs 10 and 16, comparable biomass concentration of approximately 11.33 g/L was resulted providing that, for the former, all variables supplemented were at high levels except for $(NH_4)_2SO_4$ and glucose whose concentrations were at low level, however, for the latter, all variables introduced were at high level. Additionally, it should be borne in mind that when all selected parameters were present at low level (Run 1) a slightly higher cell concentration, approximately 6.62 g/L, was obtained in comparison with experimental run 6 where sucrose, KNO_3 and pH were present at high level and, at the same time, glucose, $(NH_4)_2SO_4$ and *n*-hexadecane were supplemented at low concentration, 5.43 g/L cell concentration.

Prior to statistical analysis, normality of data was tested using the Anderson-Darling method and found that data, cell concentration, obtained distributed normally (Figure A1, Appendix) given that the p-value of 0.675 was resulted which is greater than 0.05 indicating insignificance of test. The standard deviation for cell concentration obtained at the center points were less than 5% (2.83%) which is well within acceptable region of experimental error. Subsequently, statistical analysis was conducted using Minitab 14.

Regressive analysis and analysis of variance (ANOVA) results were provided in Table 4.4 and Table 4.5, respectively.

The regressive analysis (Table 4.4) of FFD using biomass production at day 8 post inoculation as response provides coefficients, student *t* distribution, and corresponding p-values along with parameters' estimated effects. In addition, the coefficient of determination (R^2) together with adjusted R^2 obtained for cell concentration was, respectively, 99.77% and 97.91%, indicating great similarity between the observed and predicted values. Further, it is obvious that only 0.23% of the variation occurred during experiment could not sufficiently be explained by the model; therefore, the data were well fitted by model.

Table 4.3 Experimental designs required for 2_{IV}^{6-2} fractional factorial design together with responses in terms of cell and astaxanthin concentrations obtained at day 8 post inoculation.

Runs	A	B	C	D	E	F	Dry Weight (g/L)	Astaxanthin Content (μg/g $_{yeast}$)
1	-1	-1	-1	-1	-1	-1	6.62	896.53
2	1	-1	-1	-1	1	-1	7.76	713.75
3	-1	1	-1	-1	1	1	8.70	359.30
4	1	1	-1	-1	-1	1	9.51	136.12
5	-1	-1	1	-1	1	1	9.22	1,351.49
6	1	-1	1	-1	-1	1	5.43	896.82
7	-1	1	1	-1	-1	-1	6.61	209.22
8	1	1	1	-1	1	-1	8.74	158.39
9	-1	-1	-1	1	-1	1	6.83	686.29
10	1	-1	-1	1	1	1	11.34	641.78
11	-1	1	-1	1	1	-1	8.14	548.52
12	1	1	-1	1	-1	-1	9.96	300.37
13	-1	-1	1	1	1	-1	6.95	802.94
14	1	-1	1	1	-1	-1	12.35	701.17
15	-1	1	1	1	-1	1	7.21	173.29
16	1	1	1	1	1	1	11.33	78.80
17	0	0	0	0	0	0	9.59	302.55
18	0	0	0	0	0	0	9.49	315.27
19	0	0	0	0	0	0	9.09	343.85

As can be observed from the ANOVA table (Table 4.4) sucrose, KNO_3 and *n*-hexadecane were regarded statistically significant and influenced biomass production positively. It should be noted that the model provided by statistical analysis was considered significant for all the terms, i.e., main effects as well as two way and three way interactions (Table 4.5) ($p<0.05$). Moreover, curvature was also significant statistically indicating that concentration of each variable leading to maximum response was included within the chosen range. Consequently, further optimization should lead to conditions where biomass is maximized given that appropriate range of variables is carefully chosen. The result obtained in Table 4.4 demonstrated that sucrose×KNO_3 or its alias, *n*-hexadecane×pH, had a large positive effect on biomass production whose significance was confirmed by ANOVA analysis with corresponding p-value of 0.005. It should be kept in mind that this p-value was the combined contributions by the interactive terms themselves together with their aliases. Therefore, interaction plot could be applied to explain its corresponding significance. As can be seen from Figure 4.3 that the lines of interaction between sucrose and KNO_3 did not overlap (Figure 4.3A) whereas interaction between *n*-hexadecane and pH did meet (Figure 4.3B), therefore, it may be deduced that interaction between *n*-hexadecane and pH was probably more significant than that of sucrose and KNO_3 on biomass production. Further, interaction between glucose×KNO_3 and its alias, $(NH_4)_2SO_4$×pH and sucrose×pH, together with its alias, KNO_3×*n*-hexadecane showed small positive effect on biomass production (Table 4.4) given that these interactive effects were regarded statistically significant with the p-value of 0.037 and 0.045, respectively. Once again, these p-values were derived from a combined effect contributed by the interactive effect shown with alias. Hence, since glucose and KNO_3 showed weak interaction (Figure 4.3C), while, its alias, $(NH_4)_2SO_4$ and pH clearly met (Figure 4.3D), implying that interactive effect between $(NH_4)_2SO_4$ and pH was considered more significant on biomass production than that of glucose and KNO_3. In addition, when interaction between sucrose×pH, and its alias, KNO_3 and *n*-hexadecane were investigated graphically (data not shown). No clear distinctive conclusion could be drawn since, within the tested range, no interaction was observed.

Table 4.4 Regressive analyses of 2_{IV}^{6-2} fractional factorial design (FFD) using cell concentration attained at day 8 post inoculation as response

Term	Effect	Coefficient	*t*	*p*
Constant		8.5433	128.70	0.000
A	2.0159	1.0079	15.18	0.004*
B	0.4636	0.2318	3.49	0.073
C	-0.1266	-0.0633	-0.95	0.441
D	1.4414	0.7207	10.86	0.008*
E	0.9546	0.4773	7.19	0.019*
F	0.3041	0.1521	2.29	0.149
AB	0.2024	0.1012	1.52	0.267
AC	-0.0529	-0.0264	-0.40	0.729
AD	1.9461	0.9731	14.66	0.005*
AE	-0.4756	-0.2378	-3.58	0.070
AF	-0.6031	-0.3016	-4.54	0.045*
BD	-0.6711	-0.3356	-5.06	0.037*
BF	0.5216	0.2608	3.93	0.059
ABD	-1.1959	-0.5979	-9.01	0.012*
ABF	0.8509	0.4254	6.41	0.023*

$S = 0.2655$ $R^2 = 99.77\%$ R^2-(adj) = 97.91%

Note: * designates significance at $p < 0.05$

(Alias pattern: AD/EF, BD/CF, AF/DE, BF/CD, AE/BC, AC/BE, AB/CE)

Table 4.5 Analysis of Variance (ANOVA) for cell concentration by *X. dendrorhous* at 8 day post inoculation

Source	DF	Adj MS	F	*p*
Main Effects	6	4.917	69.75	0.014*
2-Way Interactions	7	2.939	41.69	0.024*
3-Way Interactions	2	4.308	61.11	0.016*
Curvature	1	1.805	25.61	0.037*
Residual Error	2	0.070		
Pure Error	2	0.070		
Total	18			

Note: * designates significance at $p < 0.05$

(A: sucrose, B: glucose, C: KNO_3, D: $(NH_4)_2SO_4$, E: *n*-hexadecane, and F: pH)

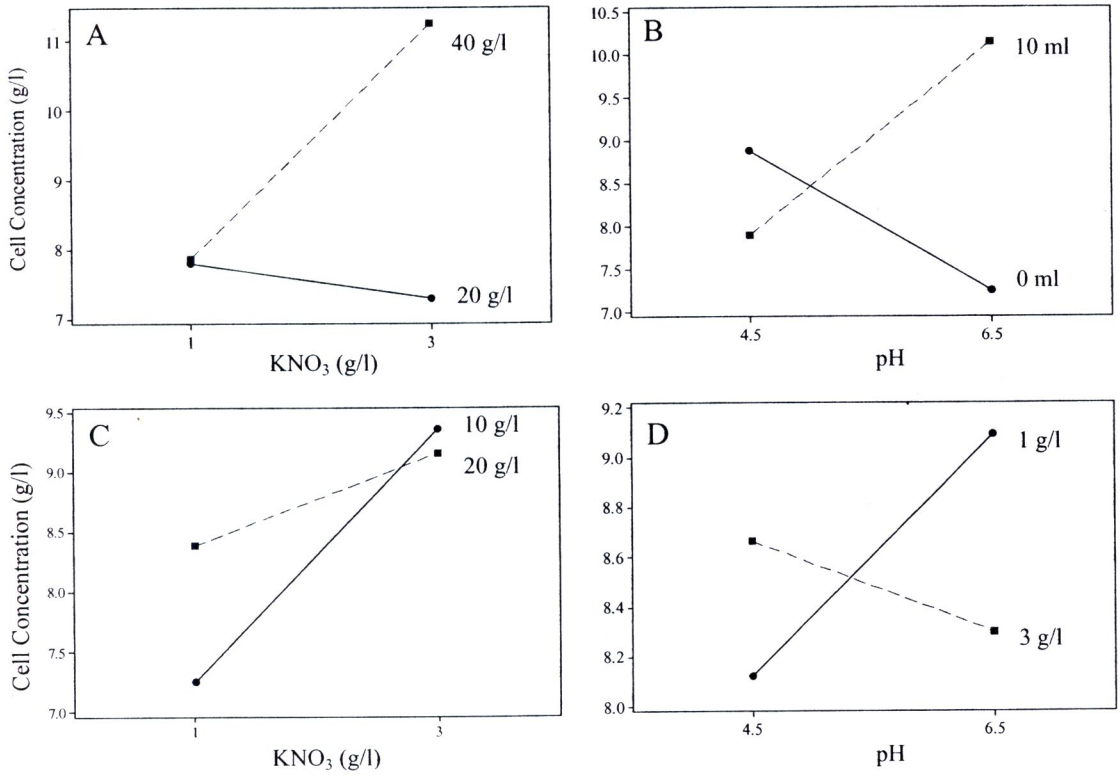


Figure 4.3 The two-way interactive effects between sucrose and KNO_3 (A), and its alias, *n*-hexadecane and pH (B) glucose and KNO_3 (C), and its alias, $(NH_4)_2SO_4$ and pH (D).

4.3.2 Factors Influencing Astaxanthin Production by *X. dendrorhous*

As is evident from Table 4.3, the highest astaxanthin concentration was obtained (1,351.49 $\mu g/g_{yeast}$) when glucose and sucrose were supplemented at low level whereas *n*-hexadecane was set at high level (Run 5). Furthermore, it is worthy to note that during cultivation of *X. dendrorhous* when glucose was at low level high astaxanthin concentration was resulted (Table 4.3). Moreover, when both glucose and sucrose were supplemented at low level astaxanthin produced was higher than those obtained when glucose and sucrose were supplemented at low and high level, respectively. Additionally, when glucose was present at high level during *X. dendrorhous* cultivation low pigment production was noted. Further, when all factors were at high levels low astaxanthin production, only 78.80 $\mu g/g_{yeast}$, was resulted (Run 16).

According to the Anderson-Darling test of normality, it was found that the data distributed normally ($p \approx 0.138$). Moreover, the standard deviation at the center points was only 6.59%, which is fairly acceptable (Figure A2, Appendix). The regressive analyses of FFD using astaxanthin content as response were given in Figure 4.4. The coefficient of determination (R^2) and adjusted R^2 for astaxanthin production by *X. dendrorhous* were 99.96 and 99.61%, respectively, indicating great similarity between the observed and predicted values of the response. It should, therefore, be noted that only 0.04 % of the variation could not be explained by the model. Therefore, it was concluded that the data were fitted well by model. As can be seen from pareto chart (Figure 4.4) that for main effect, glucose, sucrose, and KNO_3 were considered statistically significant ($p<0.05$) and, at the same time, influenced astaxanthin production negatively, while *n*-hexadecane which posed positive influence on astaxanthin accumulation also considered significant statistically at the confidence interval of 95%.

