Improved lycopene production from different substrates by mated fermentation of

Blakeslea trispora

Ayse Sevgili, Osman Erkmen[1]

Department of Food Engineering, Faculty of Engineering, University of Gaziantep, 27310 Gaziantep, Turkey, erkmen@gantep.edu.tr, Tel: +90 342 3172313

[1]Address correspondence to Prof. Dr. Osman Erkmen

Department of Food Engineering, Faculty of Engineering, University of Gaziantep, 27310 Gaziantep, Turkey   Tel: +90 3423172313   Fax: +90 3423172362

e-mail: erkmen@gantep.edu.tr

Abstract: The production of lycopene from different substrates by *Blakeslea trispora* in fermentation was investigated. Lycopene productions from 4 and 6 % glucose (pH 6.5) in shake flask fermentation were 77.7 and 28.1 mg L^{-1}. A maximum lycopene concentration of 944.8 mg L^{-1} was detected with 4 % glucose supplemented with 1.0 % sunflower oil in fermentor studies. Zygospores of *B. trispora* are the morphological forms, which are responsible for the production of the lycopene. The highest level of zygospores was correlated with the highest amount of intracellular lycopene in the total biomass dry weight. The media containing only orange peel (1 %) gave a 4.9 mg L^{-1} lycopene production in a fermentor. The presence of oils as substrates resulted in enhanced mold growth and subsequent higher lycopene production. Substrates containing linoleic acid compounds led to high lycopene production. The data showed that the biosynthesis of lycopene starts in most cases simultaneously in the early growth phase even in trace amounts and the amount of lycopene formation increased continuously from 2 to 7 days.

Keywords: Lycopene, *Blakeslea trispora*, fermentation, vegetable oil, orange peel

1. Introduction

Lycopene is an important naturally occurring intermediate metabolite in the mevalonate metabolic pathway of microorganisms and can be transformed into beta-carotene by lycopene cyclase. Thus, in order to enhance lycopene accumulation, lycopene cyclase activity should be inhibited by adding lycopene cyclase inhibitors into the fermentation medium. Lycopene has important physiological functions to prevent chronic diseases, including osteoporosis, certain types of cancer, cataract formation and cardiovascular diseases; enhance immune responses; and other biological effects in humans and animals [1,2]. In last few years, demand on lycopene has increased due to its use in the food, pharmaceutical, cosmetic and animal feed industries [3]. Moreover, lycopene is used as an antioxidant to reduce cellular or tissue damage, and as a coloring agent for food products [4]. These make lycopene very attractive in the food and feed industries, in medicine and cosmetic formulation. Lycopene used industrially is manufactured from plant extraction and chemical synthesis. Market needs for lycopene are not met using plant extraction due to the restricted number of plant sources and high cost. Chemical synthesis also has disadvantages including low yields, product instability, low product quality and high production cost. Due to decreased natural production of lycopene and its increased worldwide demand there is the potential for lycopene to be produced using fermentation. Various algae and fungi produce intracellular carotenoids, including β-carotene, lycopene and astaxanthin. *Blakeslea trispora*, *Mucor circinelloides*, *Candida utilis* and *Phycomyces blakesleeanus* have the ability to produce lycopene [5]. *B. trispora* has an advantage in that it does not need any specific fermentation conditions for growth. Mating type of (+) and (−) *B. trispora* strains together can increase production of lycopene [6].

There is much interest in biotechnological production of lycopene due to consumer demand for high quality and "natural" food additives. Efforts have been made to increase the lycopen yield and reduce process costs, by using low-cost agro-food media rich in organic compounds (such as carbon, nitrogen, mineral and other sources). The amount of lycopene production depends on the microorganism, the culture medium, substrates and the fermentation conditions (temperature, pH, aeration rate etc.). Most of the research has been aimed at optimizing the culture conditions that directly affect the growth of the microorganism. The main research is focused on optimization of the medium, extraction of lycopene from *B. trispora* and fermentation conditions [4-9].

An industrial mold over-producing lycopene is *B. trispora* which accumulates all-trans-carotene, as a secondary metabolite [10]. *B. trispora* is a saprophyte and performs vegetative cycle of spores and filamentous mycelia [6]. There has been a lot of effort to use food wastes in the production of lycopene mitigation [11]. At present, there are no research on the use of orange peel and sunflower oil in the production of lycopene. The transport cost, sales problems, low quality and susceptibility to spoilage of the food-wastes by microorganisms have led to alternative utilization approaches. Agro-food wastes (fruits and vegetables: cabbage residues, watermelon husk and peach peels) have been used in the production of carotenes by *B. trispora* [12,13]. Optimization of fermentation conditions can also be required in the production of lycopene from different nutrient sources. This report was focused on the effect of a series of media containing various substrates (glucose, refined vegetable oils and orange peel) in the accumulation of lycopene in submerged shake flasks and fermentation studies. Morphological, biomass and pH changes occurring during fermentations were also studied.

2. Materials and Methods

2.1. Microorganism and Chemicals

Blakeslea trispora mating strains ATCC 14271 (+) and ATCC 14272 (-) were obtained from the American Type Culture Collection (ATCC; Rockville, MA, USA). Lyophilized cultures were rehydrated with malt extract broth (MEB; Darmstadt, Germany) by incubation at 35°C for 3 days and were used in the stock culture preparation. Stock cultures of *B. trispora* were prepared on potato dextrose agar (PDA; Darmstadt, Germany) slant. Yeast extract, glucose, agar, asparagine, starch, lycopene, nicotine, KHPO$_4$, MgSO$_4$, HPLC grade petroleum ether, methanol, acetonitrile and dichloromethane were obtained from Sigma-Aldrich (Interlab Company, Adana, Turkey).

2.2. Preparation of B. trispora *Cultures*

B. trispora (+) and (-) cultures were prepared for fermentation studies using a yeast phosphate soluble starch (YpSS) agar medium. The composition of YpSS agar medium (g L^{-1}) was: Yeast extract 4.0, K$_2$HPO$_4$ 1.0, MgSO$_4$ 0.5, starch 15.0 and agar 20.0. The pH of the medium was adjusted to 6.5. Each of the *B. trispora* strains from stock culture was inoculated separately onto YpSS agar plates and incubated at 28°C for 5 days. After sporulation of cultures, 10 ml of sterile distilled water were added onto the cultures in the Petri plates and the spores were collected by scraping off the medium surface using a sterile cotton swab. The spore suspension containing 3.9-8.5x10^6 spores ml^{-1} was used to inoculate the fermentation media. Strains were inoculated into the fermentation medium with 2.5 and 7.5 ml of spore suspension (+) and (-) strains, respectively, to provide 1:3 ratio of inoculation.

2.3. Preparation of Fermentation Media

The basal fermentation medium (BFM) had the following composition (g L^{-1}): Yeast extract 1.0, asparagine 2.0, K_2HPO_4 1.5 and $MgSO_4$ 0.5. BFM was supplemented with different concentrations of ingredients as given in Table 1: refined vegetable oils (sunflower and corn oils), glucose and orange peel. Fermentation media were prepared at two different pH: 6.5 and 5.5. Fermentation media (50 ml) in Erlenmeyer flasks (250 ml) were sterilized at 121°C for 15 min in autoclave. Fermentation media for fermentor studies were sterilized in a sterilizable-in-place bench top fermentor (7 L; New Brunswick Scientific BioFlo 410 SIP Fermentor, Eppendorf, MA USA).

