CHAPTER 6

Mutation of oleaginous red yeast *Sporidiobolus pararoseus* KM281507 for carotenoids and lipids productions

6.1 Introduction

Carotenoids represent one of the broadest groups of natural antioxidants with significant biological effects and numerous industrial applications (Certik et al., 2009). Industrially, carotenoids are used in pharmaceuticals, neutraceuticals and as animal feed additives, as well as colorants in cosmetics and foods. Scientific interest in dietary carotenoids has increased in recent years because of their beneficial effects on human health, such as lowering the risk of cancer and enhancement of immune system function, which are attributed to their antioxidant activity (Das et al., 2007). Although most of these added carotenoids are chemically synthesized there is an increasing interest in carotenoids of biological origin because of the public concern over the safety of artificial food colorants (Iturriaga et al., 2005). The increasing interest in microbial sources of carotenoids is related to consumer preferences for natural additives, and the potential cost effectiveness of creating carotenoids via microbial biotechnology. In recent years, the interest in production of natural carotenoids by microbial fermentation has been increasing. Microbial production of carotenoids is an environmentally friendly method compared to chemical production methods, and able to meet the increasing demand for natural carotenoids (Das et al., 2007).

Among the many types of carotenoid-producing microorganisms, e.g. bacteria, microalgae, mold and yeasts, the latter seem to be more convenient than the other microorganisms, especially, in large-scale production due to their unicellular nature and high growth rate. Red yeasts of the genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* and *Phaffia*, are capable of producing carotenoids naturally (Frengova and Beshkova, 2009). Moreover, some strains of those red yeasts can accumulate both

carotenoids and lipids in their cells. The red yeasts and other yeasts that can accumulate a high level of lipids totaling more than 200 mg/g of their biomass (20% by weight), and are identified as "oleaginous red yeasts or oleaginous yeasts" (Meng et al., 2009). Under favorable conditions, some oleaginous yeasts can accumulate lipids up to 700 mg/g of their dry weight (Zhang et al., 2011).

Crude glycerol is the principal byproduct of biodiesel production. It occurs in fats and oils at a level of approximately 10% by weight (Thompson and He, 2006). Nowadays, crude glycerol is mostly obtained from the biodiesel production process via the transesterification reaction of triacylglycerol with a short chain alcohol, particularly methanol. This crude glycerol is very low in value because of its high impurities (Pachauri and He, 2006; Thompson and He, 2006). In addition, disposal of crude glycerol may be difficult. Moreover, purification of crude glycerol is almost industrially infeasible because of the high processing cost. The methanol and alkali catalyst contents require crude glycerol to be treated as hazardous waste (Chatzifragkou et al., 2011a; Gerpen, 2005; Isahak et al., 2010). Therefore, the impurities and other physico-chemical parameters can be important considerations in bioconversion of crude glycerol to higher value products, e.g. carotenoids, lipids and organic acids (Nicol et al., 2012).

Nowadays, crude glycerol is interesting as the sole carbon source for oleaginous red yeasts (Yang et al., 2012) because its price is very low (Fan et al., 2010; Nicol et al., 2012). Many aspects have been investigated to use crude glycerol as a sole carbon source for microbial growth and carotenoid production (Manowattana et al., 2012; Saenge et al., 2011; Yimyoo et al., 2011), single cell oil production (André et al., 2010), and lipids production (Galafassi et al., 2012; Saenge et al., 2011). Many researchers have focused on the utilization of crude glycerol, combined with optimization of the process to increase yeast biomass and carotenoid production. For example; Yimyoo et al. (2011) found that *Rhodosporidium paludigenum* DMKU3-LPK4 exhibited the capacity to produce carotenoids with the application of glycerol as the sole carbon source. The carotenoid concentration was 3.42 mg/L under optimal conditions. Saenge et al. (2011) reported that crude glycerol could be used as the sole carbon source for carotenoid production by *Rhodotorula glutinis* TISTR5159 and a relatively high carotenoid production of 135.25 mg/L was obtained. Moreover, a red yeast,

Sporobolomyces pararoseus TISTR5213 produced carotenoids in a basal medium supplemented with crude glycerol about 18.7% higher than pure glycerol (Manowattana et al., 2012).

Moreover, the improvement of carotenoids and lipids production by oleaginous red yeasts by mutation has also been investigated. The genetic approach to identifying new genes in the cell is to create mutants that display a particular phenotype. This strategy allows the researcher to examine the entire genome for genes of interest (Yasbin, 2002). DNA lesions can arise naturally or in the presence of a variety of genotoxic substances such as UV, ionizing radiation, or chemicals (Barbour et al., 2006). Normally the study of DNA damage and repair systems has primarily involved the effects of ultraviolet (UV) radiation. UV can be easily administered to cells under defined condition (Yasbin, 2002). Now a day, two major chemical mutagens are routinely used. These are alkylating agents and base analogs. Each has a specific effect on DNA. Alkylating agents such as ethyl methane sulfonate (EMS), ethyl ethane sulfonate (EES) and mustard gas can mutate both replicating and non-replicating DNA (Khunna, 2010). In contrast, a base analog (5-bromouracil or 5BU) and 2-aminopurine) only mutate DNA when the analog is incorporated into replicating DNA (Khunna, 2010). Each class of chemical mutagen has specific effects that can lead to transitions, transversions or deletions (Khunna, 2010).