The pareto plot (Figure 4.4) showed further that sucrose×*n*-hexadecane, and its alias, glucose×$(NH_4)_2SO_4$ and glucose×pH, together with its alias, $(NH_4)_2SO_4$×KNO_3, had a small negative effect on astaxanthin production; whose significance was confirmed by ANOVA analysis with corresponding p-value of 0.003 and 0.008, respectively. Nevertheless, as mentioned previously, these p-values were the combined contributions of the interactive terms themselves together with their aliases. Therefore, interaction plot could be employed to elucidate the corresponding significance. As can be observed from Figure 4.5 that the lines representing interaction between glucose and $(NH_4)_2SO_4$ did not intersect (Figure 4.5B) whereas interaction between sucrose and *n*-hexadecane did meet (Figure 4.5A), it may, therefore, be deduced that interaction between sucrose and *n*-hexadecane was probably more significance than that of between glucose and $(NH_4)_2SO_4$ on astaxanthin production. Additionally, by the same token, interactive effect stemmed from glucose and pH (Figure 4.5E) may be considered more significance than that of $(NH_4)_2SO_4$×KNO_3 (Figure 4.5F). Further, interactions between glucose×KNO_3, and its alias, $(NH_4)_2SO_4$×pH, and sucrose×KNO_3, and its alias, *n*-hexadecane×pH, showed small positive effect on astaxanthin production (Figure 4.5) providing that these interactive effects were considered statistically significant with the p-value of 0.004 and 0.038, respectively. Yet again, these p-values were calculated by combined effect contributed by the interactive effect shown with its alias. Hence, since

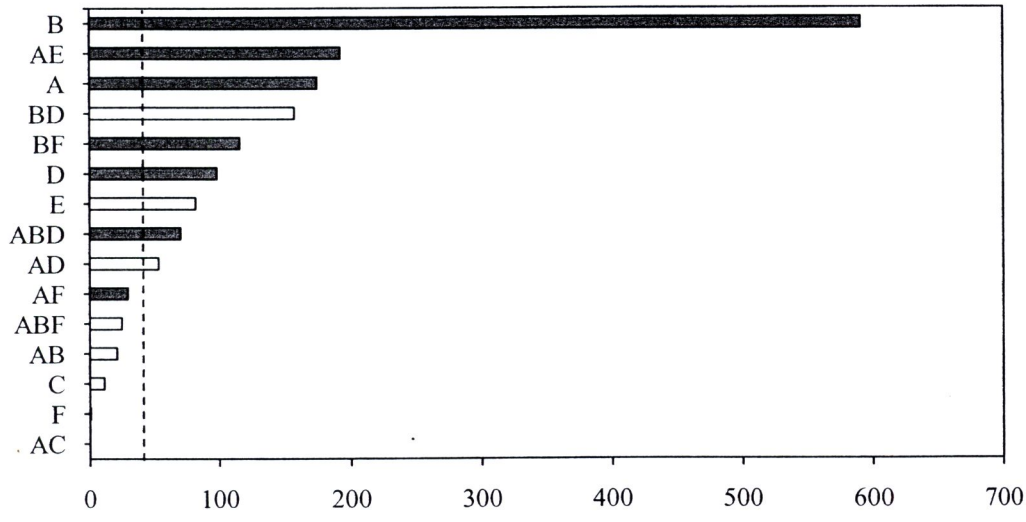


Figure 4.4 Pareto charts depicting the influence of sucrose (A), glucose (B), $(NH_4)_2SO_4$ (C), KNO_3 (D), *n*-hexadecane (E), and pH (F) on astaxanthin production by *X. dendrorhous*. Open bars represent positive effect, while close bars represent negative effect. The dot line indicates significance at 95%. (Alias pattern AD/EF, BD/CF, AF/DE, BF/CD, AE/BC, AC/BE, AB/CE)

Table 4.6 Analysis of Variance (ANOVA) for astaxanthin production by *X. dendrorhous* at 8 day of cultivation

Source	DF	Adj MS	F	p
Main Effects	6	264190	590.52	0.002*
2-Way Interactions	7	45480	101.66	0.010*
3-Way Interactions	2	10993	24.57	0.039*
Curvature	1	122682	274.22	0.004*
Residual Error	2	447		
Pure Error	2	447		
Total	18			

Note: * designates significance at $p < 0.05$

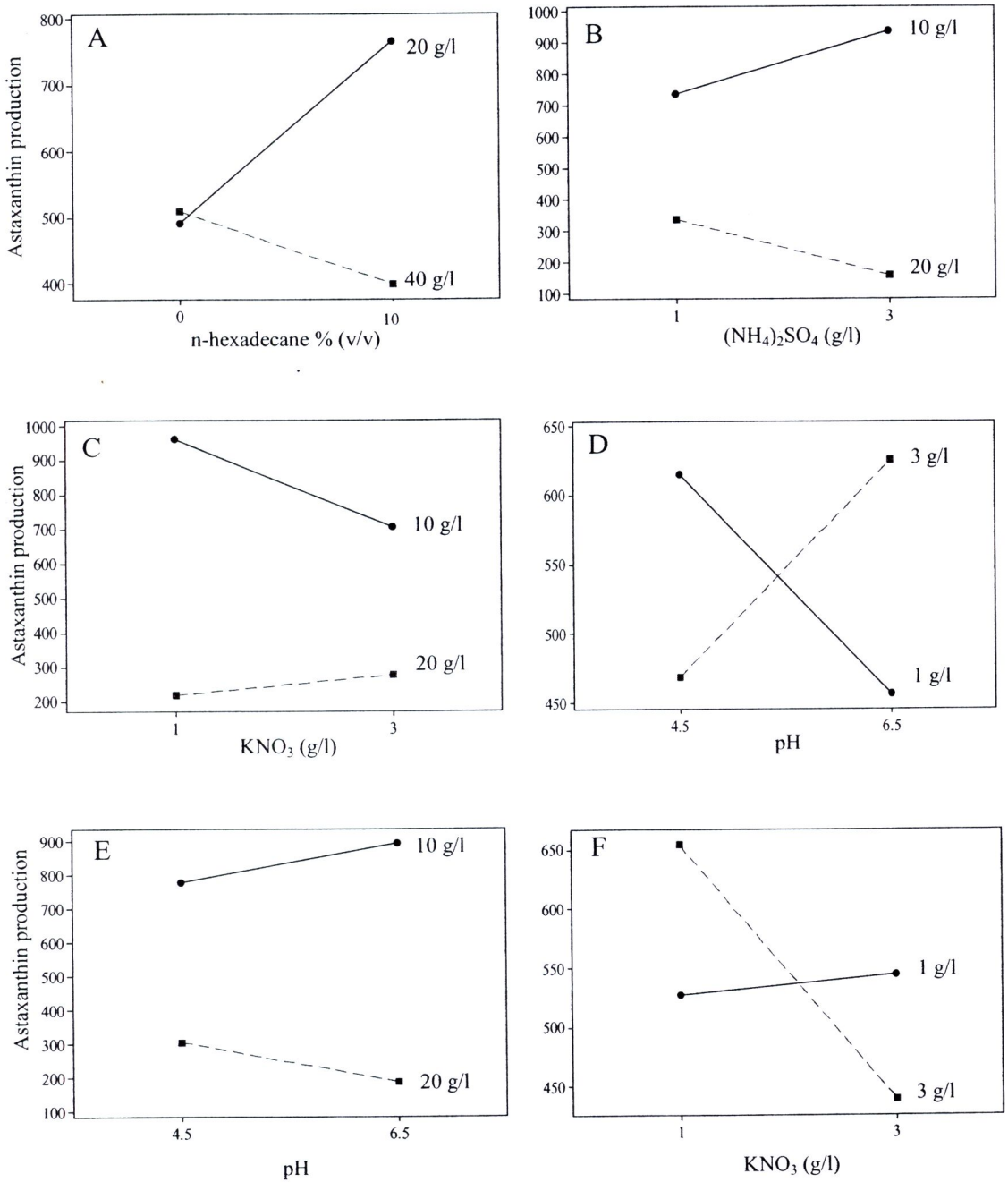


Figure 4.5 The effects of two-way interactions between sucrose and *n*-hexadacane (A), and its alias, glucose and $(NH_4)_2SO_4$ (B); glucose and KNO_3 (C), and its alias, $(NH_4)_2SO_4$ and pH (D); glucose and pH (E), and its alias, $(NH_4)_2SO_4$ and KNO_3 (F) on astaxanthin accumulation by *X. dendrorhous*

glucose and KNO_3 showed no interaction (Figure 4.5C), while, its alias, $(NH_4)_2SO_4$ and pH clearly met (Figure 4.5D) implying that interactive effect between $(NH_4)_2SO_4$ and pH was more significance on astaxanthin production than that of glucose and KNO_3. In addition, when interaction between sucrose×KNO_3, and its alias, *n*-hexadecane×pH were investigated graphically (data not shown) no clear distinctive conclusion could be drawn since, within the tested range, no interaction was observed.

It could be observed further that all variables (main effects) chosen affected astaxanthin production negatively except *n*-hexadecane ($p \approx 0.016$) whose presence appeared to favor astaxanthin production by *X. dendrorhous*. Results further showed that the model provided by statistical analysis was of significant meaning since all the terms, main effects, two way and three way interaction (Table 4.6) were considered statistically significant ($p<0.05$) providing that curvature was also evident ($p<0.05$) within the tested range of variables chosen, in other words, concentration of each variable present together leading to maximum astaxanthin was included. Therefore, further optimization should lead to conditions where astaxanthin accumulation is maximized given that appropriate range of variables is carefully selected.

In general, screening tests are employed to select the important main effects from less important ones (Nair *et al.*, 2008). In this study, the two level fractional factorial (2_{IV}^{6-2}) design was chosen to determine the influence of six operating parameters (i.e., sucrose, glucose, $(NH_4)_2SO_4$, KNO_3, *n*-hexadecane, and pH). Sucrose, potassium nitrate, and *n*-hexadecane have been identified as the significant factors affecting both cell growths and astaxanthin production by *X. dendrorhous* in shake-flask cultures. It should be noted that effect of the two main nutrients, namely, sucrose and KNO_3, on growth and astaxanthin production were contradictory in that, for better biomass production, relatively high concentrations of both sucrose and KNO_3 should be provided while, for greater astaxanthin biosynthesis, much lower concentrations of sucrose and KNO_3 should be supplemented. Results found were in good agreement with those reported in literature (An *et al.*, 1989; Haard, 1988; Kesava *et al.*, 1998; Hayman *et al.*, 1995).

Fang and Cheng (1993) reported that sucrose supplemention at 10 g/L promoted high cell growth (4.585 g/L) of *P. rhodozyma* mutant, whereas Johnson and Lewis (1979) demonstrated that cultivation of *P. rhodozyma* with carbon source of 200 mg in 50 mL

0.1 M phthalate-buffered yeast nitrogen base medium supplemented with 0.6% (w/v) Bacto-peptone and 4 g/L final sugar concentration yielded 3.72 and 3.85 g/L biomass production using sucrose and glucose, respectively. It was further found that sucrose and glucose were of comparable efficiency in promoting cell proliferation. In addition, several studies have illustrated that growth medium containing sucrose could sufficiently and effectively promote growth and pigment production by the yeast *X. dendrorhous* such as sugarcane (Fontana *et al.*, 1996), pineapple juice extract (Jirasripongpun *et al.*, 2008), peat hydrolysates (Martin *et al.*, 1993), grape juice (Meyer and du Preez, 1994), date juice (Ramírez *et al.*, 2000), Molasses, (An *et al.*, 2001 and Haard, 1988). The basidiomycetous yeast *X. dendrorhous* is known to produce an invertase (Killian *et al.*, 1996), an enzyme responsible for hydrolyzing sucrose into glucose and fructose which could be utilized for either growth or astaxanthin production. Moreover, transfructosylation activity of invertase involves in transferring a fructose moiety to sucrose molecule resulting in neokestose, a prebiotic, which, in term, could promote growth of *X. dendrorhous* (Killian *et al.*, 1996; Kritzinger *et al.*, 2003). Therefore, since pineapple juice concentrate contained high sucrose and glucose as well as fructose concentration, high biomass as well as astaxanthin production may be anticipated providing that sucrose in this study were considered statistically significant ($p<0.05$) for both growth (Table 4.4) and astaxanthin biosynthesis (Figure 4.5). Moreover, interaction between sucrose and *n*-hexadecane, an oxygen vector, was also considered significant statistically ($p<0.05$) for astaxanthin biosynthesis (Figure 4.5B). Thus, cultivating *X. dendrorhous* in the medium containing low sucrose concentration with high oxygen vector concentration may lead to high astaxanthin production due to an increase in dissolved oxygen available in the system (Yamane *et al.*, 1997b, Liu and Wu, 2006).

Glucose is generally used in microbial medium cultivation. However, *X. dendrorhous* has been demonstrated to suffer glucose or catabolite repression, in other words, crabtree effect, (Ramírez *et al.*, 2001) when glucose concentration within the growth medium exceeded 19 g/L. Further, when glucose is present at high concentration, fermentation sets in leading to a marked decrease in the astaxanthin biosynthesis and increase in the accumulation of ethanol and organic acids (Walker, 1998; Meyer and Du Preez, 1994). Even though glucose has not been found to be statistically significant on growth ($p>0.05$, Table 4.4), however, high glucose concentration largely affected

pigment biosynthesis. Therefore, glucose would be maintained at low concentration in the future experiment.