2.4. Shake Flask Fermentation Process

Fermentation media (50 ml) in Erlenmeyer flasks (250 ml) were inoculated with 10 ml *B. trispora* strains ATCC 14271 (+) and 14272 (-) by 1:3 ratio from spore stock culture. The lycopene cyclase inhibitor of nicotine (5 mM^{-1}) was added on the second day of fermentation. Fermentation media (total volume 60 ml) were incubated at 28°C for 7 days on a bench type water bath shaker ST-402 (NÜVE; Sanayi Malzemeleri Imalat ve Ticaret A. Ş., İstanbul, Turkey) with wrist shaking at 120 rpm.

2.5. Fermentation Process in Fermentor

Four liter of fermentation medium were prepared in a 7 liter fermentor. The compositions of base fermentation medium consisted of (g L^{-1}): glucose 40, asparagine 2, yeast extract 1, KH_2PO_4 1.5 and $MgSO_4$ 0.5. The initial pH of fermentation was adjusted to 6.5. Fermentation media for fermentor studies were prepared as given in Table 1. The fermentation with 4 % glucose media was also performed at two aerations and

agitations. The fermentor was incubated at 28ºC. The inoculation ratio of two strains (+/-) into the fermentation medium from plate cultures was the same as those in the shake flask. The lycopene cyclase inhibitor of nicotine (5 m/M) was added on the second day of fermentation. Dissolved oxygen tension was not controlled during fermentation.

2.6. Sampling

Fermented samples were aseptically removed from the shake flask and fermentor after 2, 3, 4, 6 and 7 days of fermentation. Initial samples were also removed at the beginning of fermentation. At every sampling interval, 5 ml of sample was taken from the fermentation media and the contents were analyzed for lycopene, pH and biomass changes. Two ml of the sample was used in lycopene analysis and 3 ml was used in pH and biomass analysis. At each sampling time and after the first day, 0.4 ml of the sample was also removed for morphological analysis.

Two samples were removed and two parallel analyses were performed from each sample. All experiments were repeated three times.

2.7. Analysis

2.7.1. Lycopene Analysis

Samples were analyzed for lycopene by the HPLC method [1]. Two ml of a 5 ml sample was mixed with 15 ml of petroleum ether. The mixture was subjected to ultrasound (Soniprep 150 Ultrasonic Disintegrator, MSE, UK) for 30 sec and then, the sample was centrifuged using a table-type centrifuge (Hettich eba III) at 6000 rpm for 15 minutes. The supernatant was passed through 4 µm filter paper. About 2.5 ml of liquid was added into a 3 ml vial and the sample was analyzed by HPLC immediately. The

presence of lycopene was detected by HPLC using a fluorescence detector. The flow rate was 1 ml min^{-1} and the column was nukleosil C18 (250 x 4.6 mm ID). Temperature was 28°C. Lycopene was detected at 450 nm and 20 µm sample was injected to HPLC automatically. The result was read for 40 minutes. The mobile phase was acetonitrile-methanol-water-dichloromethane (7:1.5:0.5:1, v:v:v:v). The mobile phase was filtered through a disposable filter unit (0.45 µm) and degassed in a degasser. The peaks in a chromatogram were evaluated according to the lycopene standard curve.

Analytical Method Validation: Linearity was determined between 0.1 to 0.0001 g ml^{-1} using five levels of calibration in triplicate. The Mandel's fitting test was used to evaluate the linearity of the straight-line regression model [14].

Limit of detection (LOD) and limit of quantification (LOQ) were determined according to the method described by Cucu et al. [15]. For this, standards of 0.1 to 0.0001 g lycopene ml^{-1} were prepared and injected three times each. The mean of the slopes (S) and standard deviation of the intercepts (σ) were calculated from the three calibration curves. The LOD and the LOQ were calculated according to the formulas below [15]:

LOD=(3.3∗σ)/S

LOQ=(10∗σ)/S

For the assessment of the matrix effect, calibration curves were prepared in the petroleum ether and analyzed in triplicates. The calibration curve of lycopene was the equations: y = ((area+intercept)/slope).

2.7.2. pH, Biomass and Morphological Analysis

8

In biomass analysis, Whatman No. 41 filter paper was dried at 105℃ to constant weight, cooled in a desiccator and weighed before use. Three ml of the remaining sample was filtered through the filter paper. The filter cake on paper was washed three times with distilled water. The filter paper was placed into an incubator at 105℃, dried until constant weight and then weighed to calculate the biomass (g L^{-1}).

In pH analysis, a filtered solution was filled to 10 ml with distilled water and pH was detected using a pH meter (EMAF EM78X model) equipped with a glass electrode.

In image analysis, the morphological changes of *B. trispora* during the fermentation was analyzed using an image analysis system consisting of a light microscope (Olympus BX51; Tokyo, Japan) equipped with a Pixera PVC 100C camera. Samples for image analysis were prepared by mixing 0.4 ml of fermentation broth with 0.1 ml of lactophenol blue. One drop of the mixed sample was pipetted on to a microscope slide, covered by a cover slip so that no air gap or bubbles were trapped between the sample and the coverslip and observed immediately with 20X objective by taking 5 different images. The morphological parameters measured were the area of zygospores and zygospores (% of total area of mycelium), the diameter of zygospores (mm), freely dispersed hyphae and clumps (aggregates) of different sizes on the basis of their projected area, and the hyphal length (mm). The microscope eyepiece is equipped with a scale micrometer that is used to measure the morphological parameters of mold. For each sample, the process was repeated at least 5 times using new positions on the same slide, and the morphological parameters were expressed as the mean values of each sample [16].

2.8. Statistical Analysis

All data were analyzed by SPSS 16 software (SPSS Inc, Chicago, IL). Statistical differences between fermentation time (days) and between substrates used in the fermentations were tested by one-way analysis of variance following Duncan's multiple-range test. A probability of 0.05 was used to determine statistical significance.

3. Results

The fermentations were carried out at 28°C for 7 days using different carbon sources and ingredients in shake flask and fermentor.

The initial pH is one of the important parameters in the production of lycopene by *B. trispora*. The pH of the fermentation media changed during the fermentation time in the medium. The pH decreased and increased during the 7 days fermentation in the shake-flask and fermentor studies. The pH of the fermentation media was decreased during the first 2 days then increased during the remaining 7 days. At 2 days, the pH reduced to approximately 5.0, and then the pH increased to over 6.0. The pH drop would be due to trisporic acid or/and other unidentified acidic formations in the lycopene production [17]. According to Nanou et al. [18], the pH of the lycopene production fermentation broth decreased slightly during the first 2 days of the fermentation from 7.6 to 5.3 and then increased slowly up to 7.0 at the end of the fermentation (10 days). The pH of the fermentation medium was probably increased by ammonia liberation during degradation of proteins of the medium by the mold. This would be indicative of amine metabolic product being released as a consequence of cell death and subsequent lysis occurring after that time.