In the recently years, Li et al. (2007) have studied the UV, EMS and NTG mutagenesis of *Rhodotorula* sp. and found that a mutant of this yeast could produce 603.93 μg/g of carotenoid, while only 213.18 μg/g of carotenoid was produced by the wild-type. Moreover, Xie et al. (2014) have improved astaxanthin production of *Phaffia rhodozyma* 2.1557 by mutagenesis. A genetically stable mutant, YZUXHONG686 was obtained and it enhanced astaxanthin production from 1.54±0.05 to 41.13±1.63 mg/L. Therefore, in this study, the improvement of carotenoids and lipids production by *Sporidiobolus pararoseus* KM281507 using mutagenesis (UV, EMS and 5BU) was investigated.

6.2 Materials and Methods

6.2.1 Microorganism and culture conditions

The oleaginous red yeast *Sporidiobolus pararoseus* KM281507 (formerly *Sporobolomyces pararoseus* TISTR5213) was obtained from the culture collection of the Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani 12120, Thailand. The inoculum preparation, production medium and cultivation condition was performed according to the method of Manowattana et al. (2012). The production medium composed of yeast extract 1.0 g, demethanolized crude glycerol 55.0 g, KH_2PO_4 5.5 g, $(NH_4)_2SO_4$ 5.3 g, K_2HPO_4 3.7 g, $MgSO_4.7H_2O$ 0.5 g, $MnSO_4.H_2O$ 0.2 g and NaCl 0.25 g (Appendix A). The initial pH of the medium was adjusted to 5.63 before sterilization at 121°C for 20 min (Manowattana et al., 2012).

6.2.2 Mutagenesis

UV mutagenesis

Ultraviolet (UV) mutagenesis was carried out using a UV lamp (18 W, 25 cm) for 0, 5, 10, 15, 20, 25 and 30 min. Cells of *Sporidiobolus pararoseus* from YM medium, were washed twice in 0.1 M potassium phosphate buffer (pH 6.0), followed by resuspension in the same solution. After that, the cell suspension was diluted by 10–fold serial dilution and spread on the basal medium agar supplemented with pure glycerol (modified method from Li et al. (2007)). Culture plates were incubated at 25°C for three days in the dark.

EMS mutagenesis

Ethyl methane sulfonate (EMS) mutagenesis was performed by washing freshly grown cells in 0.1 M sodium citrate buffer (pH 5.5), followed by resuspension in the same buffer. EMS was then added to the suspension at final concentrations ranging from 0.01 to 1.00 g/L, and the cells were incubated for 30, 60 and 90 min at 25°C, then washed twice with sodium citrate buffer. Surviving cells were diluted by 10-fold serial dilution and spread on basal medium agar supplemented with pure glycerol (modified method from Li et al. (2007)). Culture plates were incubated at 25°C for three days.

5-Bromouracil mutagenesis

5-Bromouracil (5BU) mutagenesis was performed by washing freshly grown cells in 0.1 M potassium phosphate buffer (pH 6.0), followed by resuspension in the same buffer. The 5BU was then added to the suspension at final concentrations ranging from 0.01 to 1.00 g/L, and the cells were incubated for 30, 60 and 90 min at 25°C, then washed twice with sodium citrate buffer. Surviving cells were diluted by 10-fold serial dilution and spread on the basal medium agar supplemented with pure glycerol (modified method from Li et al. (2007)).

Colonies on the plates were counted to determine the survival rate, which was expressed as the relative ratio of colony-forming units per milliliter (CFU/mL) after each mutagenesis treatment. Determinations were made in triplicate. After that, single colonies with more intensive red coloration than the wild type were selected to study carotenoid production.

6.2.3 Analytical methods

Ten milliliter of 5 day-old cultivation broth cultures were taken from each flask and centrifuged at 6,000 rpm (4,146 *g*) at 4°C for 10 min (Hettich MIKRO 22R; Germany). The cell pellet was washed twice with *n*-hexane (LabScan, Thailand) and once with distilled water. Dry cell weight (DCW) was measured by drying at 80°C overnight and transferring to a desiccator until a constant weight was reached (Manowattana et al., 2012). Next, the carotenoids in the cell pellet were extracted by a method which ruptured the yeast cells, and was carried out in a screw cap tube (25 × 150 mm), containing 10.0 mL acetone and 4.0 g glass beads (3 mm-diam.; Superior, Germany). The mixture was vigorously shaken in a vortex mixer (G560E; Scientific industries Inc., USA) for 15 min in the presence of 100 ppm ascorbic acid (Sigma, USA). The broken cells were centrifuged at 6,000 rpm (4,146 *g*) at 4°C for 10 min and the clear supernatant was collected and dried by flushing it with N_2, then re-dissolved in 1.0 mL *n*-hexane. The extract was filtered through a nylon membrane filter (0.2 μm; FilTrex, USA) and subjected to HPLC analysis. A modified method of HPLC analysis was performed on the analytical HPLC (Shimadzu, Japan) equipped with a C18-column (4.6 mm × 250 mm, 5μm; Restek, France). The mobile phase was composed of

acetonitrile:dichloromethane:methanol (80:10:10, v/v/v) with a flow rate of 1.0 mL/min. The column thermostat was set at 30°C. The detector was operated at 454 nm; in a linear gradient for 45 min, maintaining this proportion until the end of the run (Manowattana et al., 2012).