Fang and Cheng (1993) reported that inorganic nitrogen source highly supported biomass production rather than organic nitrogen source. Nitrogen source, particularly potassium nitrate, was found to significantly affect the growth and carotenoid production of the yeast *X. dendrorhous* (Table 4.4, Figure 4.4). This is in good agreement with several reports available in literature (Fang and Cheng, 1993; Parajó *et al.*, 1998; Ni *et al.*, 2007). Parajó *et al.* (1998) found that when potassium nitrate was utilized at low concentration, approximately 0.1-0.2 g/L, as sole nitrogen source for *X. dendrorhous* cultivation, high astaxanthin concentration could be achieved. In addition, when ammonium phosphate was used as sole nitrogen source, majority of carotenoids accumulated by *X. dendrorhous* was astaxanthin, however, at lower concentration in comparison with that obtained with organic nitrogen source, whose, mechanism is still unknown, nevertheless. Since KNO_3 showed large positive effect on growth, however, small negative effect on astaxanthin production, therefore, a rather high concentration of KNO_3, approximately 2 g/L, was adopted for further optimization study given the fact that center point of fractional factorial design where KNO_3 concentration was 2 g/L yielded high biomass production. Additionally, Parajó *et al.* (1998) reported that too high concentration of KNO_3 (4-5 g/L) supplemented resulted in a decline in biomass production. Therefore, for KNO_3, concentration ranging from 0.1-2.0 g/L was adopted for further optimization study.

In this study, *n*-hexadecane was found to significantly affect growth and astaxanthin production by *X. dendrorhous* which is in accordance with Liu and Wu (2006) who found that supplementation of 9% (v/v) *n*-hexadecane into growth medium led to an increase in carotenoid yield by 58% and the oxygen transfer rate by 90%. It has also been reported that high oxygen supply was advantageous to increasing astaxanthin biosynthesis and biomass production (Wang and Yu, 2009; Liu and Wu, 2006).

It has been demonstrated that pH plays a significant role on growth and astaxanthin production by *X. dendrorhous* (Johnson and Lewis, 1979; Fang and Cheng, 1993; Meyer and du Preez, 1994; An *et al.*, 1996; Ramírez *et al.*, 2001). However, pH as main effect was not considered significant statistically in this study ($p>0.05$, Table 4.4, Figure 4.4). Nevertheless, interaction effect, pH×hexadecane and pH×$(NH_4)_2SO_4$ (Figure 4.5B

and Figure 4.5D) were considered statistically significant on biomass production whereas pH×$(NH_4)_2SO_4$ (Figure 4.5D) was found to be important on astaxanthin biosynthesis, therefore, pH was maintained at 5.5 which are in good keeping with several reports available in literature (Johnson and Lewis, 1979; Fang and Cheng, 1993; Meyer and du Preez, 1994; An *et al.*, 1989).

As a consequence, since sucrose, potassium nitrate, and *n*-hexadecane were considered to significantly influence growth as well as astaxanthin production, their corresponding optimal concentrations were investigated further using Response Surface Methodology, namely, Doehlert design.

4.4 Optimization of Growth using Doehlert Design

To optimize biomass production, a response surface methodology (RSM) was employed in shake flask cultures using Doehlert design. Sucrose, KNO_3 and *n*-hexadecane (obtained from screening experiments, so-called FFD) were adopted to optimize cell growth and astaxanthin production by *X.* dendrorhous, due to their strong effects according to FFD. KNO_3 and sucrose were investigated at five levels in order to obtain most information of the system whereas *n*-hexadecane, the less importance, was studied at only three levels (Table 3.3) together with three replicates at center point (Table 3.4). Typically, the application of center points are necessary to estimate pure error in experiments (Myers and Montgomery, 2002). The experimental design of the Doehlert design with triangular face plane projection was chosen (Figure 3.1) and corresponding experimental results are given in Table 4.7. In this experiment, glucose concentration and pH were maintained at 10 g/L and 5.5, respectively, because high value of astaxanthin production could be achieved under low glucose concentration as can be seen in Pareto chart (Figure 4.4) whereas the pH at the center points also led to high dry cell weight and astaxanthin biosynthesis (Table 4.3).

As can be seen from Table 4.7, the highest biomass production of 13.28 g/L was attained when KNO_3 was supplemented at high level (+1) given that sucrose together with *n*-hexadecane were set at central level (0) (Run 2).

Table 4.7 Experimental designs and corresponding responses in terms of dry cell weight and astaxanthin accumulation obtained at day 8 post inoculation

Run No.	KNO_3	Sucrose	Hexadecane	Dry cell weight (g/L)	Astaxanthin ($\mu g/g_{yeast}$)
1	0	-1	0	12.42	643.30
2	1	0	0	13.28	718.93
3	0	1	0	13.14	654.47
4	-1	0	0	12.52	649.87
5	-0.5	-0.5	0.707	12.19	675.09
6	0.5	-0.5	0.707	12.47	721.81
7	0.5	0.5	0.707	12.74	778.29
8	-0.5	0.5	0.707	12.78	753.32
9	-0.5	-0.5	-0.707	12.56	744.58
10	0.5	-0.5	-0.707	12.20	800.32
11	0.5	0.5	-0.707	12.61	724.10
12	-0.5	0.5	-0.707	12.80	733.02
13	0	0	0	13.12	769.59
14	0	0	0	13.12	777.82
15	0	0	0	13.11	733.97

The experimental results (Table 4.7) obtained from RSM were subjected to statistical analysis and results were provided, respectively, in Table 4.8 and Table 4.9 for regressive analysis and analysis of variance (ANOVA). ANOVA results (Table 4.9) revealed that the obtained model well fitted to the experimental data because normality of data tested using the Anderson-Darling indicated normal distribution (Figure A3, Appendix) given that the p-value of 0.317 was resulted representing insignificance of analysis. The standard deviation for biomass production achieved at the center points were 0.041%, which is well within acceptable region of experimental error, less than 5%. Additionally, the coefficient of determination (R^2) for dry cell weight was 86.3% indicating that only 14.7 % of the total variation could not be explained by the model; therefore, the data were well fitted by model. The Student's *t* distribution and the corresponding p-value, along with the parameter's estimate, are given in Table 4.8. The p-values are employed as tool to ensure the significance of each of the coefficients, which, in turn, are necessary to understand the pattern of the mutual interactions

Table 4.8 Regressive analysis on biomass production by Response Surface Methodology using Doehlert design

Variables	Coefficient	SE Coefficient	*t*	p
Constant	13.1167	0.1264	103.763	0.000*
X	0.1512	0.1095	1.382	0.226
Y	0.3688	0.1095	3.368	0.020*
Z	0.0018	0.1095	0.016	0.988
X*X	-0.2167	0.1999	-1.084	0.328
Y*Y	-0.3367	0.1999	-1.684	0.153
Z*Z	-0.8694	0.2280	-3.814	0.012*
X*Y	-0.0750	0.3096	-0.242	0.818
X*Z	0.2793	0.2190	1.276	0.258
Y*Z	0.0743	0.2190	0.339	0.748

$S = 0.2189$ $R^2 = 86.3\%$ $R^2(\text{adj}) = 61.6\%$
Note: * designates significance at $p < 0.05$

Table 4.9 Analysis of Variance (ANOVA) for biomass production by *X. dendrorhous* at 8 day post inoculation

Source	DF	Seq SS	Adj SS	Adj MS	*F*	p
Regression	9	1.5098	1.5098	0.1678	3.50	0.091
Linear	3	0.6354	0.6354	0.2118	4.42	0.072
Square	3	0.7880	0.7880	0.2627	5.48	0.049*
Interaction	3	0.0863	0.0863	0.0288	0.60	0.642
Residual Error	5	0.2397	0.2397	0.0479		
Lack-of-Fit	3	0.2396	0.2396	0.0799	2396.25	0.000*
Pure Error	2	0.0001	0.0001	0.0000		
Total	14	1.7495				

Note: * designates significance at $p < 0.05$

(X: KNO_3, Y: Sucrose, and Z: *n*-hexadecane)

between the variables. The smaller the p-values, the more significant the corresponding coefficient (Li *et al.*, 2007). This implied that the main effects, sucrose and the interaction effect, *n*-hexadecane×*n*-hexadecane, are highly significant as is evident from their p-values (Table 4.8).

In order to visually observed the influence of the three chosen parameters (KNO_3, sucrose and *n*-hexadecane) on biomass production, the quadratic model was represented graphically as contour plots and response surface plots (Figure 4.6 - Figure 4.8). Such graphical tools provide useful information on the process conditions necessary to achieve the desired value of response. Figure 4.6 shows that biomass was enhanced with increasing sucrose and KNO_3 level up to around 36 and 0.6 g/L, respectively. Consequently, Figure 4.7 exhibits the response arising from the interaction between KNO_3 and *n*-hexadecane providing that dry cell weight increased by increasing KNO_3 concentration up to around 0.8 g/L while *n*- hexadecane was kept at approximately the central level (7.5-9.5 % v/v). Additionally, a further increase in sucrose above 34 g/L together with *n*-hexadecane at central level (7.5-9.0 % v/v) resulted in a rise in growth of *X. dendrorhous* as shown in Figure 4.8E. Further, Figures 4.6B, 4.7D and 4.8F shows response surfaces established using the quadratic model (Equation 4.1) which indicated that there were regions where the response could be maximized, more than 13 g/L of dry cell weight.

By applying multiple regression analysis on the experimental data accomplished using Minitab 14, the following second-order polynomial equation was found to satisfactory explain the biomass production regardless of the significance of coefficients (Table 4.8):

$$\text{Dry cell weight} = 13.1167 + 0.1512X + 0.3688Y + 0.0018Z - 0.2167X^2 - 0.3367Y^2 - 0.8694Z^2 - 0.0750XY + 0.2793XZ + 0.0743YZ$$

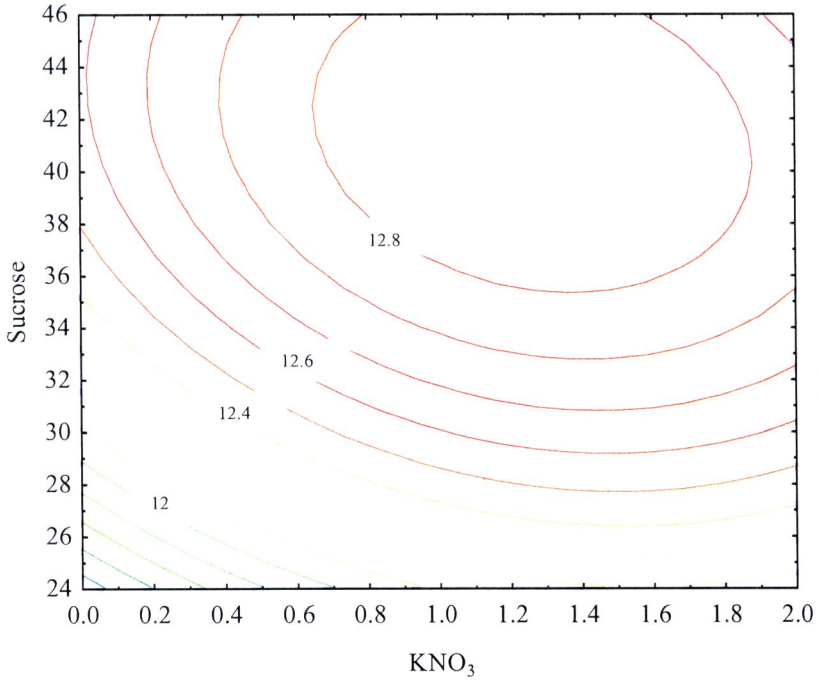
(Equation 4.1)

where X, Y, and Z are the concentration of KNO_3, sucrose, and *n*-hexadecane, respectively. In this case, the Lagrange's criteria were applied to characterize the

response function obtained in order to determine the nature of the stationary point of a function. Mathematical manipulation of Equation 4.1 according to Lagrange's criteria yielded Δ_1, Δ_2, and Δ_3 of -0.4334, 0.2862, and -0.4459, respectively. The values of $\Delta_1<0$, $\Delta_2>0$, and $\Delta_3<0$ demonstrated that there is a maximum point located within the chosen range of variables included in this study. Maximum biomass production of 13.24 g/L could be anticipated according to Equation 4.1 at the concentration of KNO_3, sucrose and *n*-hexadecane of 0.305, 0.522 and 0.072, respectively, which correspond to actual value of 1.23, 40.2 g/L and 8.2 % (v/v), respectively.

The red yeast *X. dendrorhous* appears to be the best candidate for producing a natural astaxanthin of all the strains reported so far, due to its rapid heterotropic metabolism and production of high cell densities in bioreactor (Johnson, 2003). RSM is generally employed to optimize parameters in order to increase in dry cell weight and astaxanthin production of *X. dendrorhous*. Doehlert design was chosen in order to optimize selected parameters in this study, i.e., Sucrose, KNO_3 and *n*-hexadecane, since less experimental run are required while the two more significant parameters were studied at five levels and the less important variable was studied at only three levels.

In this study, sucrose was adopted as carbon source due to its low price and abundantly present in pineapple juice concentrate. The results showed that high dry cell weight could be achieved when sucrose was supplemented above 36 g/L (Table 4.6A and Figure 4.8E). *X. dendrorhous* being specific carotenogenic yeast are capable of utilizing sucrose and, at the same time, synthesized probiotic, neokestose (Kritzinger *et al.*, 2003). According to Kongtragool (2006) who cultivated *P. rhodozyma* using 36.64 g/L sucrose together with 11.98 g/L fish soluble and found that 11.45 g/L dry cell weight could be anticipated. Moreover, it has been reported that growth medium supplemented with sucrose at high concentration (49 g/L), approximately 9.6 g/L biomass production was attained (Haard, 1988).



