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M.Sc. in Food Engineering

AYŞE SEVGİLİ

UNIVERSITY OF GAZİANTEP

GRADUATE SCHOOL OF

NATURAL & APPLIED SCIENCES

IMPROVING LYCOPENE PRODUCTION BY *BLAKESLEA TRISPORA* USING INGREDIENTS

M.Sc. THESIS

in

FOOD ENGINEERING

BY

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Improving Lycopene Production by *Blakeslea trispora* Using Ingredients

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University of Gaziantep

Supervisor

Prof. Dr. Osman ERKMEN

by

Ayşe SEVGİLİ

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Prof. Dr. Metin BEDİR
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Medeni MASKAN
Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in and quality, as a thesis for the degree of Master of Science.

Prof. Dr. Osman ERKMEN
Supervisor

Examining Committee Members

Prof. Dr. Osman ERKMEN

Prof. Dr. Hüseyin ERTEN (Chairman)

Assoc. Prof. Dr. Emine (ALBEN) ERÇELEBİ

Assist. Prof. Dr. Derya KOÇAK YANIK

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Ayşe SEVGİLİ

ABSTRACT

IMPROVING LYCOPENE PRODUCTION BY *BLAKESLEA TRISPORA* USING INGREDIENTS

SEVGİLİ, Ayşe
M.Sc. in Food Engineering Department
Supervisor: Prof. Dr. Osman ERKMEN
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In this research, the effects of initial pH, different ingredients (glucose, sucrose, orange peel) and different amount of natural oils (as sunflower oil and corn oil) on lycopene production by *Blakeslea trispora* were studied. Lycopene, pH, biomass concentrations and lycopene contents were determined during fermentation time.

Optimum initial pH value for lycopene production from *B. trispora* was found as 6.5. About 77.6, 7.7 and 3.7 mg/L of lycopene was produced from 4, 6 and 8 % glucose with initial pH 6.5, respectively. When glucose concentration increased, lycopene concentration decreased. About 12.4, 57.0 and 57.2 mg/L of lycopene was produced from 5, 6 and 7 % sucrose with initial pH 6.5, respectively. As sucrose concentration increased, lycopene concentration increased.

B. trispora can be used as an industrial microorganism for lycopene production. It can not need any specific environmental conditions for growth.

Key words: *Blakeslea trispora*, lycopene, HPLC, fermentation, fermenter

ÖZET

KATKILAR KULLANILARAK *BLAKESLEA TRISPORA* İLE LİKOPEN ÜRETİMİNİN ARTTIRILMASI

SEVGİLİ, Ayşe
Yüksek Lisans Tezi, Gıda Mühendisliği Bölümü
Tez yöneticisi: Prof. Dr. Osman ERKMEN
Ocak 2016, 87 sayfa

Bu çalışmada, *B. trispora*'dan likopen üretimi üzerine başlangıç pH'ının, farklı substratlar (glikoz,sukroz,portakal kabuğu) ve farklı doğal yağların (ayçiçek ve mısır yağı) miktarının etkisi çalışıldı. Fermentayon sırasında, likopen, pH, biyokütle konsantrasyonu ve likopen içeriği belirlendi.

B. trispora'dan likopen üretimi için optimum başlangıç pH'ı 6.5 olarak bulundu. Başlangıç pH'ı 6.5 ve 4, 6 ve 8 % glukoz içeren ortamlarda, sırasıyla 77.6, 7.7 ve 3.7 mg/L likopen üretildi. Glukoz konsantrasyonu arttıkça, likopen konsantrasyonu azaldı. Başlangıç pH'ı 6.5 ve sukroz 5, 6 ve 7 % olan ortamlarda, sırasıyla 12.4, 57.0 ve 57.2 mg/L likopen üretildi. Sukroz konsantrasyonu arttıkça, likopen konsantrasyonu arttı.

B. trispora, likopen üretimi için sanayi microorganizması gibi kullanılır, en büyük avantajı gelişimi için özel bir çevre ortamına ihtiyaç duymamasıdır.