The lipids content was extracted with a mixture of chloroform and methanol (2:1, v/v) for 1 h (Bligh and Dyer, 1959). The crude lipids extract was centrifuged at 6,000 rpm (4,146 *g*) at 4°C for 10 min, and the clear supernatant was collected. The solvent was removed by vacuum evaporation and transferred to a desiccator until a constant weight was reached. The lipids was expressed as the weight of the extracted lipids in the media (mg/L).

6.3 Results and discussion

6.3.1 UV mutagenesis

Many types of irradiation have been used to generate mutation. Ultraviolet irradiation is easily controlled and requires only comparatively inexpensive equipment (Dale and Park, 2004). UV is the most convenient radiation to use as a mutagen. The major effect of UV is to cause adjacent thymine residues in the same strand to react together to form thymine dimers (Smith and Wood, 1991). The viability of *Sporidiobolus pararoseus* KM281507 decreased as the time of UV irradiation increased (Figure 6.1). Cell concentrations of 1.9×10^7, 1.6×10^7, 1.35×10^7, 1.09×10^7, 1.0×10^7, 0.83×10^7 and 0.67×10^7 CFU/mL were observed at 0, 5, 10, 15, 20, 25 and 30 min after UV irradiation treatment, respectively.

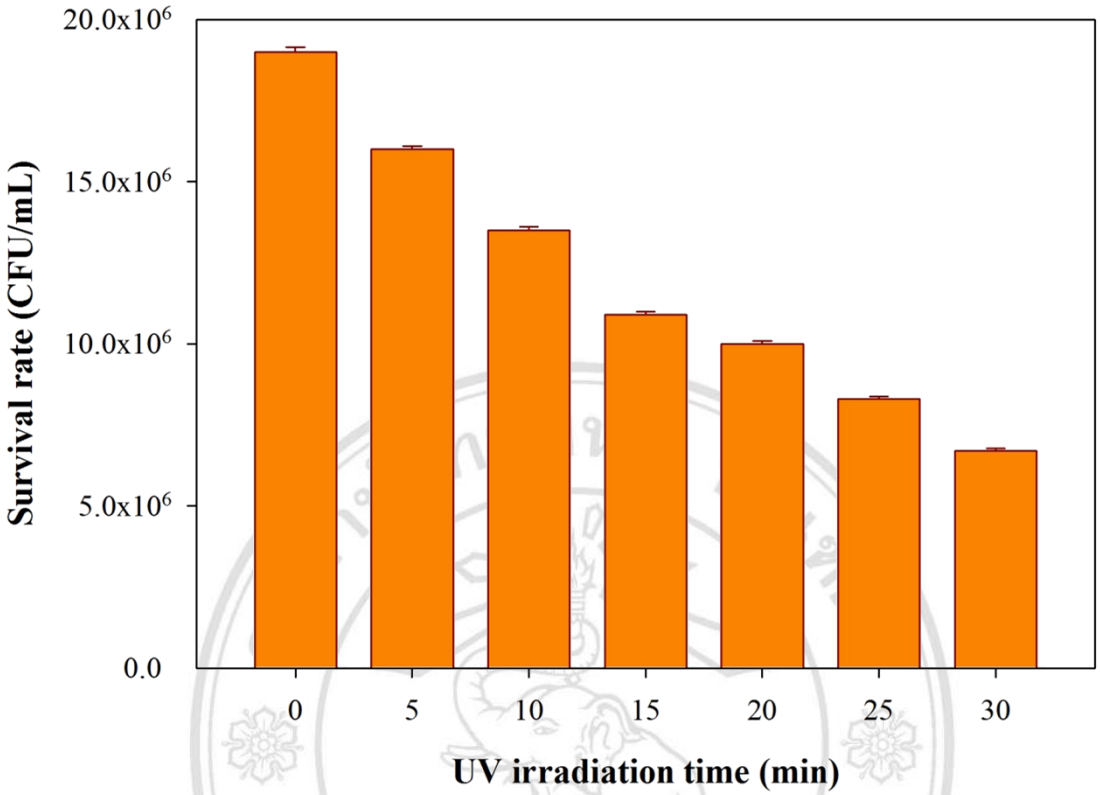

Figure 6.1 Effect of UV irradiation time on the survival of *Sporidiobolus pararoseus* KM281507

After UV treatment, more than 500 red colonies were obtained, and four of them were deeper red than the wild-type on the basal medium supplemented with pure glycerol. After cultivation with different carbon sources, demethanolized crude glycerol, pure glycerol and glucose, the total carotenoid contents of those mutants (U9, U10, U19 and U23) were determined (Figure 6.2). The mutant strain U10 produced the highest total carotenoids, 69.37±1.91 mg/L, (44.61% higher than wild-type) when demethanolized crude glycerol was used as the carbon source (Figure 6.2A), while the wild-type produced only 47.97±0.26 mg/L. In contrast, the DCW was not significantly different (Figure 6.2B). These results were similar to those of Bhosale and Gadre (2001) who found that after UV mutagenesis of *Rhodotorula glutinis* NCIM3353, the mutant strain 32 grew well in a medium prepared with seawater and produced total carotenoids of 86 mg/L, which was higher than in a medium prepared with distilled water (70 mg/L). Moreover, Li et al. (2007) reported that after UV mutagenesis, a mutant produced 211.51 μg/g of total carotenoids, while only 124.55 μg/g was produced by the wild-type.