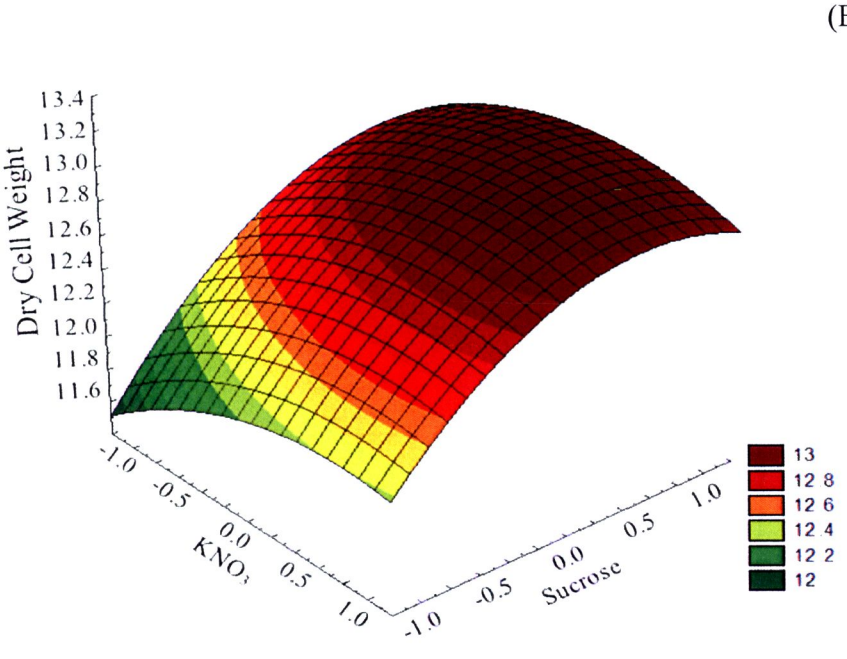
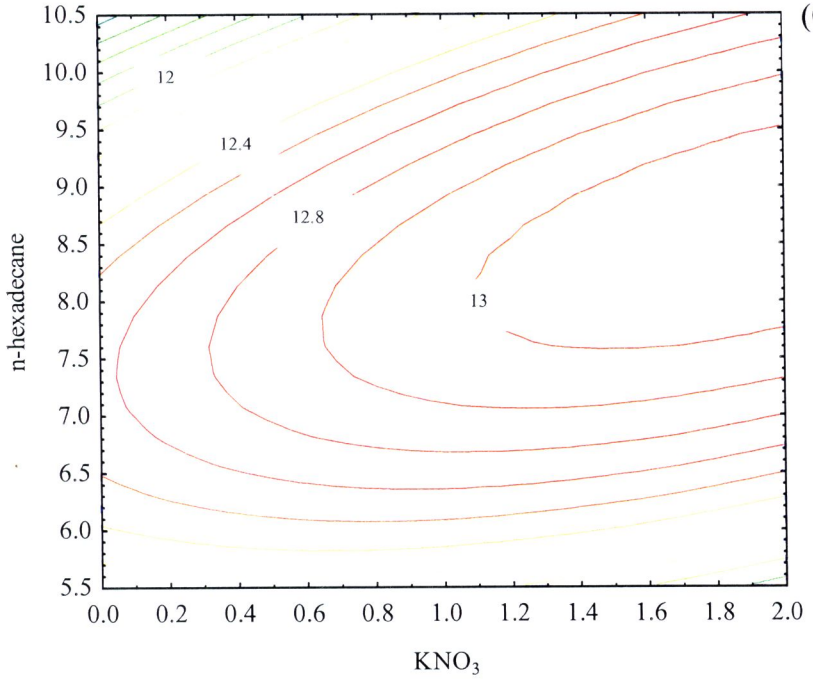


Figure 4.6 Contour (A) and surface plots (B) representing influence of KNO_3 and sucrose on biomass production



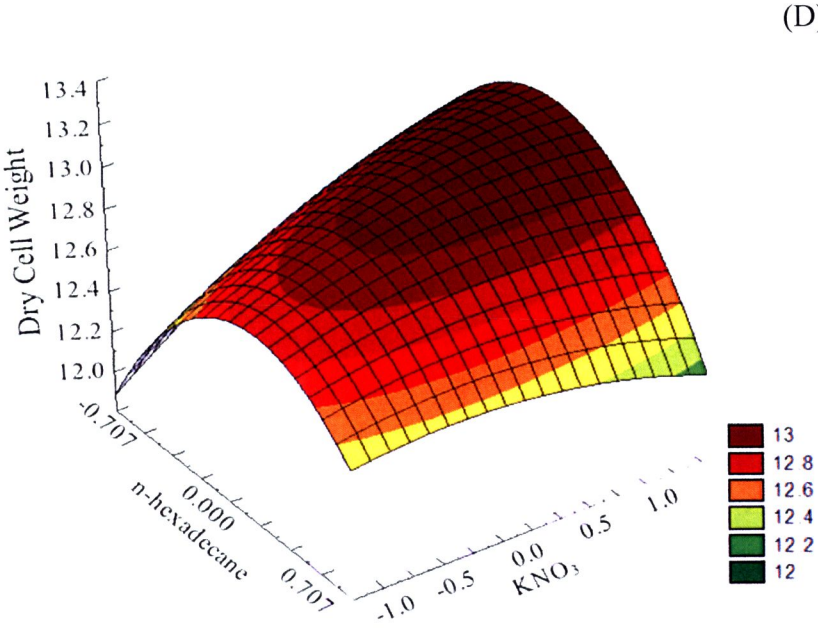
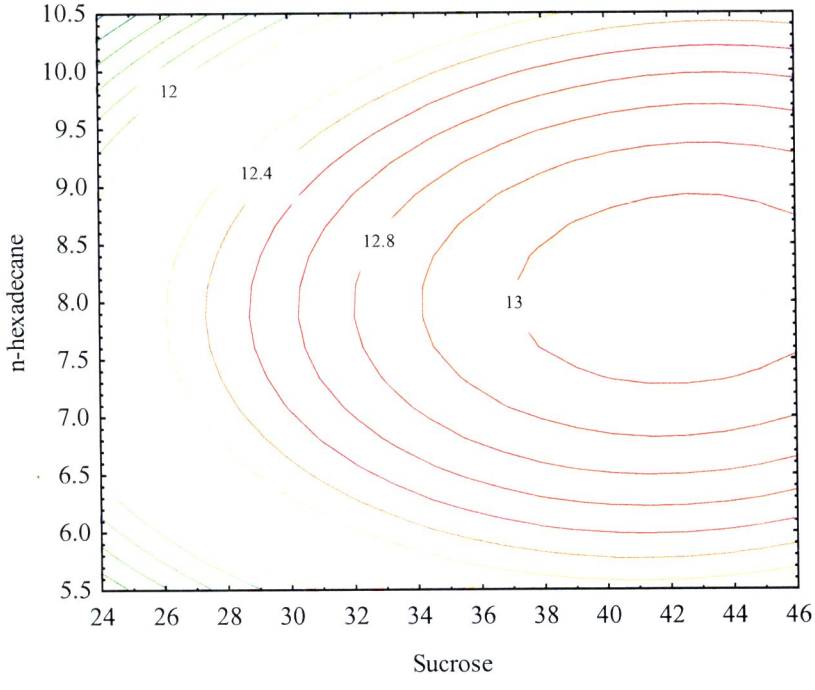


Figure 4.7 Contour (C) and surface plots (D) depicting influence of KNO_3 and *n*-hexadecane on biomass production



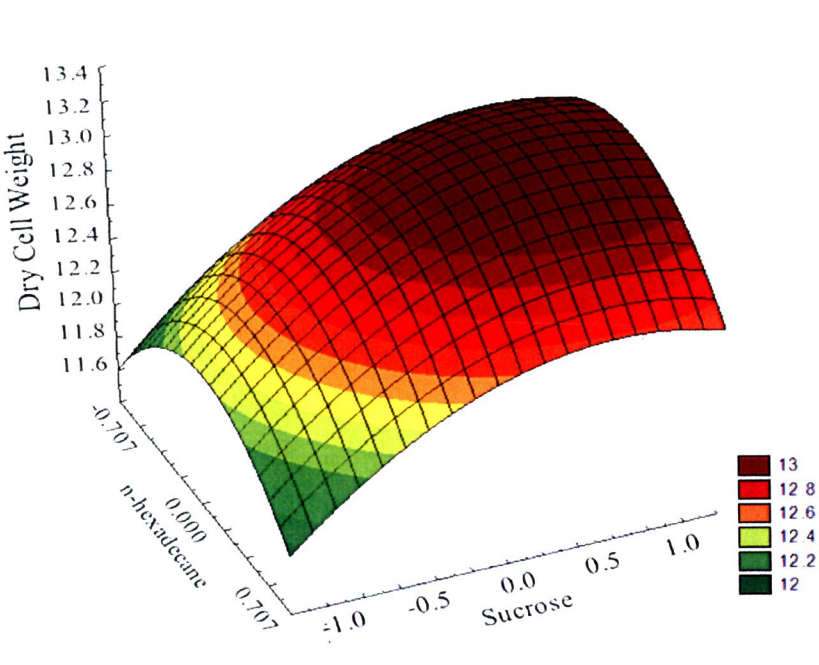


Figure 4.8 Contour (E) and surface plots (F) demonstrating influence of sucrose and *n*-hexadecane on biomass production

This study showed that 40.2 g/L sucrose concentration resulted in high biomass production (13.24 g/L) which are in good agreement with that reported by Park *et al.*, (2008) who found that sucrose was an excellent carbon source for growth and astaxanthin production by *X. dendrorhous* with which the biomass achieved was 1.3-folds higher than that obtained with glucose and fructose. Additionally, results further demonstrated that the suppression of growth was noted when glucose concentration were present above 19 g/L (Johnson and Lewis, 1979) which does not occur with sucrose. Therefore, it may be concluded that sucrose is more favorable carbon source when cultivation using high sugar concentration is of interest, in other words, no catabolite repression.

KNO_3 is an inorganic nitrogen, which can be easily utilized by *P. rhodozyma* in comparison with organic nitrogen (Fang and Cheng, 1993) and could significant increase in total carotenoid production when supplented at appropriate concentration (Cruz and Parajó, 1998; Fang and Cheng, 1993). As can be seen from Figure 4.6 and Figure 4.7, the highest biomass production was obtained when KNO_3 was supplemented at the concentration higher than 0.8 g/L. Results showed further that higher level of KNO_3 could support better growth of *X. dedrorhous*. For instance, 13.24 g/L biomass production was resulted when 1.23 g/L KNO_3 was supplied. In contrast, Parajó *et al.*, (1998) reported that the biomass production steadily decreased as the higher level concentrations of nitrogen sources was supplemented providing that 5 g/L KNO_3 supplementation decrease biomass production.

It has also been reported that oxygen supply was a critical parameter affecting the growth and carotenoid accumulation by *X. dendrorhous*. Cultivation of the red yeast at low oxygen condition led to reduced growth due to the oxygen limitation in the broth (Wang and Yu, 2009). Therefore, this study chose oxygen vector, namely, *n*-hexadecane, as a mean to supply oxygen into the medium. The outcome was that the higher the *n*-hexadecane volume supplemented into the medium, approximately, 7.0-9.5 % (v/v), the higher the biomass produced. Concentration of *n*-hexadecane lower than 7.0 % (v/v) and higher than 9.5 % (v/v) caused limitation of growth. Further, it was found in this study that the optimum concentration of 8.2 % (v/v) *n*-hexadecane was established which is in good agreement with that reported by Liu and Wu (2006) who found that supplementation of *n*-hexadecane at 9% was optimal.

In summary, the base growth medium, pineapple juice concentrate diluted to contain 10 g/L final glucose concentration, supplemented with KNO_3, sucrose, and *n*-hexadecane at the concentration of 1.23, 40.2 g/l and 8.2 % (v/v), respectively, led the maximum biomass production of 13.24 g/l. Since objectives of this study were to enhance both biomass and astaxanthin production, therefore, factor affecting astaxanthin production was investigated further using Fractional Factorial Design while optimal concentration of selected factors was also elaborated further using Response Surface Methodology, the so-called Doehlert Design, once again.

4.5 Screening of Factors Affecting Astaxanthin Biosynthesis of *X. dendrorhous* by The Fractional Factorial Design

As is evident in literature that medium supplementation, in other words, chemical inducer, may enhance astaxanthin production (Gu *et al.*, 1997; Johnson, 2003; Flores-Cotera *et al.*, 2001; An, 2001; Wang *et al.*, 1999). Therefore, effectiveness of supplements such as ethanol, pyruvate, metal ions, and surfactant on improving astaxanthin accumulation by *X. dendrorhous* was investigated. Table 4.10 provides experimental runs required for 2^{4-1}_{IV} fractional factorial design together with three additional center points employed in this study. Responses chosen in this study were biomass concentration and astaxanthin content attained at day 10 post inoculation.