3.1. Shake Flask Studies

Lycopene production of two glucose concentrations (4 and 6 %) were investigated at two initial pH values (6.5 and 5.5). Figs. 1 and 2 show the effect of glucose concentrations on lycopene production at the initial at pH 6.5 and 5.5 respectively. Lycopene production at pH 6.5 with both glucose concentrations was higher than at pH 5.5. The amount of lycopene production was 77.7 and 28.1 mg L^{-1} with 4 and 6 % glucose, respectively, after 7 days fermentation at pH 6.5. The biomass dry weight (DW) increased with the increase in glucose concentration from 4 to 6 %. The lowest amount of lycopene content (0.3 mg g^{-1} DW) was obtained at pH 5.5 with glucose concentration 6 %. There is an indirect relationship between biomass and lycopene concentration. Subsequently, increasing the glucose concentration to 6 % resulted in a decrease in lycopene production by 36.2 %. The decreased concentration of lycopene encountered with the highest concentration of glucose was probably due to osmotic effects. It has been reported that above a critical substrate concentration, the decreased water activity and the onset of plasmolysis combine to cause a decrease in the rates of fermentation and product formation [19]. In all cases, the biomass DW increased during the first two days of the fermentation and then remained constant until the maximum concentration of the lycopene was obtained. The maximum lycopene content was 2.4 and 0.6 mg g^{-1} DW with 4 and 6 % glucose, respectively, at pH 6.5 after 7 days while they were 0.7 and 0.1 mg g^{-1} DW after 4 days, respectively. There are significant differences ($p<0.05$) in lycopene production during fermentation. There are also significant differences ($p<0.05$) in lycopene production between 4 and 6 % glucose concentrations, and pH 6.5 and 5.5 at both glucose concentrations.

Figs. 3 and 4 show the effect of sunflower and corn oils, respectively, on the production of lycopene in 4 % glucose medium at initial pH 6.5. The maximum lycopene was

produced 795.1 and 714.2 mg L^{-1} with 1 % sunflower and corn oils respectively. The highest amount of lycopene (335.4 mg L^{-1}) was produced with 3 % sunflower oil rather 3 % corn oil (275.3 mg L^{-1}). Lycopene content was 131.4 and 115.0 mg g^{-1} DW at the end of fermentation for 1 % sunflower and corn oils respectively. There are significant differences ($p < 0.05$) in lycopene production during the 7 days of fermentation. There are also significant differences ($p < 0.05$) in lycopene production with and without oil, and between both oil types.

Lycopene productions from 1 and 2 % orange peel during 7 days of shake flask fermentation were given in Fig. 5. Lycopene productions after 7 days were 2.9 and 1.7 mg L^{-1} with 1 and 2 % orange peel respectively. Biomasses formations were 8.6 and 11.6 g L^{-1} for orange peel respectively. Lycopene content was 0.3 and 0.1 mg g^{-1} DW respectively at the end of fermentation. There were significant differences ($p < 0.05$) in lycopene production during the 7 days of fermentation, between the two orange peel concentrations.

3.2. Fermentor Studies

Effects of 4 and 6 % glucose concentration (pH 6.5) on the production of lycopene were studied at 3 L/min aeration with 500 rpm agitation (Fig. 6). The highest amount of lycopene was produced (92.3 mg L^{-1}) with 4 % glucose concentration rather than 6 % glucose (57.8 mg L^{-1}). Biomass DW formations after 7 days of fermentation were 154.9 and 196.7 mg L^{-1} with 4 and 6 % glucose concentration respectively. Lycopene content was 3.8 and 1.9 mg g^{-1} DW at the end of fermentation for 4 and 6 % glucose concentrations respectively. There is an indirect relationship between biomass and lycopene formation similar to the shake flask. The production of lycopene in the

fermentor was higher than in the shake-flask from glucose. This would be due to the better aeration of the medium in the fermentor. There were significant differences ($p<0.05$) in lycopene production during the 7 days of fermentation. There were also significant differences ($p<0.05$) in lycopene production between 4 and 6 % glucose concentrations.

The effect of the high level of aeration (12 L min^{-1}) and agitation (500 rpm) on the production of lycopene in a 4 % glucose medium during the 7 days of fermentation is given in Fig. 7. The highest amount of lycopene was produced at low level of aeration (3 L min^{-1} aeration and 500 rpm agitation) rather than at the high levels (12 L min^{-1} and 750 rpm). The highest amount of biomass was formed at the higher aeration (12 L min^{-1}) and agitation (750 rpm) rather than at the lower levels. Lycopene content of DW (3.8 mg L^{-1}) was higher at 3 L min^{-1} aeration and 500 rpm agitation rather than 12 L min^{-1} at 750 rpm (1.8 mg L^{-1}). Generally, the results showed that the biomass increased with increasing aeration and agitation. This was due to the better air supply to the cells. This is especially important for high biomass concentrations. *B. trispora* is a strictly aerobic microorganism. Thus, moderate aeration rates in combination with high impeller agitation improved the growth of mold. There are significant differences ($p<0.05$) in lycopene production during the 7 days of fermentation and between the two fermentation conditions (3 and 12 L min^{-1} aeration).

Fig. 8 shows the effect of 1 % sunflower and corn oils on the lycopene production in the fermentor. Lycopene production was 944.8 and 859.8 mg L^{-1} respectively. Biomass formations were 29.5 and 19.6 g L^{-1}. Lycopene content was 65.0 and 48.9 mg g^{-1} DW. Lycopene production in the presence of sunflower and corn oils in the fermentor was 12.2 and 11.1 times higher, respectively, then without oil from 4 % glucose in a shake flask. This would be due to retaining higher amount of oxygen in the media. Since oil slightly

increases the density of the fermentation media. There are significant differences ($p<0.05$) in lycopene production during fermentation periods. There are also significant differences ($p<0.05$) in lycopene production with and without oil, and between sunflower and corn oils.

Lycopene production was studied from different concentrations of orange peel in 5 L fermentation media with aeration 3 L min^{-1} and agitation 500 rpm (Fig. 9). Lycopene was produced 4.9 mg L^{-1} with 1 % orange peel. Biomass was 9.7 g L^{-1} and lycopene content was 0.05 mg g^{-1} DW at the end of fermentation. Lycopene production from orange peel was two times higher in the fermentor than in the shake flask. There are significant ($p<0.05$) difference in lycopene production during the 7 days of fermentation.

3.3. Morphological Analysis

The morphology of *B. trispora* was studied in the shake-flask and fermentor studies (3 L min^{-1} aeration and 500 rpm agitation) in 4 % glucose medium containing 1 % sunflower oil using an image analysis system. During the first day of fermentation, microscopic examination showed that *B. trispora* formed a great amount of mycelium in the fermentation broth. After one day of fermentation, the two opposite mating types of the microorganism produced zygospores which grew towards each other and produced pro-gametangia at their tips. Septation in the pro-gametangia led to the production of terminal gametangia, which fused to form the zygospores. The area of zygospores to the total area of mycelium remained almost constant up to 4 days of fermentation and then decreased significantly and disappeared on the 7th days of fermentation. On the other hand, the area of zygospores to the total area of mycelium increased significantly with the increase of fermentation time from 4 to 7 days. The hyphal length increased from the first day of

fermentation up to 4 days and then slightly increased until the end of the fermentation. The diameter of zygospores increased from the first 2 days of incubation up to 4 days and then decreased slightly. Generally, morphological measurements using image analysis showed that *B. trispora* formed zygophores, zygospores and mycelium in which the hyphae appeared as a homogeneous dispersed suspension through the fermentation medium.