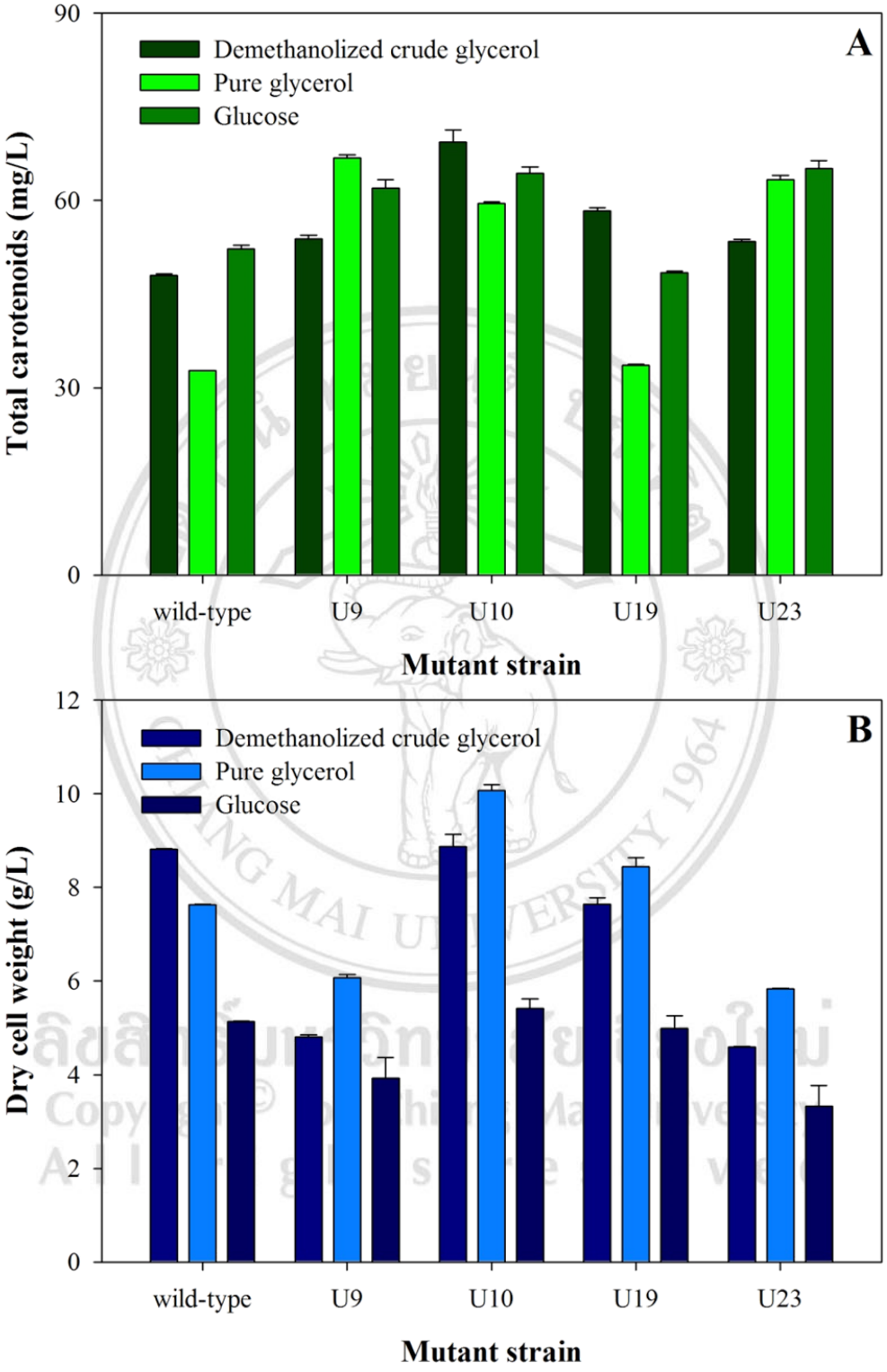

Figure 6.2 Effect of different carbon sources on total carotenoids (A) and dry cell weight (B) produced by the wild-type and UV-induced mutant strains of *Sporidiobolus pararoseus* KM281507

6.3.2 EMS mutagenesis

Ethyl methane sulfonate (EMS) is an agent that causes the removal of purine rings from DNA by a multistep process that begins with the ethylation of a purine ring and ends with the hydrolysis of the glycosidic (purine-deoxyribose) bond, causing the loss of the base (Tamarin, 1996). The results showed that the initial cell concentration of 1.9×10^7 CFU/mL decreased as the EMS concentration increased (Figure 6.3). A similar inverse relationship was observed between cell concentration and EMS incubation time.