It is evident from Table 4.10 that the highest astaxanthin biosynthesis of 1068.94 $\mu g/g_{yeast}$ was obtained in Run 2 where ethanol and pyruvate were set at high level while $ZnCl_2$ and Tween20 were provided at low level. Furthermore, as can be observed that when high level of pyruvate was presented (Run 2, 4, 6, and 8) higher astaxanthin production was obtained in comparison with experimental runs 1, 3, 5, and 7 where low level of pyruvate was provided. Additionally, supplementation of Tween20 at low level (Runs 1, 2, 3, and 4) led to higher astaxanthin production than those supplemented with high level of Tween20 (runs 1, 3, 5, and 7). These results were confirmed by coefficient of regression equation (Table 4.11).

As is evident from statistical analysis, normality of data was tested using the Anderson-Darling method and found that the data of astaxanthin production obtained distributed normally (Figure A4, Appendix) along with the p-value of 0.719 which is greater than 0.05 indicating insignificance of test. The standard deviation for astaxanthin

biosynthesis attained at the center points were less than 5 % (1.98 %) which is well agreeable with range of experimental error acceptable. Additionally, regressive analysis and analysis of variance (ANOVA) results were provided in Table 4.11 and Table 4.12, respectively.

The regressive analysis (Table 4.11) of FFD using astaxanthin biosynthesis obtained at day 10 post inoculation as response provides coefficients, student's *t* distribution, and corresponding p-values along with parameters' estimated effects. In addition, the coefficient of determination (R^2) together with adjusted R^2 obtained for astaxanthin accumulation was 98.04 and 93.48 %, respectively, representing great similarity between the observed and predicted values. Further, it is obvious that only 1.96 % of the variation occurred during experiment could not sufficiently be explained by the model; therefore, the data were well fitted by model.

Table 4.10 Experimental designs required for 2_{IV}^{4-1} fractional factorial design together with responses in terms of astaxanthin concentrations obtained at day 10 post inoculation

Run No.	A	B	C	D	Astaxanthin (μg/g $_{yeast}$)	Astaxanthin (mg/L)
1	-1	-1	-1	-1	886.41	11.11
2	1	-1	-1	1	1068.94	12.46
3	-1	1	-1	1	946.71	11.11
4	1	1	-1	-1	978.99	10.32
5	-1	-1	1	1	778.17	9.05
6	1	-1	1	-1	772.47	7.54
7	-1	1	1	-1	606.72	6.67
8	1	1	1	1	725.69	7.62
9	0	0	0	0	823.35	9.76
10	0	0	0	0	815.29	9.68
11	0	0	0	0	792.43	8.97

Table 4.11 Regressive analyses 2^{4-1}_{IV} fractional factorial design (FFD) using astaxanthin production attained at day 10 post inoculation as response

Term	Effect	Coefficient	*t*	P
Constant		835.9	84.74	0.000*
A	82.0	41.0	3.55	0.038*
B	-62.0	-31.0	-2.68	0.075
C	-249.5	-124.8	-10.78	0.002*
D	68.7	34.4	2.97	0.059
AB	-6.4	-3.2	-0.28	0.800
AC	-25.4	-12.7	-1.10	0.353
AD	-47.1	-23.6	-2.04	0.134

$S = 32.7165$ $R^2 = 98.04\%$ $R^2(\text{adj}) = 93.48\%$
Note: * designates significance at $p<0.05$
(Alias pattern: AB/CD, AC/BD, AD/BC)

Table 4.12 Analysis of Variance (ANOVA) for astaxanthin production by *X. dendrorhous* at 10 day of cultivation

Source	DF	Adj MS	*F*	p
Main Effects	4	38770.8	36.22	0.007*
2-Way Interactions	3	1938.6	1.81	0.319
Residual Error	3	1070.4		
Curvature	1	2696.6	10.48	0.084
Pure Error	2	257.3		
Total	10			

Note: * designates significance at $p<0.05$

(A: Pyruvate, B: $ZnCl_2$, C: Tween20, and D: Ethanol)

As can be observed from Table 4.12, pyruvate and ethanol positively influenced astaxanthin production while $ZnCl_2$ and Tween20 affected astaxanthin production negatively. It should be noted that the model obtained by statistical analysis indicated that main effects but not two-way interactions were considered statistically significant (Table 4.12) ($p<0.05$). Additionally, only pyruvate and Tween20 were considered statistically significant at the confidence interval of 95% (Table 4.12) whereas no two-interactions were found to be significant statistically ($p>0.05$). Therefore, pyruvate and Tween20 were subsequently selected for further study. Furthermore, statistical analysis showed that curvature was insignificant statistically ($p>0.05$) indicating that concentration of each variable investigated leading to maximum response was not included within the chosen range (Table 4.12).

4.6 The Steepest Ascent

According to FFD previously conducted, curvature was found to be insignificant statistically indicating that the optimal region for the experimental condition lies out of the present design range. Therefore, steepest ascent was subsequently employed to determine the next set of experiments with which optimum conditions may be found more rapidly.

The steepest ascent procedure takes advantage of the magnitude and sign of the linear effects of the simple regression model to determine the direction towards higher predictive response to find the proper direction of changing variables. In this study, since only pyruvate and Tween20 were considered statistically significant ($p<0.05$) therefore, only these two variables were taken into consideration in the steepest ascent experiment, whilst $ZnCl_2$ and ethanol were maintained at 1 mM and 35 mM, respectively, regardless of the concentrations of both pyruvate and Tween20 adopted. As can be seen in Table 4.11, the coefficients of pyruvate and Tween20 were 41.0 and -124.8, respectively. Therefore, experiments included in steepest ascent were constructed in such a way that concentrations of both pyruvate and Tween20 were increased and decreased proportionally to its corresponding coefficient providing that such changes were based upon those of the center point included in the previously conducted FFD. As could be observed from Table 4.13, the first experimental run was conducted at concentrations of both pyruvate and Tween20 of 25 mM and 0.55%,

Table 4.13 Experimental designs required for steepest ascent together with responses in terms of astaxanthin concentrations obtained at day 9 post inoculation

Steps	Path	Pyruvate	Tween20	Responses	
				Astaxanthin ($\mu g/g_{yeast}$)	Astaxanthin (mg/L)
	X_0	25	0.55	-	-
	ΔX	5	-0.076	-	-
1	X_0	25	0.55	651.72	7.46
2	$X_0+\Delta X$	30	0.47	730.00	10.00
3	$X_0+2\Delta X$	35	0.40	805.57	9.29
4	$X_0+3\Delta X$	40	0.32	904.84	11.27
5	$X_0+4\Delta X$	45	0.25	1023.71	11.98
6	$X_0+5\Delta X$	50	0.17	1095.00	13.02
7	$X_0+6\Delta X$	55	0.09	1116.48	13.25
8	$X_0+7\Delta X$	60	0.02	1107.38	12.70

Note: concentration of $ZnCl_2$ and ethanol were kept at 1 and 35 mM, respectively

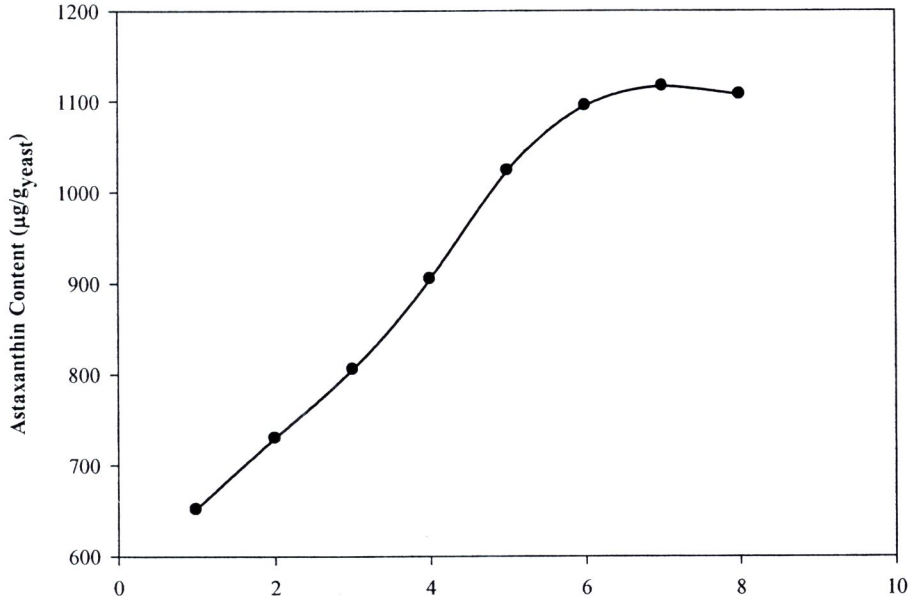


Figure 4.9 Astaxanthin concentration obtained at day 9 post inoculation along the path of steepest ascent

respectively, while the last step (eighth step) was conducted at pyruvate and Tween20 concentrations of 60 mM and 0.02%, respectively.

Astaxanthin production obtained from steepest ascent experiment was provided in Table 4.13. It is evident that astaxanthin production increased rapidly and continuously from the first step (651.72 μg/g $_{yeast}$) to the sixth step (1095.00 μg/g_{yeast}) (Figure 4.9, Table 4.13). Subsequently, astaxanthin concentration increased slightly after the sixth step from 1095.00 to 1107.38 μg/g_{yeast} in step six to step eight. It may be deduced that further manipulation of both pyruvate and Tween20 would no longer result in an increase in astaxanthin production. Hence, it may be anticipated that optimization conducted with pyruvate and Tween20 at 50 mM and 0.17 %, respectively, as center point may eventually lead to maximization of astaxanthin accumulation. Therefore, optimization using Doehlert design was conducted at the factor levels of run 6, where pyruvate and Tween20 concentrations were, respectively, 50 mM and 0.17 %.

4.7 Optimization of Astaxanthin Biosynthesis by *X. dendrorhous* using Doehlert Design

As shown in Table 4.14, the highest astaxanthin biosynthesis of 1184.69 μg/g $_{yeast}$ was accomplished when the concentrations of pyruvate and Tween20 achieved in steepest ascent experiment were set at central level (0) (Run 9). In addition, while, for the main effects, pyruvate (X) were considered insignificant statistically ($p>0.05$), Tween20 (Y) was, on the contrary, found to influence astaxanthin production statistically significant at the confidence level of 95% (Table 4.15). Further, the second order interaction effects, namely, pyruvate×pyruvate and Tween20×Tween20 were found to play a significant role ($p<0.05$) on astaxanthin production whilst interaction between pyruvate and Tween20 affected astaxanthin accumulation statistically insignificant ($p>0.05$).

The experimental results (Table 4.15) attained according to Doehlert design were fitted to a second-order polynomial model using Minitab 14 and results are given in Table 4.15 and Table 4.16.

Table 4.14 Experimental designs and corresponding responses in terms of astaxanthin concentration obtained at day 9 post inoculation

Runs No.	Pyruvate (X)	Tween20 (Y)	Astaxanthin ($\mu g/g_{yeast}$)	Astaxanthin (mg/L)
1	0.5	0.866	908.43	10.00
2	0.5	-0.866	962.00	11.11
3	-0.5	0.866	869.90	10.08
4	-0.5	-0.866	1048.82	11.59
5	1	0	971.81	11.27
6	-1	0	925.17	10.79
7	0	0	1176.35	13.57
8	0	0	1155.83	13.25
9	0	0	1184.69	13.81

Further, it was established that the data obtained experimentally distributed normally ($p>0.05$) according to Anderson-Darling test indicating that there was no bias involved when data were collected. The standard deviation for astaxanthin production achieved at the center points were 1.24%, which is well within acceptable range of 5%. Statistical analysis revealed further that the obtained model was significant since the 'lack-of-fit' was found to be insignificant with the p-value of 0.121. Additionally, the coefficient of determination (R^2) for astaxanthin production was 98.4% indicating that only 1.6 % of the total variation could not be explained by the model. Moreover, the value of the adjusted determination coefficient (R^2-adj), a measure of the goodness of fit of the regression model, of 97% was attained suggesting that the experimental data are in good agreement with predicted values, therefore, the all data were well fitted by model.