Anahtar kelimeler: *Blakeslea trispora,* likopen, HPLC, fermentasyon, fermentör

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LIST OF ABBREVIATIONS

µg/kg	Microgram/kilogram
Acetyl-CoA	Acetyl coenzyme A
ADI	Acceptable daily intake
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BFM	Basal fermentation medium
CO_2	Carbon dioxide
DMAPP	Dimethylallyl pyrophosphate
DW/L	Dry cell weight
FAO	Food and Agriculture Organization
g/L	Gram/Liter
GGPP	Geranylgeranyl pyrophosphate
h	Hours
HCI	Hydrogen chloride
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzymeA
IPP	Isopentenyl pyrophosphate
i.d.	Identification
JECFA	The joint FAO/WHO expert committee on food additives
L/min	Liter/minute

LOD	Limit of detection
LOQ	Limit of quantification
$MgSO_4.7H_2O$	Magnesium sulfate
mL	Milliliter
mL/min	Milliliter/minute
Mn^{2+} and Ca^{2+}	Manganese and Calcium
MVA	Mevalonate
NADH/NAD^+	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
PDA	Potato dextrose agar
SCFA	Short-chain fatty acids
SSF	Solid-stage fermentation
subsp.	Subspecies
WHO	World health organization
YpSS	Yeast phosphate soluble agar

CHAPTER 1

INTRODUCTION

Lycopene is a carotenoid pigment that is red in color. It is the most common carotenoid in plant foods and the most powerful antioxidant. Lycopene is obtained from red fruits and vegetables, like tomato, watermelon, pink grapefruit, pink guava, papaya and apricots. Lycopene can also be produced from algae and fungi. Tomatoes and their products are the most important sources of lycopene.

Lycopene (2,6,10,14,19,23,27,31-octamethyl-2,6,8,10,12,14,16,18,20,22,24,26,30-dotriacontatridecaene) is a 40 carbon atom unsaturated aliphatic hydrocarbon (Mantzouridou and Tsimidou, 2008).

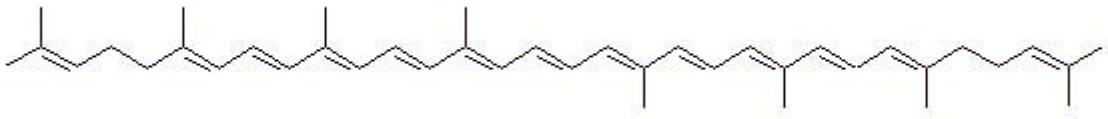

Figure 1.1 Molecular structure of all-trans-lycopene (Molecular weight = 536.89 Molecular Formula = $C_{40}H_{56}$)

Lycopene provides some of advantages against diseases, like diabetes, cardiovascular disease, cancer and osteoporosis. It is used as food additives, like food coloring. Lycopene doesn't dissolve in water. It is obtained from plant extract, chemical synthesis and microbial fermentation. Market needs are not met from plant extraction due to the low content of lycopene.

Chemical synthesis has disadvantages including low yields, product instability, low product quality and high production cost. The low natural production of lycopene and its increased worldwide demand have necessitated that a new large scale production strategy is developed and submerged fermentation is one potential solution. *Blakeslea trispora, Mucor circinelloides, Candida utilis* and *Phycomyces blakesleeanus* have been reported to produce lycopene (Wang et al., 2012). *B. trispora* produces higher amount of lycopene than other microorganisms.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) had previously evaluated lycopene (both natural and synthetic) to be used as a food colour (FAO/WHO, 1978). JECFA indicated that both synthetic lycopene and lycopene extracted from *B. trispora* were acceptable as food colors and established a group ADI of 0-0.5 mg/kg bodyweight/day for both preparations (FAO/WHO, 2007) (Rath et al., 71st).

Lycopene is produced by aerobic fermentation with *B. trispora*. The lycopene production pathway is mevalonate (MVA). During fermentation, MVA is converted to isopentenyl pyrophosphate (IPP) with acetyl-CoA. At the last stage, phytoene is converted to lycopene after that it is carotene. Nicotine is used in the production and it inhibits lycopene cyclase.

The aim of this research is to improve lycopene production by *B. trispora* with shake flask and fermenter studies using different ingredients (glucose, sucrose, sunflower oil and corn oil). Different pH and different substrate concentrations are compared with respect to ingredients, lycopene production, biomass formation and changes of pH were detected during fermentation.