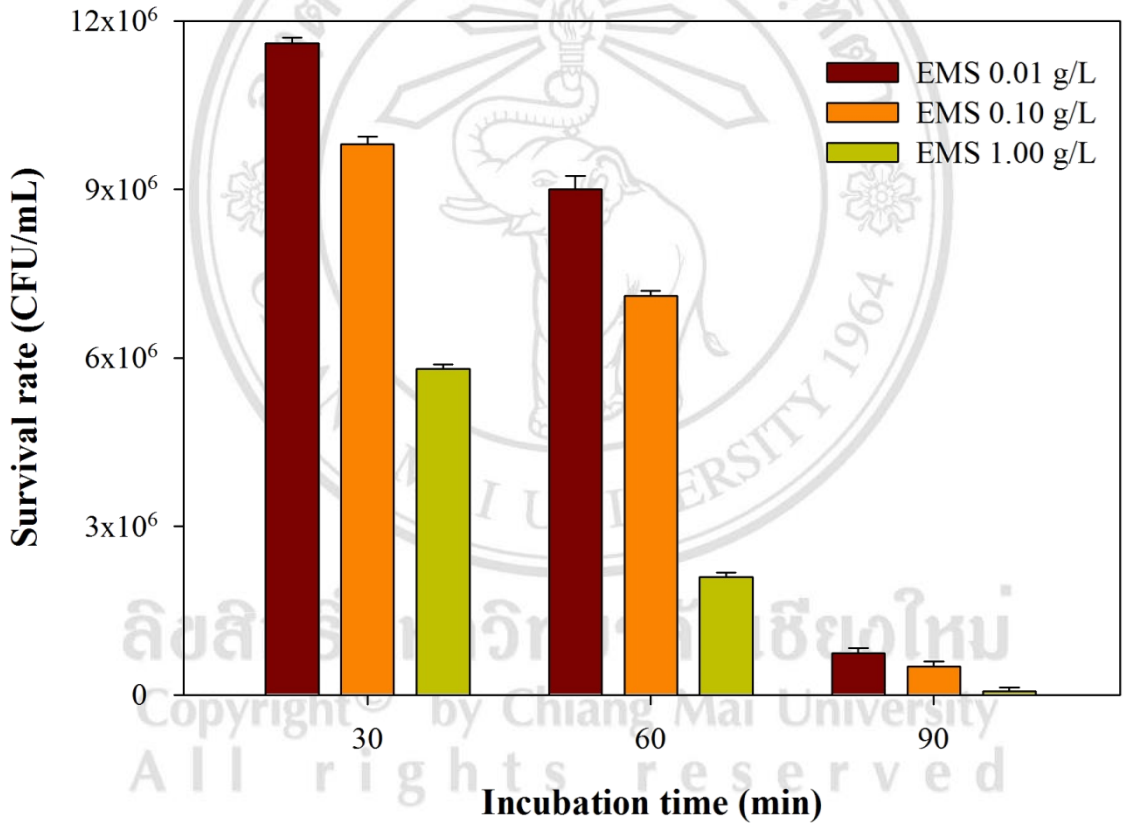

Figure 6.3 Effect of EMS concentration and incubation time on the survival of *Sporidiobolus pararoseus* KM281507

After EMS mutagenesis, more than 500 red colonies were obtained. Thirteen of those mutants, E1, E29, E41, E43, E47, E53, E81, E174, E188, E194, E245, E246 and E277, showed deeper red than the wild-type. Among those thirteen mutants, E47 had the highest total carotenoid content (67.99±1.80 mg/L) when cultivated using demethanolized crude glycerol as the carbon source, which represented a 41.73% increase over the wild-type (47.97±0.26 mg/L) (Figure 6.4A). The DCW of E47 (7.77±0.38 g/L) was less than wild-type (8.81±0.01 g/L) (Figure 6.4B). These results were similar to those reported by Li et al. (2007), who found that after EMS mutagenesis, total carotenoids was increased by 50.19%.