By applying multiple regression analysis on the experimental data, the following second-order polynomial equation (Equation 4.2) was obtained representing the correlation between pyruvate and Tween20 on astaxanthin production.

$$\text{Astaxanthin Concentration } (\mu g/g_{yeast}) = 1172.29 + 7.50X - 67.12Y - 223.80XX - 225.42YY + 72.37XY$$

(Equation 4.2)

Table 4.15 Regressive analysis on astaxanthin biosynthesis (astaxanthin content) obtained at 9 day post inoculation by response surface methodology using Doehlert design

Variables	Coefficient	SE Coefficient	*t*	p
Constant	1172.29	14.69	79.782	0.000*
X	7.50	14.69	0.510	0.645
Y	-67.12	14.69	-4.568	0.020*
X*X	-223.80	23.23	-9.633	0.002*
Y*Y	-225.42	23.23	-9.702	0.002*
X*Y	72.37	29.39	2.463	0.091

$S = 25.45$ $R^2 = 98.4\%$ $R^2\text{-}(adj) = 95.7\%$
Note: * designates significance at p <0.05

Table 4.16 Analysis of Variance (ANOVA) for astaxanthin production by *X. dendrorhous* obtained at 9 day post inoculation

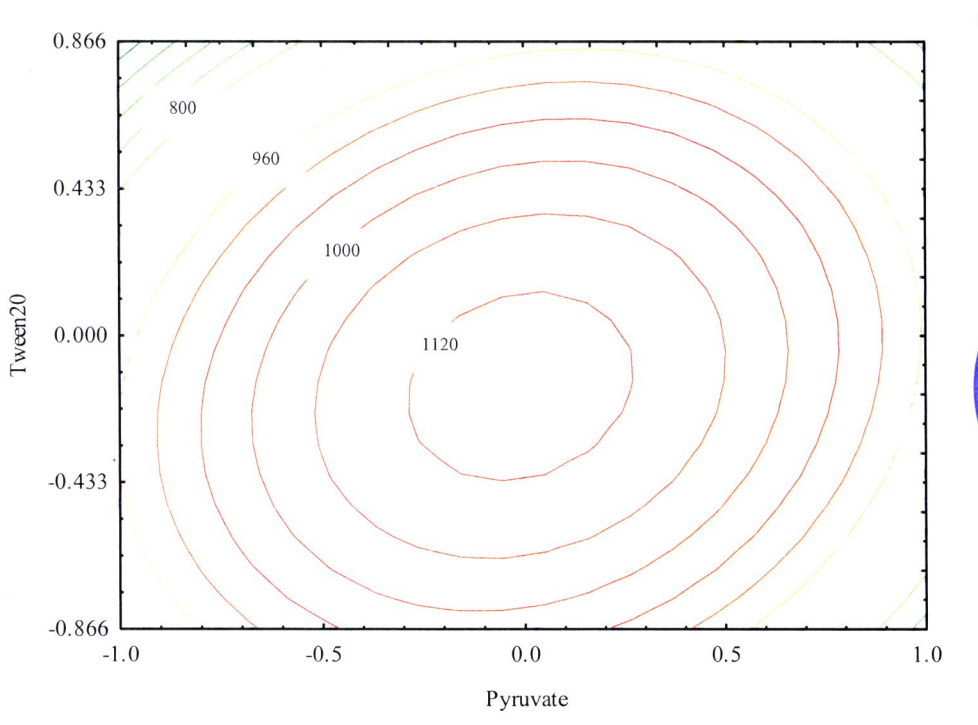
Source	DF	Seq SS	Adj SS	Adj MS	*F*	p
Regression	5	118503	118503	23700.7	36.59	0.007*
Linear	2	13682	13682	6840.8	10.56	0.044*
Square	2	100894	100894	50446.9	77.89	0.003*
Interaction	1	3928	3928	3928.2	6.06	0.091
Residual Error	3	1943	1943	647.7		
Lack-of-Fit	1	1502	1502	1502.0	6.81	0.121
Pure Error	2	441	441	220.6		
Total	8	120447				

Note: * designates significance at p <0.05

(X: pyruvate and Y: Tween20)

Where X and Y are the concentration of pyruvate and Tween20, respectively. Subsequently, the Lagrange's criteria were applied to characterize the response function obtained in order to determine the nature of the stationary point of a function. It was found that the value of Δ_1 and Δ_2 were, respectively, -447.60 and 1.965 $\times 10^5$. The values of $\Delta_1<0$ and $\Delta_2>0$ indicate that there is a maximum point lies within the experimental region endeavored. Maximum astaxanthin production of 1177.30 $\mu g/g_{yeast}$ could be achieved according to Equation 4.2 at pyruvate and Tween20 were -0.0075 and -0.15, respectively, which correspond to actual value of 49.78 mM and 0.145 %, respectively.

The quadratic model could further be represented graphically as contour plots (two-dimensional plots) and response surface plots (three-dimensional plots) (Figure 4.10) to illustrate the influence of both chosen parameters, i.e., pyruvate and Tween20, on astaxanthin biosynthesis. Such graphical tools provide useful information on the process conditions necessary to achieve the desired value of response. Figure 4.10A shows that astaxanthin biosynthesis was enhanced with increasing pyruvate level up to -0.3 to 0.25 corresponding to the real concentration of 41 to 57.5 mM, thereafter, when the concentration of pyruvate was higher than 57.5 mM, astaxanthin concentration tended to decrease. In addition, an increase in astaxanthin accumulation was attained with Tween20 concentration ranging from -0.433 to 0.170 corresponding to actual Tween20 concentration of 0.1-0.2%, where astaxanthin concentration reduced when concentration of Tween20 was higher. Additionally, Figure 4.10B shows response surfaces established using the quadratic model (Equation 4.2) which indicated that there were regions where the response could be maximized, more than 1000 $\mu g/g_{yeast}$ of astaxanthin production.



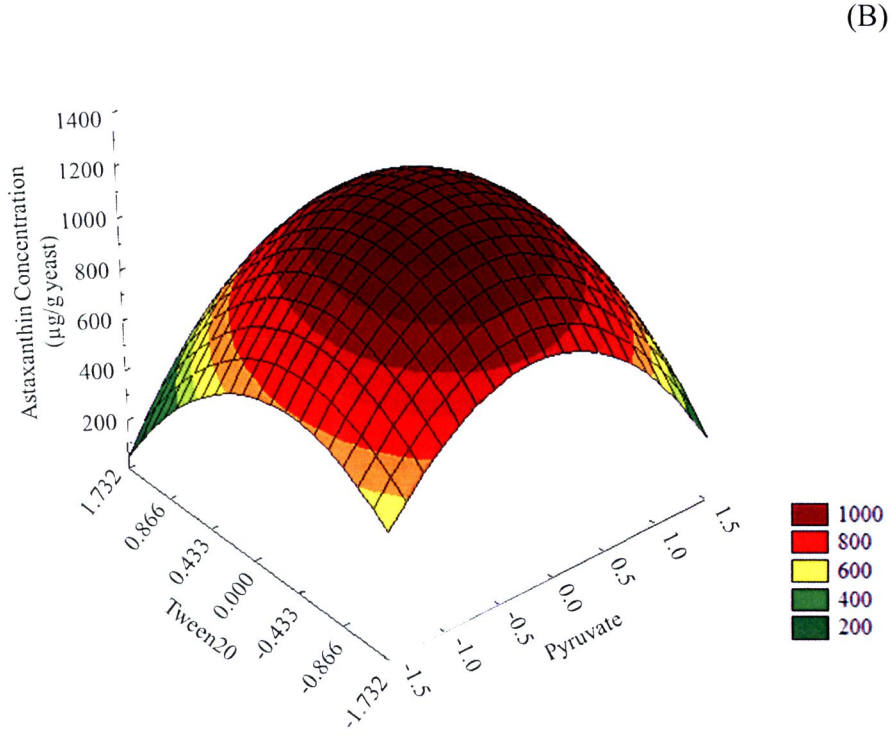


Figure 4.10 Contour (A) and response surface (B) diagrams of astaxanthin biosynthesis as a function of the concentrations of Pyruvate and Tween20

Non-ionic surfactants such as spans, tweens, Triton X-100, and pluoronic F-68 are reported to increase the cell growth and production of various enzymes and primary products including ethanol (Laouar *et al.*, 1996). Several studies demonstrated that surfactant favorably influenced cell growth, β-carotene, tricosporic acid, and lycopene production (Kim *et al.*, 1997; Xu *et al.*, 2007; Choudhari *et al.*, 2008). Kim *et al.* (1997) investigated effects of nonionic surfactants, i.e., Span 20 and 85, Tween 20 and 85, and Triton X-100, on growth and β-carotene production in *Blakeslea trispora* and found that with Span 20 supplementation; a high yield of β-carotene (2.16 g/L) of approximately 14 times in comparison with that of the control could be achieved. The authors anticipated that Span 20 could reduce the aggregation of *B. trispora* and, simultaneously, increase the cell wall permeability, and hence improve the substrate uptake and β-carotene formation leading to better β-carotene production. In addition, surfactants such as Tween80, Tween20 and gum arabic at 1% concentration have also been utilized to increase lipid and carotenoid production in *Rhodotorula glutinis* (Saenge *et al.*, 2011). It was consequently found that high biomass, lipid content, and carotenoid production were noted when surfactant especially Tween20 was introduced given that an improvement in biomass, and carotenoid yield as high as 1.8 and 2.2 times, respectively, could be anticipated.

Results found in this study are in good agreement with that reported in literature (Saenge *et al.*, 2011) in that Tween20 was also found to be able to enhance biomass and astaxanthin production. Nevertheless, negative effect of Tween20 on astaxanthin accumulation was highlighted when high concentration of Tween20 was employed. It was suggested by several studies that surfactants could alter the cell membrane permeability due to their ability to combine with lipid and/or proteins in the membranes (Xu *et al.*, 2007; Choudhari *et al.*, 2008; Kim *et al.*, 1997; Saenge *et al.*, 2011; Laouar *et al.*, 1996). The possible mechanisms of surfactant on biological membranes are proposed to follow a three-stage process of membrane-detergent interaction which are (i) incorporation into the lipid bilayer, (ii) bilayer disruption and (iii) separation of lipid from protein components leading to the formation of transmembrane pores, which would in turn facilitate substrate uptake into cell (Laouar *et al.*, 1996). Moreover, the surfactant emulsified hydrocarbon-based compounds, then breaking down into more manageable molecules, so that the microbes can then more efficiently digest; thus enhancing growth (Bodour *et al.*, 2003).

Since the building block of carotenoids was of the form of isoprene units, which subsequently produced from acetyl-CoA (Gu *et al.*, 1997). Supplementation of the precursor of acetyl-CoA into culture medium, Pyruvate, may enhance astaxanthin accumulation. In this study, pyruvate was found to be statistically significant and positively enhanced astaxanthin production. Yamane *et al.* (1997a) reported that utilization of pyruvate at 8 g/L as carbon source for *X. dendrorhous* yielded comparatively low growth (2.8 g/L) and astaxanthin content (250 $\mu g/g_{yeast}$) than that obtained with glucose at the same concentration which led to biomass and astaxanthin content of 5.7 g/L and 300 $\mu g/g_{yeast}$, respectively. On the contrary, this study employed pyruvate as enhancer to improve astaxanthin production by supplementing into cultivation medium with sucrose as principle carbon source. In addition, it is evident that supplementation of pyruvate into the cultivation medium of Sf-9 cells resulted in enhancement of carbon flux through the TCA cycle and resulting in a higher efficiency in generation of acetyl-CoA (Carinhas *et al.*, 2010). Nevertheless, pyruvate has never been employed to enhance carotenoids before. This study was the first to use pyruvate to enhance astaxanthin production and found that results were satisfactory.

It has also been demonstrated that ethanol play a significant role on growth and astaxanthin accumulation by *X. dendrorhous*. Addition of low quantities of ethanol (0.2%) led to an increase in carotenoid production by *X. dendrorhous* while posed no adverse effect on growth (Gu *et al.*, 1997). In addition, Yamane *et al.* (1997a) found that ethanol as carbon source yielded higher astaxanthin content (520 $\mu g/g_{yeast}$) than that obtained with glucose (300 $\mu g/g_{yeast}$) at the same concentration (8 g/L); however, resulted in a much lower growth than that of glucose (5.7 g/L) in comparison with 1.5 g/L for ethanol. Further, Yamane *et al.* (1997a) reported that growth medium supplemented with ethanol at low concentration (3 g/L) yielded higher growth than that supplemented with ethanol at higher concentration (5 and 10 g/L). Gu *et al.* (1997) reported that, with the presence of ethanol in culture medium, an increase in alcohol dehydrogenase and hydroxy-methyl-glutaryl-CoA (HMG-CoA) reductase activities was detected which may increase the carbon flux through the isoprenoid pathway. However, ethanol was not considered significant statistically in this study.

It was found that carotenogenesis in other carotenogenic microorganisms was induced in the presence of trace elements. Wang *et al.* (1999) reported that heavy metal ions

such as lanthanum, cerium, and neodymium could enhance the carotenoid biosynthesis of the yeast *X. dendrorhous*. Moreover, Flores-Cotera and Sánchez (2001) reported that addition of cofactor such as Cu^{2+} and Fe^{2+} into the cultivation medium could enhance astaxanthin production of *X. dendrorhous*. For example, Kobayashi *et al.* (1992) found that ferric salts could improve astaxanthin accumulation by *H. pluvialis*. In addition, Mahattanatavee and Kulprecha (1991) reported that the supplementation of the growth medium with iron, zinc, and copper ions could also improve the carotenoid production of *Rhodotorula* strains. Several studies showed that copper, cobalt, calcium, and barium ions stimulated the carotenogenesis of the pink yeast *R. rubra* (Atamanyuk and Razumorskii, 1974; Daushvili and Elisashvili, 1990; Gammal and Rizk, 1989). In contrast, this study found that Zn^{2+} had no significant effect on astaxanthin production of *X. dendrorhous*.