CHAPTER 2

LITERATURE REVIEW

2.1 Fermentation

2.1.1 History of Food Fermentation

Fermentation was the process through which energy was produced from organic compounds (Bobick et al., 2004). Millions of years before humans appeared on earth, all the chemical and enzyme reactions needed for food fermentations were present as part of the recycling reactions used by microorganisms to digest and recycle plant components; for example, fermentation of fruits and fruit juices to wine and vinegar, germination of grains as the first step in alcoholic beverages, and souring of milk. When humans and other animals evolved on Earth, they had to consume the food supply either before it was invaded by microorganisms and recycled or while in various stage of recycling- the fermented foods. When micro organisms produced unpleasant aroma sour flavors in the food or produced toxins that caused illness or death, the food was spoiled and humans learned to avoid it. If the invasion of the food components by microorganisms yielded attractive aromas, flavours and textures, humans learned to appreciate and desire such foods. These different fermented foods are produced sour milk, cheeses, wines, beers, vinegar, lactic acid products (such as sauerkraut) and hundreds of other fermented foods consumed today (Hui et al., 2004).

2.1.2 Description of fermentation

The slow chemical change produced in an organic compound by the action of enzymes, leading to the formation of smaller molecules is called fermentation (Singh and Kaur, 2006). Fermentation carried out in the presence of air/oxygen is known as

aerobic fermentation, whereas that in the absence of air is called anaerobic fermentation. Industrially, a few processes demand total absence of oxygen. Many process require a nominal amount of oxygen or at least they are not affected by oxygen (Rao, 2010).

2.1.2.1 Aerobic fermentation

Sparging air/oxygen is a very common phenomenon in fermentation processes to supply oxygen for cells to meet their specific oxygen demands. Such fermentation processes which are associated with the bubbling of oxygen are termed as aerobic fermentation. There are normally three categories of aerobic fermentation:

- For growing organisms on solid or semi-solid medium in the presence of air, though air is not specifically blown. This is also known as solid stage fermentation.
- For growing organism in liquid medium, air is not specifically blown or sparged, but air helps the growth of organisms. The air enters into the system through a cotton plug kept on the top, either in static condition on in shake flask experiments.
- Air is dispersed in the form of fine bubbles through a sparger or some other kind of bubbling mechanism, so that the oxygen gets dissolved in the liquid medium and is available for the cells or microorganisms (Rao, 2010).

2.1.2.2 Anaerobic fermentation

Yeast fermentation process to produce alcohol requires a small amount of aeration for the cells to multiply. Afterwards, no air is required. On the contrary, air is detrimental for the process, which will otherwise oxidise the substrate. Most of the anaerobic fermentations produce carbon dioxide. Many times, the gas covers the surface and acts as a blanket to prevent the effect of oxygen. The evolved carbon dioxide will also help in better mixing conditions, which is more evident in large industrial tanks because of longer pathways for the gas bubbles to go before they leave the fermenter. Mechanical agitation, though improves the degree of mixing, does not seem to have any significant effect (Rao, 2010).

2.1.3 Fermentation products

Fermentation is important in the production of wine, beer, soy sauce, baked products pickles and others (Bobick et al., 2004):

2.1.3.1 Fermented dairy products

Fermented dairy products take account of 20% of the total economic value of fermented foods throughout the world. Compared with the perishable milk, fermented dairy products have advantages of long shelf-life, high biological value of protein as well as unique organoleptic attributes. Microorganisms naturally present in air, raw dairy material, and equipments are responsible for the fermentation. Dairy cultures ferment lactose to lactic acid, providing a low pH environment for prevention of spoilage microorganisms, coagulation or calcium solubilization and generating desirable flavor and texture (Ray and Montet, 2014). Fermented dairy products can be grossly divided into three big categories: cheeses, yogurts and fermented liquid milks (Hui, 2012).