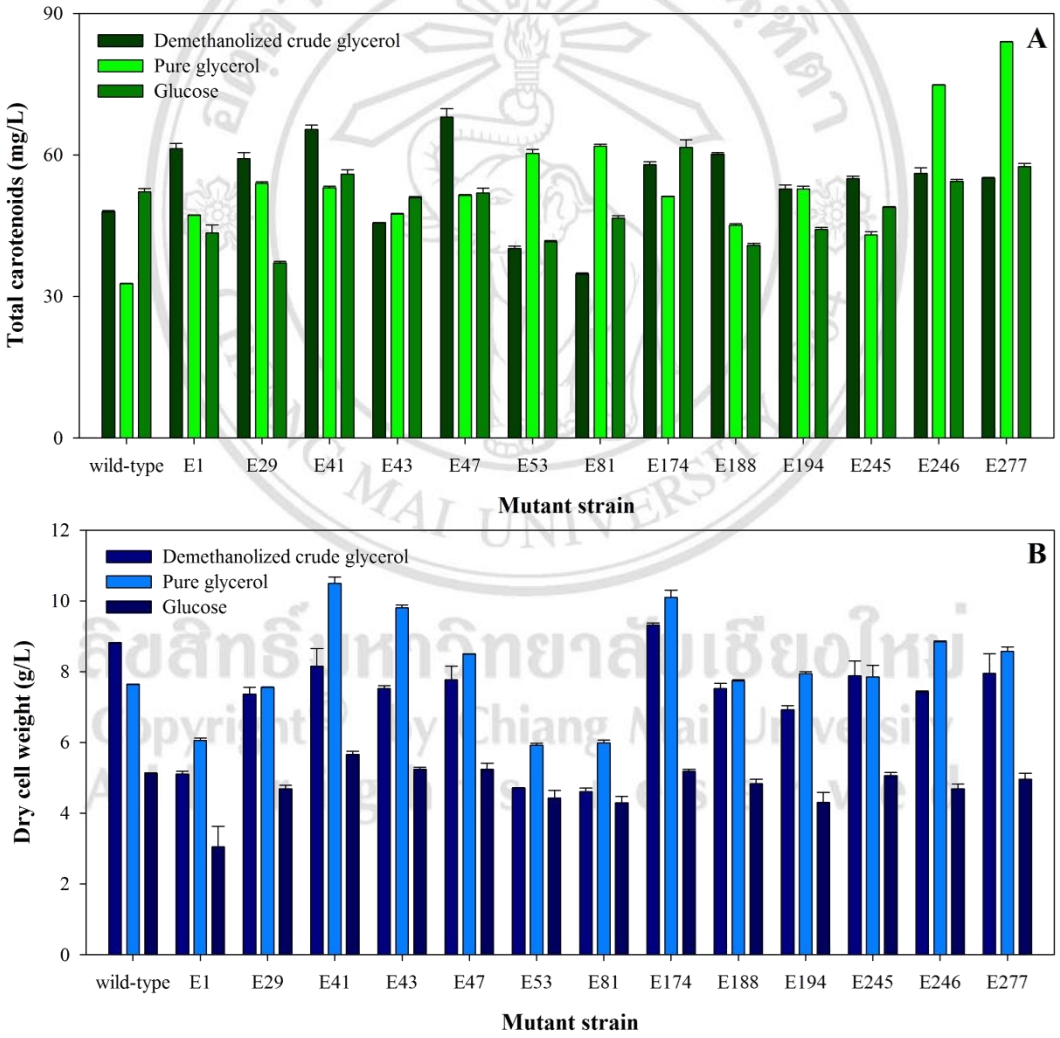

Figure 6.4 Effect of different carbon sources on total carotenoids (A) and dry cell weight (B) produced by the wild-type and EMS-induced mutant strains of *Sporidiobolus pararoseus* KM281507

6.3.3 5-Bromouracil mutagenesis

The chemical 5-bromouracil (5BU) is incorporated into DNA in place of thymine; it acts like thymine in DNA replication and, since hydrogen bonding is not changed, it should induce no mutation. However, it seems that the bromine atom causes 5BU to tautomerize more readily than thymine does. Thus 5BU goes from the keto form to the enol form more readily than thymine. Frequent transitions result when the enol form of 5BU pairs with guanine (Tamarin, 1996). The results showed that the initial cell concentration of 1.9×10^7 CFU/mL was decreased by increasing the 5BU concentration (Figure 6.5). A similar inverse relationship was observed between cell concentration and 5BU incubation time.