Although several studies reported the stimulatory effect of trace elements on carotenogenesis, their distinct function in carotenoid biosynthesis has not yet been elucidated. Nevertheless, it is postulated that trace elements affect the extent of enzyme activity involved in carotenogenesis (Goodwin, 1980). For example, some trace elements such as Fe^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+} ions are known to act as cofactors for enzymes, i.e., Phytoene synthase, Cytochrome oxidase, and Alcohol dehydrogenase, involved in the carotenoids biosynthetic pathway and, therefore, capable of enhancing carotenoid production at certain concentrations (Sandmann, 1994; 2001, Goodwin, 1984; Walker, 1998). Flores-Cotera and Sánchez (2001) proposed that Cu^{2+} involved in cytochrome-c-oxidase in the main respiratory chain. Catabolism along with low activity of cytochrome-c-oxidase should bring about an increase in $NADH/NAD^+$ ratio in cells. This could be a basic event for triggering carotenoid biosynthesis in *X. dendrorhous*, because, in oleaginous yeast, the enzyme isocitrate dehydrogenase requires NAD^+ for activity. Low enzyme activity promotes citrate accumulation in mitochondria and transport into the cytosol (Evans *et al.* 1983). Evidence suggests that cytosolic citrate could be a precursor for carotenoid accumulation in *X. dendrorhous* (Flores-Cotera *et al.* 2001). ATP citrate lyase, a key enzyme actively involved in production of Acetyl-CoA, has been detected in *X. dendrorhous*, giving further support to this possibility. In yeasts and bacteria, Zn^{2+} acts as cofactor of alcohol dehydrogenase enzyme, which plays an important role in break down alcohol molecule to acetaldehyde. Acetaldehyde,

then, catabolized into, (1) acetate by aldehyde oxidase and (2) pyruvate by pyruvate decarboxylase, which are substrates capable of triggering carotenoid production.

In summary, results revealed that supplementation of pyruvate, Tween20, ethanol, and $ZnCl_2$ into growth medium led to significant stimulatory effect on the astaxanthin biosynthesis of *X. dendrorhous* even though only pyruvate and Tween20 were considered significant statistically ($p<0.05$). Optimal concentration of 49.78 mM and 0.145 % for pyruvate and Tween20, respectively, led the maximum astaxanthin production of 1177.30 $\mu g/g_{yeast}$ which is three folds higher than that reported by Ramírez *et al.* (2000) who cultivated *X. dendrorhous* in growth medium containing date juice and urea as sole carbon and nitrogen source, respectively. Results found in this study were comparable to those reported by Domínguez-Bocanegra (2004), 850 $\mu g/g_{yeast}$, and Haard (1988), 1,182 $\mu g/g_{yeast}$. While the former cultivated *X. dendrorhous* using coconut milk alone, the latter employed molasses and peptone as carbon and nitrogen source, respectively. Therefore, pineapple juice concentrate, a by-product from canned pineapple industry, supplemented with sucrose, KNO_3, and *n*-hexadecane alone may be effectively employed to cultivate *X. dendrorhous.*

4.8 Batch Cultivation in 2 Liter Fermentor

Since optimization by Doehlert design indicated that 1177 $\mu g/g_{yeast}$ of astaxanthin could be attained under optimized conditions; therefore, it is of significant interest to validate the predictive efficiency of the obtained model. Subsequently, validation study was conducted at the previously established optimal conditions which are pineapple juice, a base medium, containing 10 g/L glucose supplemented with 40.2 g/L sucrose, 1.23 g/L KNO_3, 8.2 % (v/v) *n*-hexadecane, 0.15% Tween20, 49.8 mM pyruvate, 1 mM $ZnCl_2$, and 35 mM ethanol. Results showed that an average of astaxanthin content obtained was 883 $\mu g/g_{yeast}$ in shake flask culture which was slightly lower than that predicted by the model. However, it should be noted that astaxanthin obtained under optimized conditions was 3-3.5 folds higher than those obtained with YM and pineapple juice.

In addition, validation in 2 liter fermentor was also carried out. The cultivation was conducted using 1.5 L fermentation medium supplemented with all compositions as described above. Initial pH was adjusted to 5.5 whilst the aeration and agitation were

maintained at 1 vvm and 200 rpm, respectively, in order to maintain the dissolved oxygen concentration above 50%. Temperature was kept constant at 22 °C throughout experiment.

As is illustrated in Figure 4.11 that there appeared to be a lag phase of approximately 12 hours, then, a rapid growth was observed during 12 - 72 hours post inoculation. Afterward, biomass production increased continually and reached a maximum (approximately 17.43 g/L) at 9 days post inoculation. It is worthy to note that biomass concentration of *X. dendrorhous* observed in shake flask cultivation was only 12.94 g/L indicating an increase in biomass of 35% in comparison with that of fermentor. This may be due possibly to the fact that *X. dendrorhous* is aerobe in nature; therefore, cultivation in fermentor where oxygen supply was provided in excess may led to better growth; thus, higher biomass produced. Liu *et al.* (2006) reported that when cultivating *X. dendrorhous* in 250 mL Erlenmeyer flask containing 50 mL medium under 250 rpm agitation, oxygen partial pressure in the liquid phase dropped steeply from 100% to near zero (3.5% air saturation) within 30 hours after inoculation suggesting poor oxygen transfer and lower DO level than the critical DO value in the culture which may be responsible for poor growth.

The rapid sugar consumption took place simultaneously with rapid growth of *X. dendrorhous* particularly during the exponential phase (2-4 days post inoculation). It was further found that sucrose was rapidly depleted from growth medium within 48 hours which is coincided with an increase in concentration of glucose, fructose, and a new sugar, 1-kestose (Figure 4.12). It is not surprising since it has previously been demonstrated that *X. dendrorhous* is capable of producing invertase, an enzyme responsible for digesting sucrose into glucose and fructose, together with transglycosylase (Killian *et al.*, 1996), an enzyme actively involved in transfructosylation resulting in formation of 1-kestose, a trisaccharide which is considered prebiotic (Hidaka *et al.*, 1986; Mitsouka *et al.*, 1987). Previous studies also showed that cultures of the astaxanthin-producing yeast *X. dendrorhous* accumulated kestose as transfructosylation product when cultivated on medium containing sucrose (Killian *et al.*, 1996). An increase in glucose concentration may stem from the fact that for each mole of 1-kestose produced, one mole of glucose was also formed since

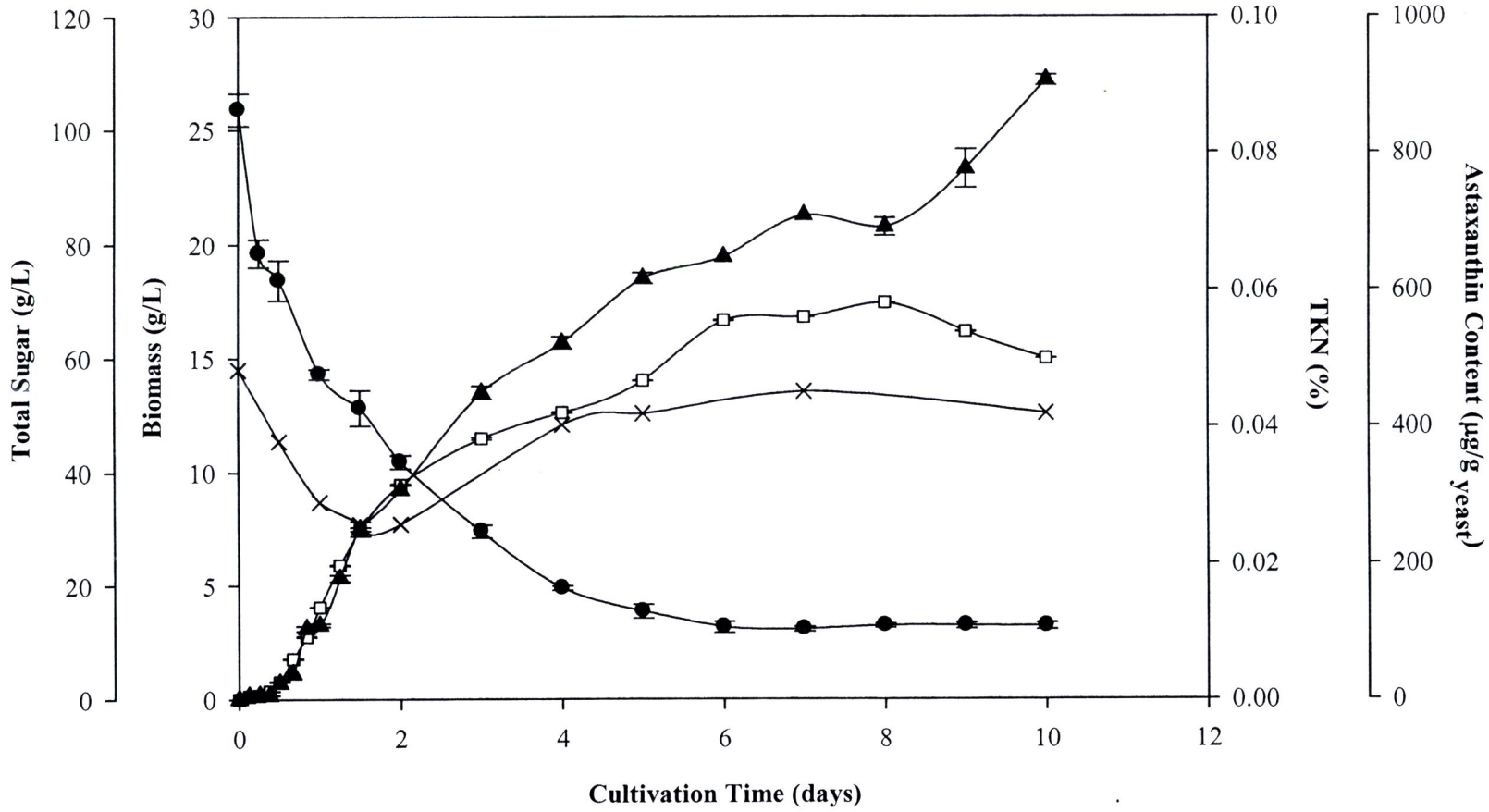


Figure 4.11 Cultivation of *X. dendrorhous* in 2 liter fermentor containing 1.5 L fermentation medium, initial pH at 5.5, inoculated with 10 % (v/v) inoculums culturing at 22 °C, 200 rpm agitation, and 1 vvm aeration providing that (●) Total sugar, (□) Cell dry weight, (×) TKN, and (▲) Astaxanthin production

transglycosylase is responsible for cleaving sucrose into glucose and fructose and subsequently, transfered fructose recently formed together with sucrose available and enzymatically converted into 1-kestose resulting in 1 molecules of glucose remained in the medium; thus; increasing glucose concentration. Further, a slight increase in fructose concentration may be due to the action of invertase upon hydrolyzing sucrose into fructose and glucose (Kilian *et al.*, 1996). Moreover, the highest glucose concentration detected at 12 hours of cultivation was approximately 19 g/L which was lower than that specified by Johnson and Lewis (1979) that the suppression of growth was noted when glucose concentration present in growth medium exceeded 19 g/L. However, this study found that with presence of glucose at 19.39 g/L no adverse effect on growth of *X. dendrorhous* was observed. Additionally, the presence of kestose, a probiotic in its own right (Kilian *et al.*, 2002), at the concentration of 18 g/L, could, in turn, promote growth of *X. dendrorhous* (Killian *et al.*, 1996; Kritzinger *et al.*, 2003) leading to better growth of *X. dendrorhous*. It should be noted that 1-kestose rapidly produced during the first 24 hours post inoculation was as well rapidly metabolized and exhausted from growth medium at 72 hours post inoculation. Moreover, it could be clearly seen in the Figure 4.12 that when glucose is present at higher concentration than that of fructose, *X. dendrorhous* preferentially metabolized glucose rather than fructose (0-48 hours). However, when both sugars are present at comparable concentration, it appeared that *X. dendrorhous* consumed both sugars at the same rate, from 48 hours onwards. Results found in this study are in part in agreement with that of Park *et al.* (2008) who found that glucose was more preferably than fructose when cultivated in growth medium containing sucrose.