4. Discussion

Various concentration of carbon sources and refined natural vegetable oils as co-substrates were used in the production of lycopene. The concentration of carbon sources and vegetable oils greatly affected the final amount of lycopene. Furthermore, the use of orange peel components gives a first indication that *B. trispora* is able to metabolize and produce lycopene.

The highest concentration of lycopene was produced from a low concentration of glucose (4%) rather than at the high concentration (6 %) in both the shake flask and fermentor studies. High concentrations of glucose may cause the inhibition of the activity of mevalonate kinase [20], which is one of the key enzymes of the lycopene biosynthesis pathway. The carbon source can act as a major constituent for the building of cellular material and as an important energy source during the microbial fermentations [21]. Lycopene production in all fermentation conditions was lower than 1.0 mg L^{-1} during the first 2 days of fermentation. Lycopene production continuously increased during the next 5 days. It reached maximum concentration after 7 days. On the other hand, biomass formation increased continuously during the 6 days of fermentation and it slowed on last day. When lycopene production increased, biomass formation decreased. Dry weight

increased continuously up to 6 days and then slowed down. But lycopene production rate was higher on the last day. The maximum lycopene content in cells occurred after 7 days of fermentation when compared with 6 days in all fermentation conditions. This indicates that lycopene and biomass are inversely proportional. The biomass rapidly increased after 2 days of fermentation and this continued up to 6 days of fermentation. Nanou et al. [18] reported that the concentration of residual sugars fell rapidly during the first 4 days of the fermentation after which it decreased slowly and this was accompanied by a rapid increase of biomass concentration.

The refined vegetable oils stimulate different biosynthetic pathways in *B. trispora*. In this study, about 944.7 mg L^{-1} of lycopene was produced in fermentor studies from 4 % glucose supplemented with 1 % sunflower oil. With 1 % corn oil, about 859.8 mg L^{-1} of lycopene was produced in a 4 % glucose medium. These results indicate that sunflower oil has a greater influence on lycopene production than corn oil due to a higher amount of vitamin E and linoleic acid in sunflower oil. Beside triacylglycerol, the main component of oils can be used in the lipid biosynthesis (involving acetyl-CoA formation resulting from β-oxidation of fatty acids) of the mold mycelium. Oils can be hydrolyzed to fatty acids and glycerol by mold exolipases [22]. When the concentration of natural oils increases (from 1 to 3 %), the production of the lycopene decreases while the biomass dry weight increases significantly. This may be related to the consumed amount of the oils converted to biomass instead of lycopene. The production of lycopene without oils in the medium was lower while biomass formation was higher.

Two different aerations (3 and 12 v min^{-1}) and agitations (500 and 750 rpm) were studied in a 7 L fermentor (pH 6.5). The highest amount of lycopene 944.8 mg L^{-1} was produced with 3 V min^{-1} aeration and 500 rpm agitation in 4 % glucose medium

containing 1 % sunflower oil. The concentration of lycopene was higher in fermentor studies than in the shake flask. Aeration could be beneficial to the growth and performance of microbial cells. Agitation of the fermentation medium creates shear forces which affect microorganisms in several ways: causing morphological changes, variation in their growth and product formation, and damaging the cell structure [19]. *B. trispora* is an aerobic microorganism and requires the oxygen in the production of lycopene.

Orange peel was used as a raw material for production of lycopene in a shake flask and fermentor. The highest amount of lycopene (4.8 mg L^{-1}) was produced in the fermentor with 1 % orange peel rather than in the shake flask (2.9 mg L^{-1}). Citrus peels are rich in cellulose, hemicelluloses, proteins and pectin, but the fat content is low. Pourbafrani et al. [21] reported that more than 95 % of the peel oil is D-limonene which is extremely toxic to fermenting microorganisms. The addition of tomato sauce as a waste into the fermentation medium enhanced the production of lycopene and its production was 3.52 mg/100 g cell DW [23].

B. trispora is a micro-fungus with a life-cycle involving hyphae, zygospores and zygospores in submerged fermentation. The zygospores are the precursors of zygospores, which are responsible for the production of lycopene. Morphological forms of *B. trispora* zygospores are responsible for the production of the lycopene [19]. The highest percentage of zygospores in an observed microscope field was correlated with the highest percentage of intracellular lycopene in the total biomass dry weight after 7 days of fermentation. High percentages of vacuolated hyphae, evacuated cells and degenerated hyphae of *B. trispora* were observed at higher aeration (12 V/min). The percentage of the vacuolated hyphae, evacuated cells and the degenerated hyphae formed during the first 2 days of fermentation were higher than for those during the remaining fermentation time.

The growing mycelium was composed primarily of intact hyphae (such as 96.8% of the biomass dry weight), and zygospores on the 2nd day of fermentation, whereas on the next day the concentration of the intact hyphae was reduced to 67.9 of the biomass dry weight and the concentration of the zygospores was about 8.3 %. On the 7th day of fermentation, the mycelium was composed mainly of evacuated cells. The addition of 1 % oils to the fermentation medium increased the concentration of the intracellular lycopene during fermentation and reached to maximum level after 7 days, whereas the percentage of zygospores to the biomass dry weight increased significantly. The highest concentration of lycopene correlated with the highest percentage of zygospores in the biomass DW. When the natural oils were used in the medium with 4 % glucose in the fermentor, a great increase in the amount of lycopene occurred after the days 7 of fermentation, while the concentration of evacuated cells and the number of zygospores increased significantly.

The effect of aeration rate and agitation speed on culture morphology was also observed. At an aeration rate of 3 V min^{-1} with 500 rpm agitation, the area of zygospores increased significantly and then remained constant. On the other hand, at aeration rates of 12 V min^{-1} with 750 rpm agitation, the area of zygospores decreased and then remained constant. The maximum concentration of lycopene was observed in the same conditions where a large number of zygospores were observed. This means that the zygospores were responsible for the synthesis of lycopene.

5. Conclusions

In this study, various substrates are compared with lycopene production in a shake flask and a fermentor. The amount of lycopene production is based on the concentration of carbon sources and natural oil content. The amount of orange peel used in the

fermentation as carbon sources affected the formation of lycopene. The use of natural oil is important for stimulation of the biosynthetic pathway in *B. trispora*. The medium with natural oils led to more lycopene than only carbon sources in the medium. The oils degraded into acetyl-CoA, which is the precursor of lycopene derived from mevalonic acid. The mated fermentation process for lycopene production may be useful and a reference to the other fermentation. Also, lycopene production from *B. trispora* is better than other alternative plant extracts which is one of the other methods of lycopene production. For the future, this study is provided to contribute to the development in the lycopene production industry from this method. Oils improved lycopene production and enhanced vacuolation and evacuation of the hyphae. The morphological characteristics of the hyphae and the proportion of zygospores to the biomass dry weight were changed significantly with in the medium composition and fermentation conditions. Moreover, there was a parallel relationship between the area of zygospores and production of lycopene. Generally, the results showed that *B. trispora* is a micro-mold that produces lycopene with a life-cycle involving hyphae, zygospores and zygospores. The hyphae do not play a role in the biosynthesis of lycopene. The zygospores are the precursors of zygospores, which are responsible for the production of the lycopene. Maximum lycopene concentration was obtained when the medium was supplied with sunflower and corn oils.