2.1.3.2 Fermented meat products

Meat fermentation emerged as a preservation strategy and make use of salting and drying to prevent spoilage of nutritious fresh meat. This technology leads to lower water activity value, low pH value, and thus inhibits the growth of spoilage and pathogenic microorganisms. Usually, salted materials are stuffed together in casings. In the anaerobic environment, lactic acid fermentation is the predominant process followed by a drying phase to further stabilize and mature the product. Different sort of sausage and smoke-cured meat products are produced in this way. During fermentation, lactic acid, pyruvic acid, alcohols, aldehydes, ketones, and carboxylic acids are yielded, contributing to the quality and storage stability of the final products (Ray and Montet, 2014). Fermented meat products such as ham and sausages (Hui, 2012).

2.1.3.3 Fermented vegetable products

Vegetables including cabbage, radishes, cucumbers, turnips and beets are great sources of fermented foods. In the traditional way, vegetables are put into clean containers. Salt and other ingredients are added. Fermentation takes places in an anaerobic environment by putting weights on the top of the containers. This economic way for food storage is popular in areas where vegetables are limited in winter. Fermented vegetable products have antimicrobial activities including bacteriocins and nisin production as reported in fermented olives, sauerkraut, fermented carrots, fermented cucumbers (Tamang and Kailasapathy, 2010).

2.1.3.4 Alcoholic fermentation

Alcoholic fermentation, also referred to as ethanol fermentation, is a biological process in which molecules such as glucose, fructose and sucrose are converted into cellular energy and thereby produce ethanol and carbon dioxide as metabolic waste products. Because yeast perform this conversion in the absence of oxygen, alcoholic fermentation is an aerobic process (Wikipedia, 2015). In alcoholic fermentation, the pyruvate from glycolysis is converted to ethyl alcohol or ethanol. First, 3-carbon pyruvate is split into carbon dioxide and 2-carbon acetaldehyde. Then electrons and hydrogen are transferred from nicotinamide adenine dinucleotide (NADH) to the acetaldehyde, forming NAD^+ and ethanol (Starr et al., 2013).

2.1.3.5 Lactic acid fermentation

Several bacteria, known as lactic acid bacteria, form large quantities of lactic acid. Species of several genera, such as *Lactobacillus, Lactococcus, Sporolactobacillus, Leuconostoc, Streptococcus* and *Bifidobacterium*, ferment sugars (Rao, 2006). Pathways formation are:

- The homofermentation
- The heterofermentation
- The bifidum fermentation

2.1.3.5.1 Homofermentation

Lactic acid bacteria are obligate fermenters, and cannot obtain energy by oxidative or respiratory process. Technically, the precursor-product exchange systems, provide an alternate way for these organisms to earn ATP 'credits' by conserving the energy that would ordinarily be used to perform metabolic work. However, the substrate level phosphorylation reactions that occur during fermentation are by far the major means by which these cells make ATP. For homofermentative lactic acid bacteria, hexoses are metabolized via the enzymes of the glycolytic Embden-Meyerhoff pathway (Hutkins, 2006).

One of the key enzymes of this pathway is aldolase, which commits the sugar to the pathway by splitting fructose-1,6-diphosphate into two triose phosphates that eventually serve as substrates for ATP-generating reactions. The Embden-Meyerhoff pathway yields two moles of pyruvate and two moles of ATP per mole of hexose. The pyruvate is then reduced to L- or D-lactose by the enzyme, lactate dehydrogenase. More than 90% of the substrate is converted to lactic acid during homofermentative metabolism (Hutkins, 2006).

Importantly, NADH formed during the glyceraldehydes-3-phosphate dehydrogenase reaction must be re-oxidized by lactate dehydrogenase, so that the [NADH]/[NAD^+] balance is maintained. Homofermentative lactic acid bacteria include *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus belveticus* and *L. delbrueckii* subsp. *bulgaricus* (used as a dairy starter organisms); *Pediococcus* sp. (used in sausage cultures); and *Tetragenococcus* (used in soy sauces) (Hutkins, 2006).

2.1.3.5.2 Heterofermentation

Heterofermentative lactic acid bacteria metabolize hexoses via the phosphoketolase pathway. In obligate heterofermentative bacteria, aldolase is absent, and instead the enzyme phosphoketolase is present. Approximately equimolar amounts of lactase, acetate, ethanol and CO_2 are produced, along with only one mole of ATP per hexose. Oxidation of NADH and maintenance of the [NADH]/[NAD^+] balance occurs via the two reductive reactions catalyzed by acetaldehyde dehydrogenase and alcohol

dehydrogenase. Many of the lactic acid bacteria used in food fermentations are heterofermentative which are *L. mesenteroides* subsp. *mesenteroides* and *Leuconostoc kimchii* (used in fermented vegetables fermentation), *O. oeni* (used in wine late fermentations), and *Lactobacillus sanfranciscensis* (used in sourdough bread production) (Hutkins, 2006).