In addition, according to Figure 4.11, nitrogen was rapidly consumed by *X. dendrorhous* immediately after inoculation until 48 hours post inoculation which was coincided with the exponential phase of cultivation. At this stage, *X. dendrorhous* rapidly uptook both nitrogen and sugars in order to proliferate more efficiently. However, after 48 hour of cultivation, nitrogen seemed to increase which also coincided with a slower rate of an increase in biomass production. Concentration of nitrogen continued to increase and reached maximum at day 4 post inoculation and, from them on, remained rather constant. This may be due to that fact that even though high nitrogen (TKN) was still present in the system; however, readily available nitrogen may be exhausted since TKN represents both organic and inorganic nitrogen compounds present in the system (Fang

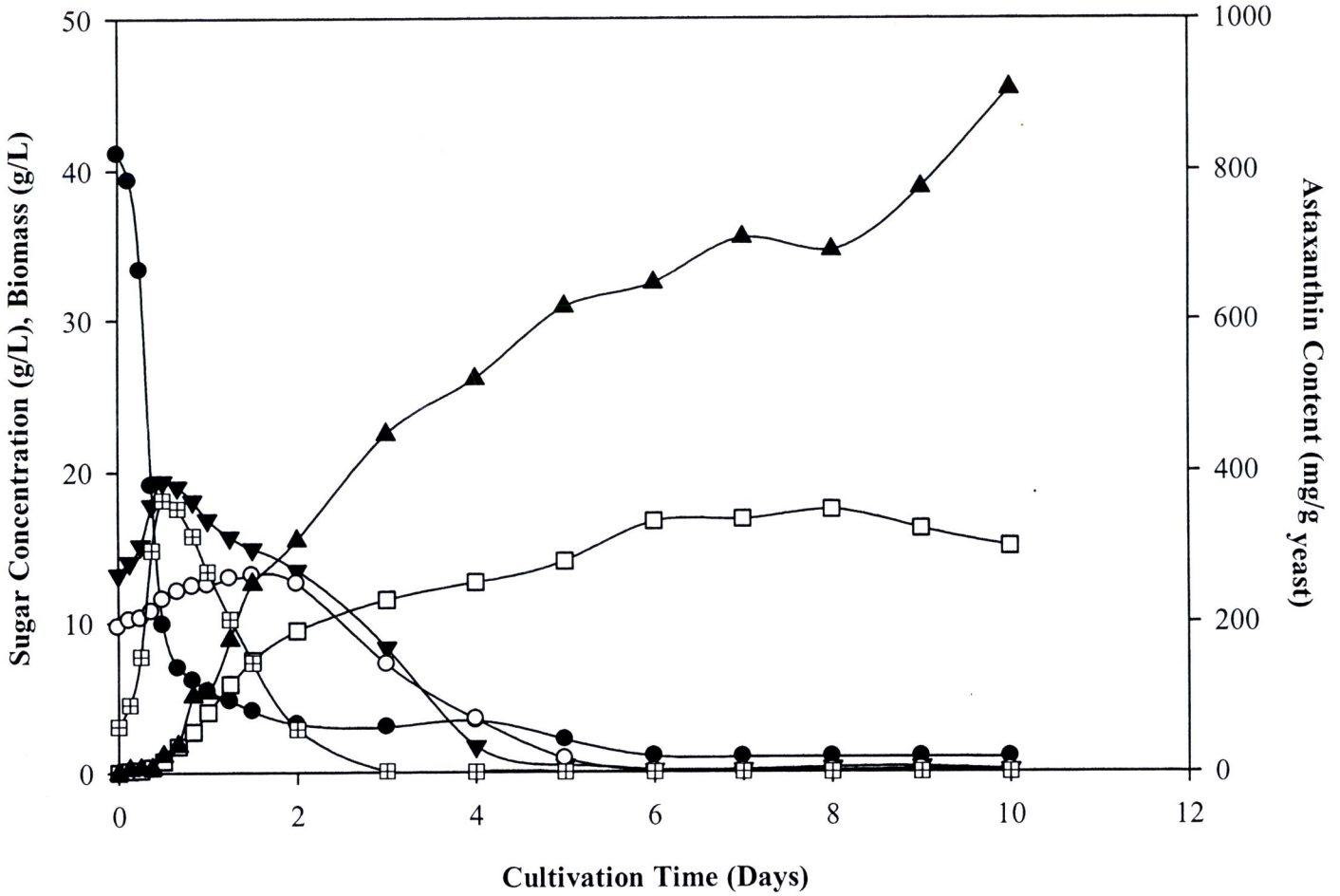


Figure 4.12 Cultivation of *X. dendrorhous* in 2 liter fermentor containing 1.5 L fermentation medium, initial pH at 5.5, with 10 % (v/v) inoculums culturing at 22 °C, 200 rpm agitation, and 1 vvm aeration: (□) Cell Dry Weight, (▲) Astaxanthin Content, (●) Sucrose, (▼) Glucose, (○) Fructose, and (⊞) 1-kestose.

and Cheng, 1993). Therefore, an increase in nitrogen may be due to cell lysis taking place resulting in a much slow rate of biomass production (24-48 post inoculation).

Astaxanthin accumulated by *X. dendrorhous* was rather low (4.7 $\mu g/g_{yeast}$) initially and gradually increased within 24 hours (Figure 4.13) which is in good agreement with that reported by Park *et al.* (2008) who found that during early stage of fermentation (10-30 hours), relatively younger yeast cells were dominant in the culture resulting in low astaxanthin content since, in this stage of cultivation, yeast required more energy and nutrient for cell proliferation; therefore, astaxanthin accumulation is rather low (Johnson and Lewis, 1979). In addition, after 48 hours or post-exponential phase when total sugar was depleted astaxanthin content and concentration increased more rapidly until the end of experiment. Again, Park *et al.* (2008) reported that when adult yeast cells were dominant a rapid increase in astaxanthin content was resulted (Figure 4.13). Astaxanthin started to increase after exponential phase suggesting that the astaxanthin production was associated to the age and lower growth rate of the culture (Fang and Cheng, 1993) since most substrate uptaken was channeled primarily to astaxanthin biosynthesis rather than formation of new generation of cells.

Additionally, cultivation of wild type strain of *X. dendrorhous* in this study conducted using 2-Liter fermentor under optimized conditions obtained previously resulted in astaxanthin production of 906.57 $\mu g/g_{yeast}$ or 13.57 mg/L and 17.43 g/L biomass concentration. It is worthy to note that pineapple juice concentrate together with optimum conditions established could efficiency and satisfactorily support both growth and astaxanthin accumulation when comparing with other studies reported in literature. For example, Kongtragool (2006) reported that 2 liter batch cultivation of *X. dendrorhous* with 36.69 g/L sucrose and 11.78 g/L fish soluble as carbon and nitrogen source, respectively, together with 43 mM citrate as enhancer led to 15 g/L biomass, and cellular astaxanthin concentration of 317 $\mu g/g_{yeast}$, in other word, 4.7 mg/L astaxanthin concentration. In addition, with fed-batch cultivation using 100 g/L sucrose concentration and 11.98 g/L fish soluble, astaxanthin content and concentration 234 $\mu g/g_{yeast}$ and 4.6 mg/L were resulted along with biomass concentration of 19 g/L. Moreover, Moriel *et al.* (2004) reported that when 100 g/L sugar cane and 1 g/L urea were employed as substrate, continuous fed-batch fermentation yielded cellular

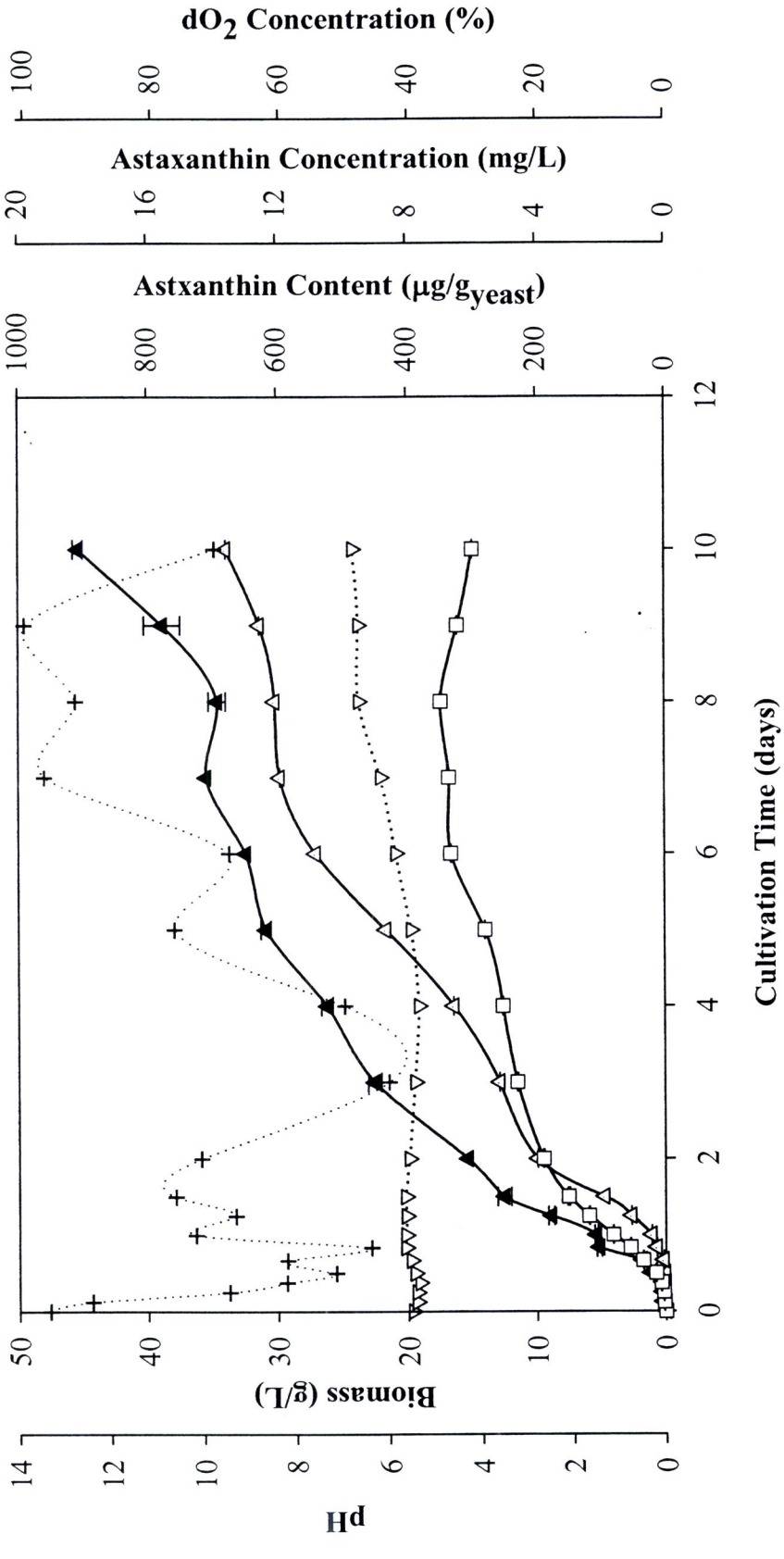


Figure 4.13 Cultivation of *X. dendrorhous* in 2 liter fermentor containing 1.5 L fermentation medium, initial pH at 5.5, inoculated with 10 % (v/v) inoculums by culturing at 22 °C, 200 rpm, 1 vvm. Providing that (□) Cell Dry Weight, (▲) Astaxanthin Content, (△) Astaxanthin Concentration, (▽) pH, and (+) dO_2 Concentration.

astaxanthin concentration of 383.73 $\mu g/g_{yeast}$, volumetric astaxanthin concentration of 7.44 mg/L, and biomass concentration of 19.35 g/L. Considering both volumetric astaxanthin and biomass production, shake flask culture led to 11.4 mg/L and 12.94 g/L, respectively, in comparison with 13.57 mg/L and 17.43 g/L obtained with 2-Liter fermentor. An increase in both biomass and volumetric astaxanthin production found in batch cultivation may stem from the fact that during batch cultivation oxygen supply (dO_2) was provided in excess, higher than 40-50%, throughout experiment (Figure 4.13) since O_2 is necessary for both growth and astaxanthin production. Further, Yamane *et al.* (1997b) suggested that production of one mole of astaxanthin required 4 moles of oxygen together with 12 moles of glucose (Equation 4.3); therefore, higher level of dO_2 available favored astaxanthin accumulation. In addition, manipulation of pH during cultivation of *X. dendrorhous* was considered of critical importance since it has been reported that optimum pH for astaxanthin production was 4.0 while the pH of 6.0 was deemed optimum for growth (Hu *et al.*, 2006). Hu *et al.* (2006) further found that low astaxanthin production was resulted when the pH of growth medium was uncontrolled during cultivation of *X. dendrorhous* providing that pH of growth medium would gradually and continuously fall from 5.0 to 2.6 towards the end of fermentation. In this study, cultivation of *X. dendrorhous* was accomplished without pH control (Figure 4.13) given that the pH rose slightly from 5.5 initially to approximately 6.3 at the end of cultivation at which considered optimum for growth rather than astaxanthin production.

$$12C_6H_{12}O_6 + 4O_2 + 14NADPH + 48NAD^+ = 1C_{40}H_{52}O_4 + 32CO_2 + 14NADP^+ + 48NADH + 12H_2O + 34H^+$$

(Equation 4.3)