References

1. Shi, Y.; Xin, X.; Yuan, Q. Improved lycopene production by *Blakeslea trispora* with isopentenyl compounds and metabolic precursors. *Biotechnol. Letter* **2002**, *34*, 849-852.

2. Liu, X.-J.; Liu, R.-S.; Li, H.M.; Tang, Y.-J. Lycopene production from synthetic medium by *Blakeslea trispora* NRRL 2895 (+) and 2896 (-) in a stirred-tank fermenter. *Biopro. Biosyst. Eng.* **2012**, *35*, 739-749.

3. Valduga, E.; Valério, A.; Treichel, H.; Júnior, A.F.; Di Luccio, M. Kinetic and stoichiometric parameters in the production of carotenoids by *Sporidiobolus salmonicolor* (CBS 2636) in synthetic and agroindustrial media. *Appl. Biochem. Biotechnol.* **2009**, *157*, 61-69.

4. Wang, J.F.; Liu, X.J.; Liu, R.S.; Li, H.M.; Tang, Y.J. Optimization of the mated fermentation process for the production of lycopene by *Blakeslea trispora* NRRL 2895 (+) and NRRL 2896 (-). *Biopro. Biosys. Eng.* **2012**, *35*, 553-564.

5. Mehta, B.J.; Obraztsova, I.N.; Cerdá-Olmedo, E. Mutants and intersexual heterokaryons of *Blakeslea trispora* for production of beta-carotene and lycopene. *Appl. Env. Microbiol.* **2003**, *69*, 4043-4048.

6. Tereshina, V.M., Memorskaya, A.S., Feovilova, E.P. Dormant cells in the development cycle of *Blakeslea trispora*: distinct patterns of the lipid and carbohydrate composition. *Microbiol.* **2002**, *71*, 684-689.

7. Mantzouridou, F.; Roukas, T.; Kotzekidou, P.; Liakopoulou, M. Optimization of beta-carotene production from synthetic medium by *Blakeslea trispora*: A mathematical modeling. *Appl. Biochem. Biotechnol.* **2003**, *101(2)*, 153-175.

8. Tereshina, V.M., Memorskaya, A.S., Feovilova, E.P. Zygote formation in *Blakeslea trispora*: morphological peculiarities and relationship with carotenoid synthesis. *Microbiol.* **2003**, *72*: 448-454.

9. Lopez-Nieto, M.J.; Costa, J.; Peiro, E.; Mendez, E.; Rodriguez-Saiz, M.; de la Fuente, J.L.; Cabri, W.; Barredo, J.L. Biotechnological lycopene production by mated fermentation of *Blakesiea trispora*. *Appl. Microbiol. Biotechnol.* **2004**, *66*: 153-159.

10. Jeong, J.C.; Lee, J.; Park, Y.H. A unique pattern of mycelial elongation of *Blakeslea trispora* and its effect on morphological characteristics and beta-carotene synthesis. *Curr. Microbiol.* **2001**, *42(3)*, 225-228.

11. Stabnikova, O., Wang, J.Y., Ding, H.B., Tay, J.H. Biotransformation of vegetable and fruit processing wastes into yeast biomass enriched with selenium. *Biores. Technol.* **2005**, *96*, 747-751.

12. Bendini, A.; Lecce, G.D.; Valli, E.; Barbieri, S.; Tesini, F.; Toschi. T.G. Olive oil enriched in lycopene from tomato by-product through a co-milling process. *Int. J. Food Sci. Nutr.* **2015**, *66(4)*, 371-377.

13. Hernandez-Almanza, A.; Montanez, J.; Martínez, G.; Aguilar-Jiménezc, A.; Contreras-Esquivel, J.C.; Aguilar, C.N. Lycopene: progress in microbial production. *Trend Food Sci. Technol.* **2016**, *56*, 142-148.

14. Loco, J.V.; Js Elskens, M.; Croux, C.; Beernaert, H. Linearity of calibration curves: use and misuse of the correlation coefficient. *Accred. Qual. Assur.* **2002**, *7*, 281-285.

15. Cucu, T.; Huvaere, K.; Bergh, M.-A.V.D.; Vinkx, C.; Loco, J.V. A simple and fast HPLC method to determine lycopene in foods. *Food Anal. Met.* **2012**, *5(5)*, 1221-1228.

16. Paul, G.C; Thomas, C.R. Characterization of mycelial morphology using image analysis. *Adv. Biochem. Eng./Biotechnol.* **1998**, *60*, 1-59.

17. Papaioannou, E.H.; Liakopoulou-Kyriakides, M. Substrate contribution on carotenoids production in *Blakeslea trispora* cultivations. *Food Bioprod. Pro.* **2010**, *88(2-3)*, 305-311.

18. Nanou, K., Roukas, T., Papadakis, E. Improved production of carotenes from synthetic medium by *Blakeslea trispora* in a bubble column reactor. *Biochem. Eng. J.* **2012**, *67*, 203-207.

19. Mantzouridou, F., Roukas, T., Kotzekidou, P. Effect of the aeration rate and agitation speed on β-carotene production and morphology of *Blakeslea trispora* in a stirred tank reactor: mathematical modeling. *Biochem. Eng. J.* **2002**, *10*, 123-135.

20. Mantzouridou, F., Roukas, T., Kotzekidou, P. Production of beta-carotene from synthetic medium by *Blakeslea trispora* in fed-batch culture. *Food. Biotechnol.* **2004**, *18(3)*, 343-361.

21. Pourbafrani, M.; Talebnia, F.; Niklasson, C.; Taherzadeh, M.J. Effect of encapsulation in fermentation of limonene-containing media and orange-peel hydrolysate. *Inter. J. Molec. Sci.* **2007**, *8*, 777-787.

22. Vereschagin, O.A.; Memorskaya, A.S.; Tereshina, V.M. The role of exogenous lipids in lycopene synthesis in the Mucoraceous fungus *Blakeslea trispora*. *Microbiol.* **2010**, *79*, 593-601.

23. Radu, G.L.; Litescu, S.C.; Albu, C.; Truica, G. Beta-carotene and lycopene determination in new enriched bakery products by HPLC-DAD method. *Romanian Biotechnol. Let.* **2012**, *17(1)*, 7005-7012.