In general, the product yields for both pathways may vary during actual fermentation process, and depend on the type and concentration of substrate, the growth temperature and atmospheric conditions, and the growth phase of the cells. This so-called heterolactic fermentation may provide cells with additional ATP or serve as a way to deal with excess pyruvate (Hutkins, 2006).

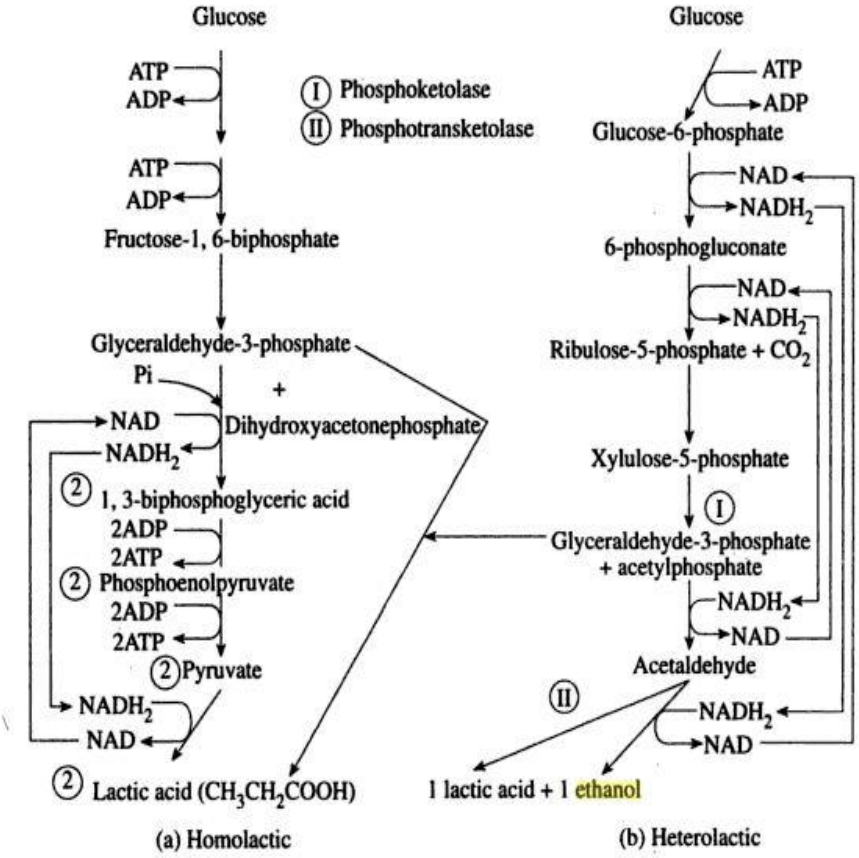

Figure 2.1 Homolactic and heterolactic fermentations (Rao, 2006)