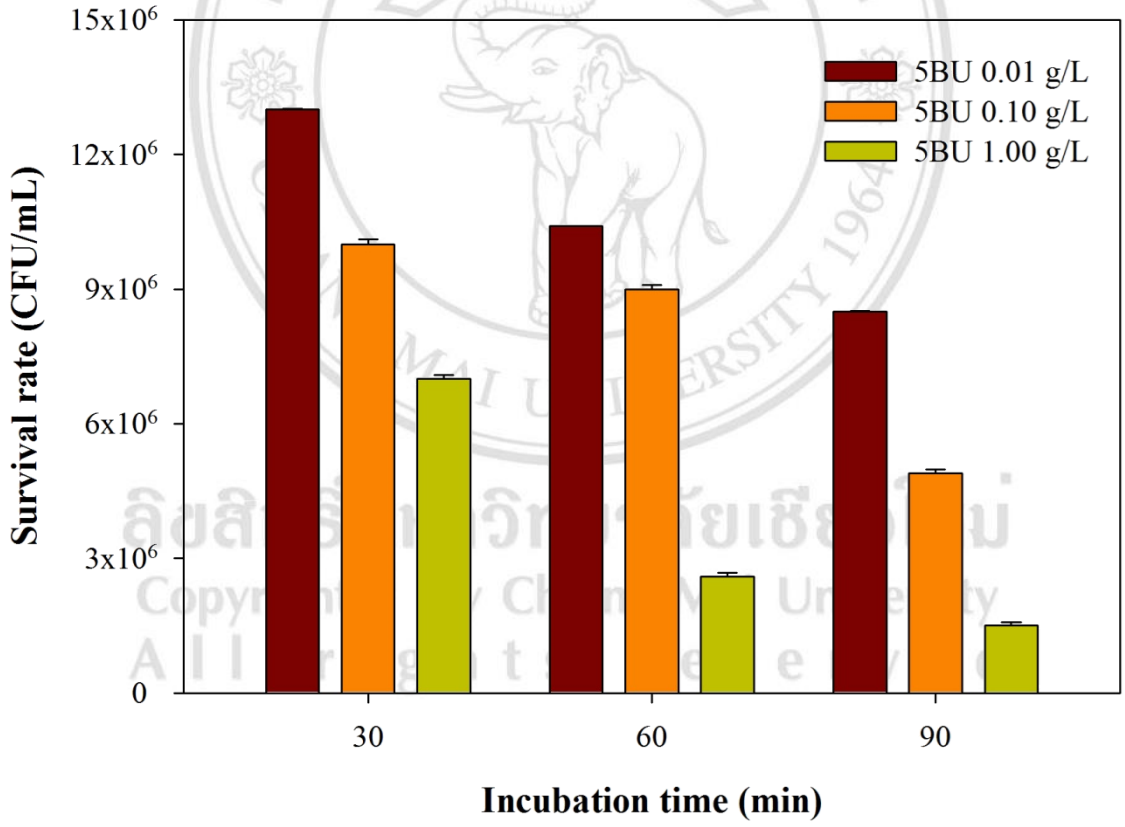

Figure 6.5 Effect of 5BU concentration and incubation time on the survival of *Sporidiobolus pararoseus* KM281507

After 5BU mutagenesis, more than 500 red colonies were obtained. Eight of those mutants, B35, B37, B39, B75, B89, B142, B151 and B239, showed deeper red than the wild-type on the basal medium supplemented with pure glycerol. The total carotenoid contents of the mutants grown using different carbon sources are shown in Figure 6.6. Among those eight mutants, B151 had the highest total carotenoid content (74.01±0.16 mg/L) when cultivated using demethanolized crude glycerol as the carbon source, which represented a 54.28% increase over of the wild-type (47.97±0.26 mg/L) (Figure 6.6A). The DCW of B151 (9.82±0.06 g/L) was significantly higher than wild-type (8.81±0.01 g/L) (Figure 6.6B). production yield of 0.62±0.05 mg/L, 1.72±0.16 mg/L and 1.59±0.01 g/L in BMP and 1.17±0.01 mg/L, 1.77±0.01 mg/L and 2.05±0.02 g/L in BMC, respectively. In comparison, BMC increased the β-carotene, total carotenoids and lipids content over BMP by approximately 47.00, 2.82 and 22.44%, respectively. Crude glycerol could enhance β-carotene synthesis because it contained trace elements e.g. Ca, K, Mg, Na, P, and S in term of ash content (Thompson and He, 2006). The percentage of ash content in demethanolized crude glycerol used in this study was 6.12±0.05%. Trace elements have been demonstrated to act as stimulants for growth of red yeasts, which had a stimulatory effect on β-carotene and γ-carotene synthesis. The observed effect of trace elements on the biosynthesis of specific lipids and carotenoids in oleaginous red yeasts may be explained by an activation or inhibition mechanism by selected metal ions on specific lipogenesis and carotenogenic enzymes, in particular, on specificity of the desaturases which are involved in β-carotene, other carotenoids and lipids biosynthesis (Frengova and Beshkova, 2009).