Table 1. Medium composition

Medium number	Composition			
	Glucose (g L^{-1})	Sunflower oil (ml L^{-1})	Corn oil (ml L^{-1})	Orange peel (g L^{-1})
In shake flask studies				
1	40	-	-	-
2	60	-	-	-
3	40	10	-	-
4	40	30	-	-
6	40	-	10	-
7	40	-	30	-
8	-	-	-	10
9	-	-	-	20
In fermentor studies				
10	40[a,6]	-	-	-
11	60[a]	-	-	-
12	40[a]	10	-	-
13	40[a]	-	10	-
14	-	-	-	10[a]

[a]At 3 V min^{-1} aeration and 500 rpm agitation

[b]At 12 V min^{-1} aeration and 750 rpm agitation
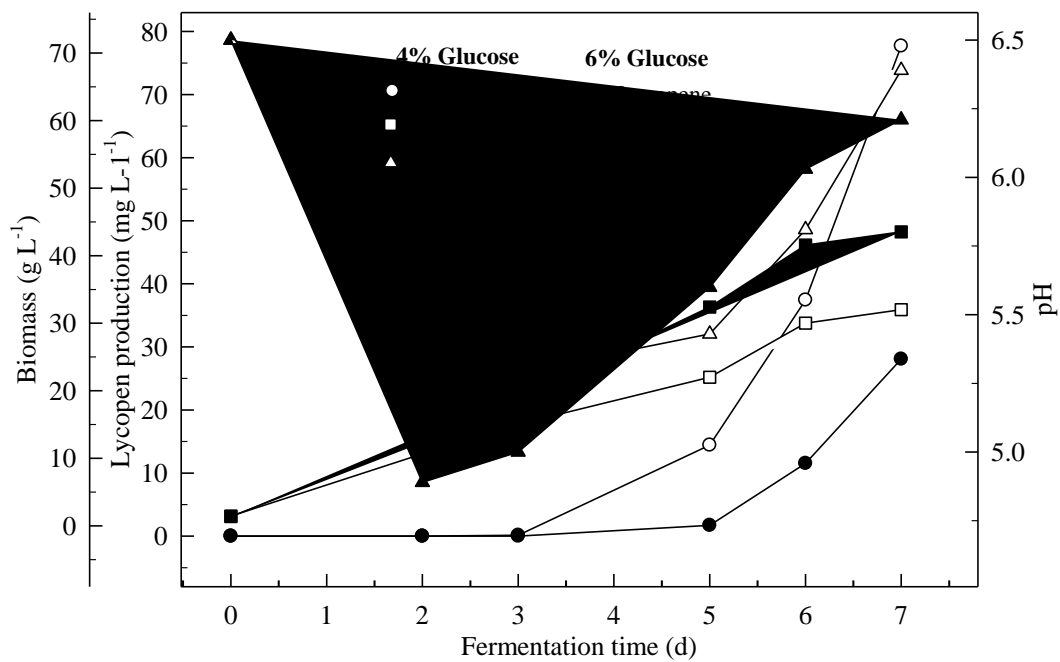
Figure 1. Changes in lycopene production, biomass formation and pH with initial pH

6.5 in a medium containing 4 and 6 % glucose
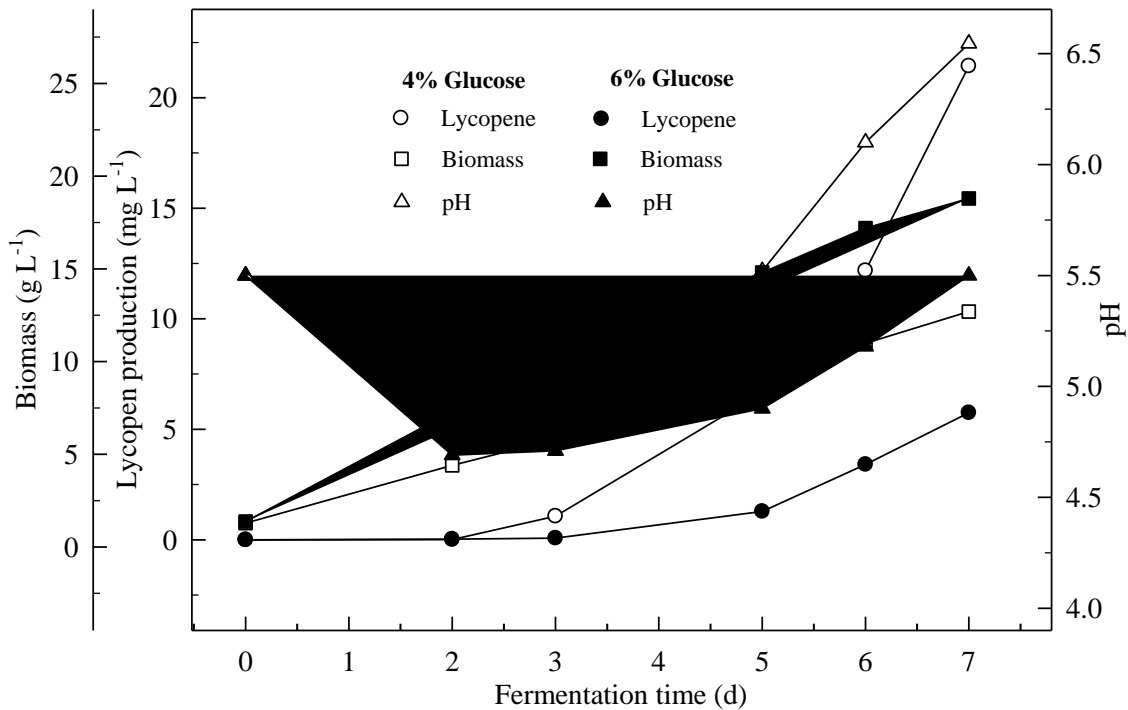
Figure 2. Changes in lycopene production, biomass formation and pH with initial pH

5.5 in a medium containing 4 and 6 % glucose
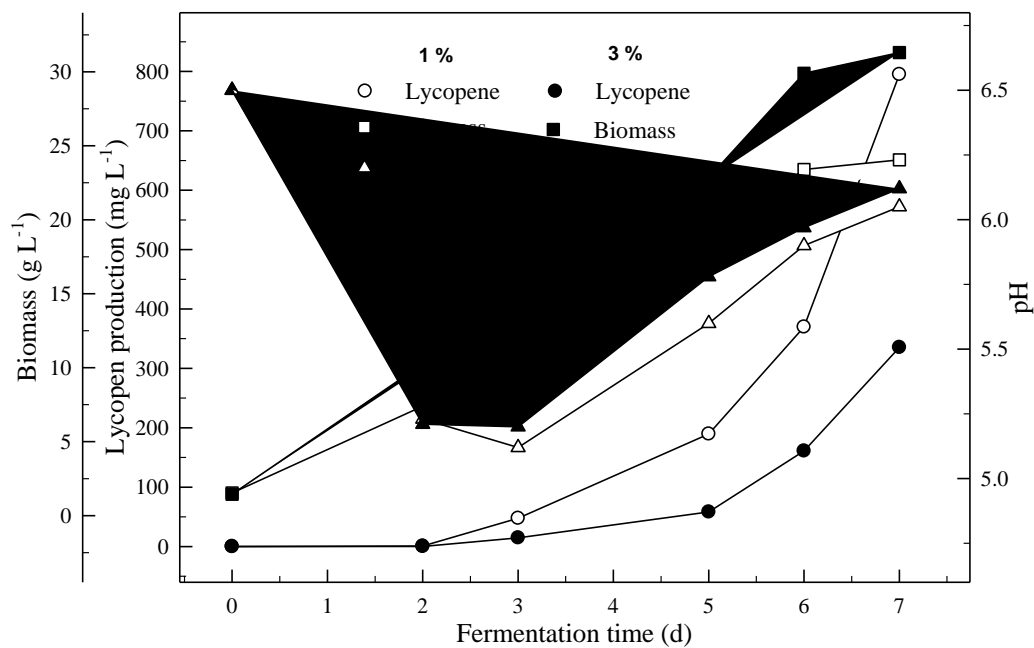
Figure 3. Changes in lycopene production, biomass formation and pH in fermentation

with an initial pH 6.5 in a 4 % glucose medium containing 1 and 3 % sunflower oil
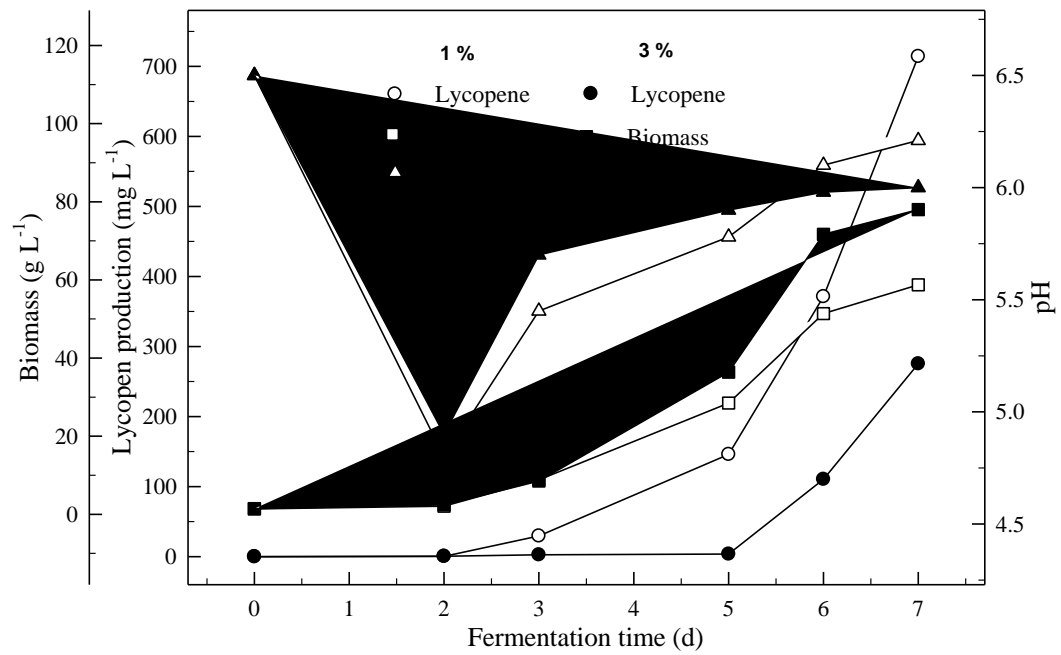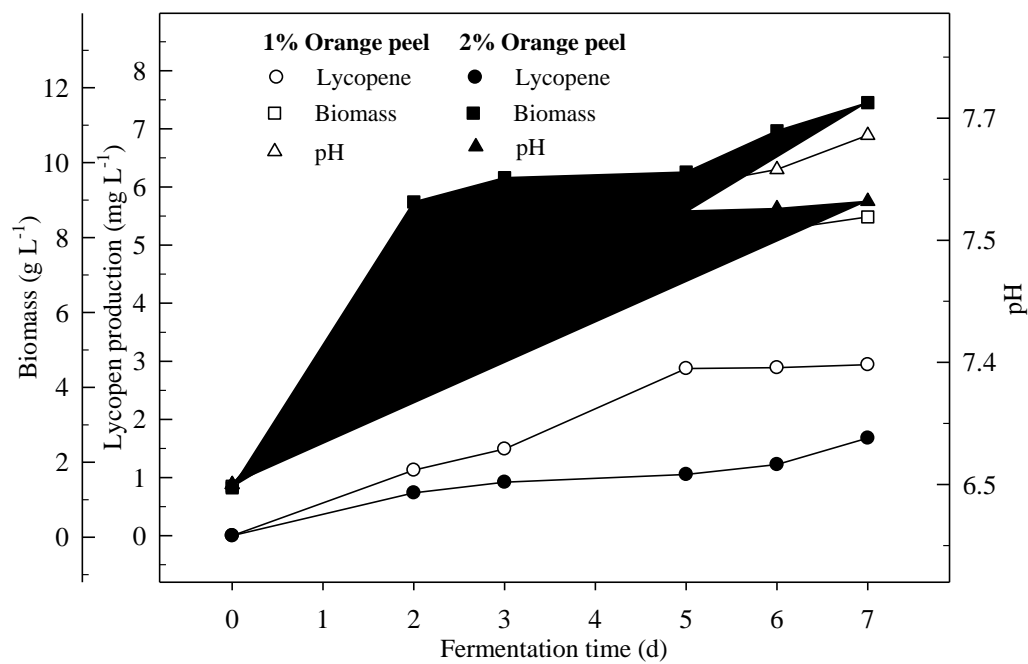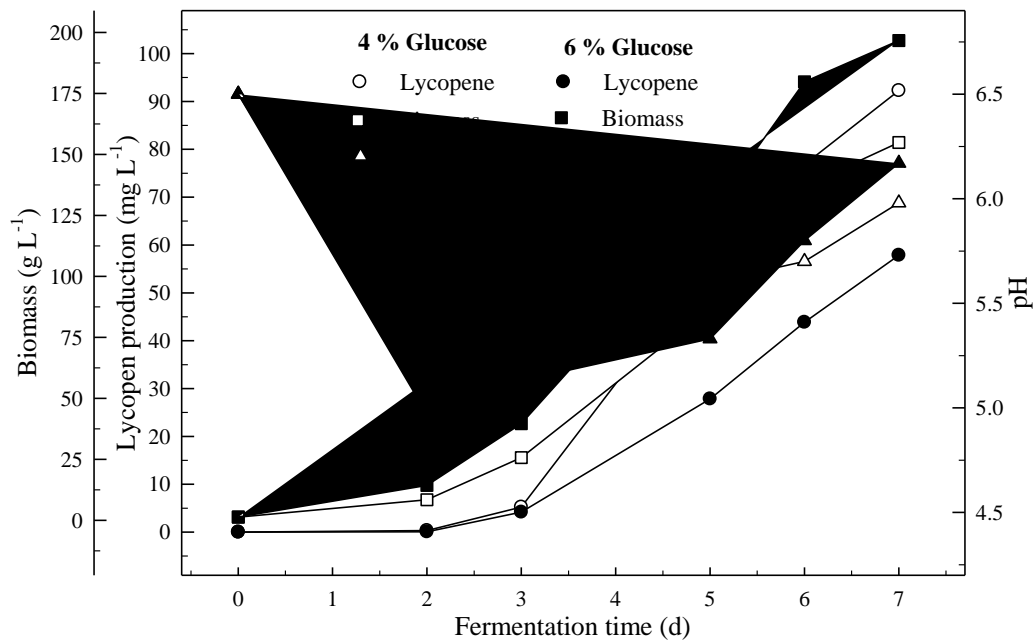
27