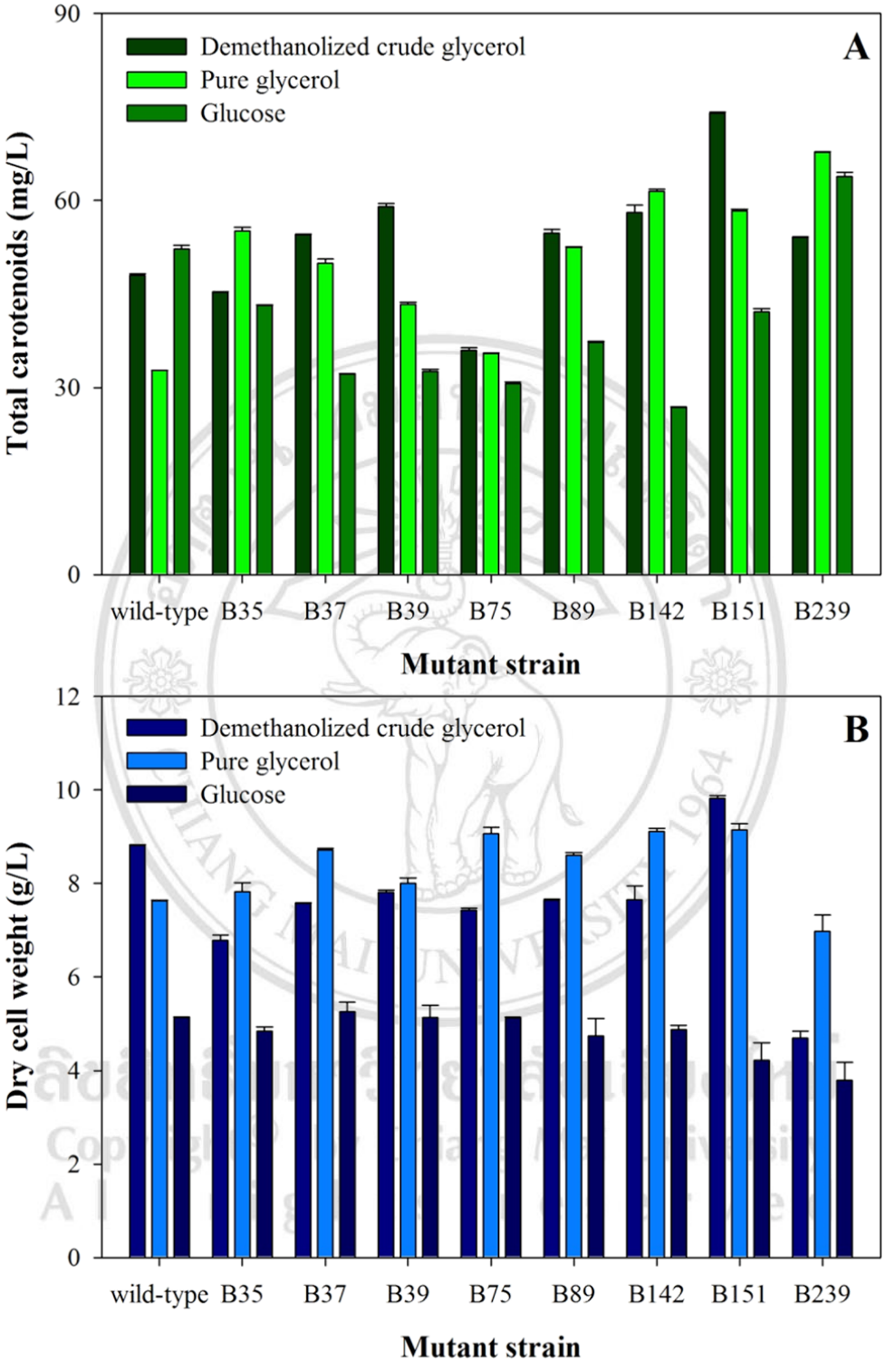

Figure 6.6 Effect of carbon source on total carotenoids (A) and dry cell weight (B) produced by the wild-type and 5BU-induced mutant strains of *Sporidiobolus pararoseus* KM281507

6.3.4 Carotenoids and lipids productions by mutant strains

The results of a comparison of DCW, total carotenoids and total lipids among *Sporidiobolus pararoseus* KM281507 (wild-type) and mutants strains U10 (UV mutagenesis), E47 (EMS mutagenesis), B151 (5BU mutagenesis) are indicated in Table 6.1. We found that the B151 strain could use demethanolized crude glycerol as a source of energy and exhibited relatively high DCW, total carotenoid and total lipid production. Only enhancement carotenoid production was observed with the E47 strain. These results are similar to those of Li et al. (2007) who found that after serial UV, EMS and NTG mutagenesis, a mutant strain MM of the red marine yeast strain *Rhodotorula* sp. produced 603.93 μg/g of carotenoids, while only 213.18 μg/g were produced by the wild-type under the same conditions.

Table 6.1 Comparison of DCW, total carotenoids and lipids produced by the wild-type and mutant strains of *Sporidiobolus pararoseus* KM281507 using demethanolized crude glycerol as the carbon source

Mutant strain	**DCW (g/L)**	**Total carotenoids (mg/L)**	**Lipids (g/L)**	**Lipids content (% g/g)**
Wild-type	8.81±0.01[*]	47.97±0.26	4.13±0.01	46.88±0.06
U10	8.87±0.27 (0.68%)[a]	69.37±1.91 (44.61%)[a]	4.88±0.03 (18.16%)[a]	55.04±1.34 (17.42%)[a]
E47	7.77±0.38 (-11.80%)[b]	67.99±1.80 (41.73%)[a]	3.06±0.04 (-25.91%)[b]	39.43±1.42 (-15.89%)[b]
B151	9.82±0.06 (11.46%)[a]	74.01±0.17 (54.28%)[a]	5.28±0.06 (27.85%)[a]	53.77±0.28 (14.69%)[a]

[*]Means and standard deviations of triplicate samples

[a]Increasing over the wild-type, %

[b]Decreasing compared to the wild-type

The U10 and B151 strains grew well in demethanolized crude glycerol, and produced 0.68% and 11.46% more DCW than wild-type, respectively, while the DCW of E47 was less than wild-type (-11.80%). Similarly, the total lipids and lipids content of U10 and B151 were 4.88±0.03 g/L (18.16%), 5.28±0.06 g/L (27.85%) and 55.04±1.34 (17.42%), 53.77±0.28 (14.69%) higher than wild-type, respectively, while the total lipids and lipids content of E47 was less than wild-type -25.91 and -15.89%, respectively. However, U10, E47 and B151 produced relatively high total carotenoids of 44.61, 41.73 and 54.28%, respectively, compared to the wild-type.

6.4 Conclusions

Demethanolized crude glycerol was found to be a good carbon source and considered to be a cost effective raw material for carotenoid production by the red yeast *Sporidiobolus pararoseus* KM281507. A mutant B151 obtained by 5BU mutagenesis produced the highest level of DCW (9.82±0.06 g/L), total carotenoids (74.01±0.17 mg/L) and total lipids (5.28±0.06 g/L), which were 11.46%, 54.28% and 27.85% higher than wild-type, respectively, when demethanolized crude glycerol was used as the sole carbon source. Furthermore, this study demonstrated that B151 shows high potential in the biotransformation of crude glycerol to high value-added products, carotenoids and lipids, and represents efficient renewable resource utilization.