Figure 4. Changes in lycopene production, biomass formation and pH in fermentation

with an initial pH 6.5 in a 4 % glucose medium containing 1 and 3 % corn oil

Figure 5. Changes in lycopene production, biomass formation and pH in a shake-flask fermentation with an initial pH 6.5 in a medium containing 1 and 2 % orange peel
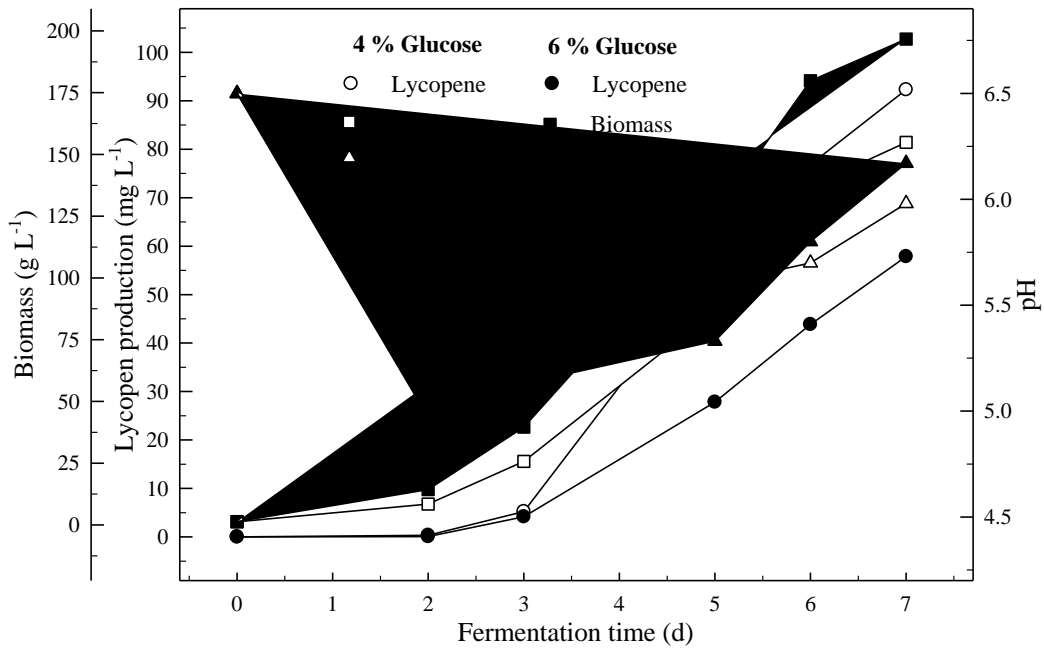
Figure 6. Changes in lycopene production, biomass formation and pH in a fermentor with an initial pH 6.5 in a 4 and 6 % glucose medium at 3 L min^{-1} aeration and 500 rpm agitation

Figure 7. Changes in lycopene production, biomass formation and pH in a fermentor with an initial pH 6.5 in a 4 and 6 % glucose medium at 12 L min^{-1} aeration and 750 rpm agitation
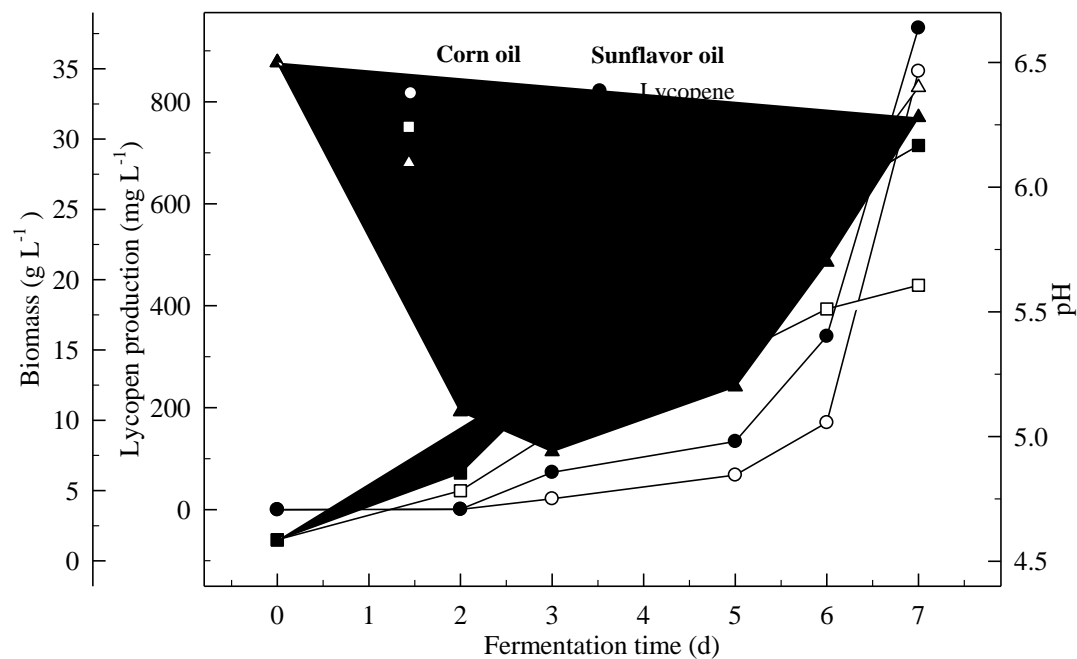
Figure 8. Changes in lycopene production, biomass formation and pH in a fermentor

with an initial pH 6.5 in a 4 % glucose fermentation medium 1 % corn and sunflower

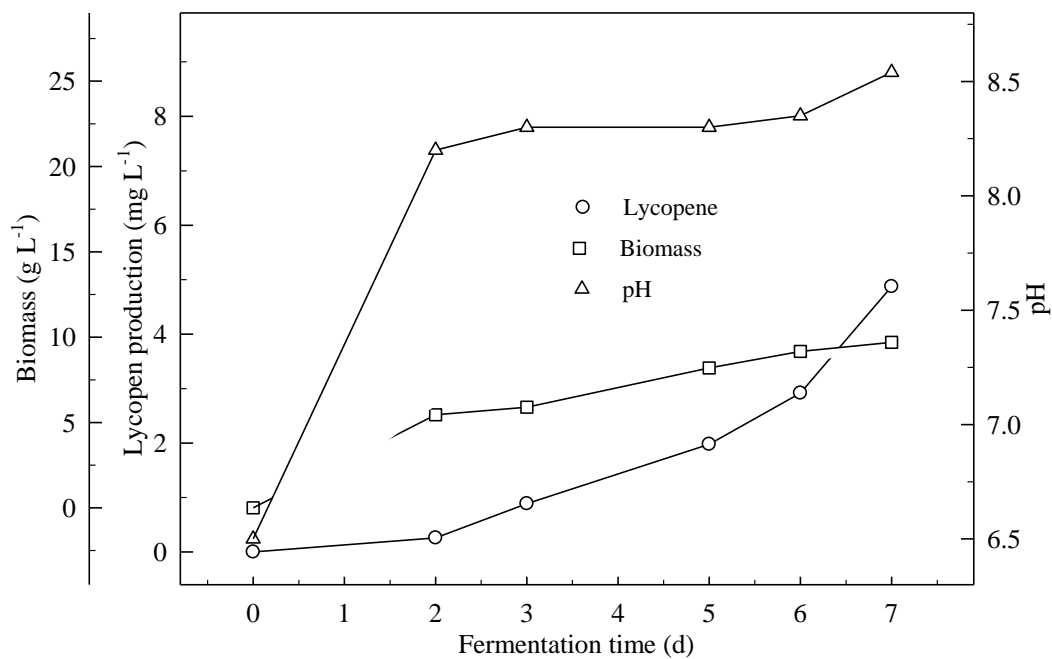oils (at aeration 3 L/min with 500 rpm agitation)

Figure 9. Changes in lycopene production, biomass formation and pH in a fermentor

with an initial pH 6.5 from a 1 % orange peel in a fermentor with aeration 3 L/min at

agitation 500 rpm