2.1.3.5.3 Bifidum fermentation

Species of *Bifidobacterium* are involved in food fermentations. They have a fermentative metabolism, these bacteria are not used in the manufacture of any fermented food, nor are they even found in most raw food materials. Rather, bifidobacteria are added to certain foods, mostly milk and fermented dairy products, strictly for their probiotic functions. The intestinal tract is their primary habitat, and their elevated presence in the human gastrointestinal tract is correlated with a reduced incidence of enteric infections and overall in testinal health. *Bifidobacterium* spp. are now so frequently used as probiotic adjuncts in foods that they have become a commercially important product line for starter culture companies as ingredients in yoghurt and culture formulations. There are more than twenty- five recognized species of *Bifidobacterium,* although only some of them are ordinarily used commercially as probiotics. These include *Bifidobacterium bifidum, Bifidobacterium adolescentis, Bifidobacterium breve, Bifidobacterium infanttis, Bifidobacterium lactis* and *Bifidobacterium longum.* For many years (until the 1970s), bifidobacteria were classified in the genus *Lactobacillus.* It is now clear that they are phylogenetically distinct from the lactic acid bacteria. Bifidobacteria are Gram positive, non-motile, non-sporing rods with a high G+C content (55 to 67 %). Cells often occur in pairs with a V- or Y-like appearance. They are strictly anaerobic and catalase negative, with a temperature optima between 37 and 41°C and a pH optima (for growth initiation) between 6.5 and 7.0. *Bifidobacterium* spp. are nutritionally fastidious and require vitamins and other nutrients for growth. Their ability to use a wide array of carbonhydrates, including non-digestible oligosaccharides that rich the colon, may provide selective advantages in the colonic environment. Sugar metabolism occurs primarily via the 'bifidum' fermentation pathway that yields acetic acid and lactic acid. Bifidobacteria (except for *B. longum*) rarely contain plasmids (Hutkins, 2006).

2.1.3.6 Vinegar- the acetic acid fermentation

Vinegar is industrially produced by two main methods: a slow process involving static surface acetic acid fermentation, and a fast-producing, submerged fermentation process. Generally the static fermentation method is used in traditional vinegar

production. This technique is not costly in terms of factory investment and product quality is good, although a relatively long period is required to complete fermentation (Solieri and Giudici, 2009).

2.1.4 Chemical change by fermentation

2.1.4.1 Changes in carbohydrates

Fermenting microorganisms are able to degrade digestible carbohydrate polymers, including starch and different types of fiber, into mono- and oligosaccharides. *Bacillus* species are known to possess carbohydrate- hydrolyzing enzymes (such as amylases, galactanases, galactosidases, glucosidases and fructofuranosidases). These enzymes hydrolyze carbonhydrates into sugars that are partly consumed by the microflora itself and partially converted to other products including ethanol and pyruvate. The end products of fermentation include gases (methane, carbon dioxide, hydrogen and hydrogen sulfide), short-chain fatty acids (SCFA, mostly acetic, propionic and butyric acids) and lactic acid in the case of *Lactobacillus* spp. (Mehta et al., 2012).

2.1.4.2 Changes in proteins

Fermentative yeast and bacteria use their enzymatic activities in addition to the available endogenous enzymes to induce several changes in the fermented raw cereal and grain materials. The change in pH-induced sourdough fermentation may catalyze the action of some grain enzymes such as endogenous prolamin-degrading cereal proteases and enhance the hydrolysis of gliadins, glutenins, glutamins, glubulins and secalins as well as the depolymerization and solubilization of the gluten networks (Mehta et al., 2012).

2.1.5 Types of fermentation

The fermentation process mainly divided into three broad categories: submerged fermentation, deep fermentation and solid-stage fermentation. The former has been readily employed in industries for production of alcohol, organic acids, enzymes,

antibiotics, vitamins and amino acids. Solid-stage fermentation has been used for the production of microbial metabolites from fungi, but suffers from limitation of operation at large scales due to operational difficulties (Shetty et al., 2006).

2.1.5.1 Solid-Stage fermentation

Solid-stage fermentation (SSF) involves the growth of microorganisms on moist solid particles, in situations in which the spaces between the particles contain a continuous gas phase and a minimum of visible water. Although droplets of water may be present between the particles, and there may be thin films of water at the particle surface, the inter-particle water phase is discontinuous and most of the inter-particle space is filled by the gas phase. The majority of the water in the system is absorbed within the moist solid-particles (Mitchell et al., 2006).

SSF is used for the production of bioproducts from microorganisms under condition of low moisture content for growth. The medium used for SSF is usually a solid substrate (e.g., rice bran, wheat bran, or grain), which requires no processing. In order to optimize water activity requirements, which are of major importance for growth, it is necessary to take into account the water sorption properties of the solid substrate during the fermentation. In view of the low water content, problems due to contamination are observed. The power requirements are lower than submerged fermentation. Inadequate mixing, limitation of nutrient diffusion, metabolic heat accumulation, and ineffective process control renders SSF generally applicable for low value products with less monitoring and control. There exists a potential for conducting SSF on inert substrate supports impregnated with defined media for the production of products (Shetty et al., 2006).

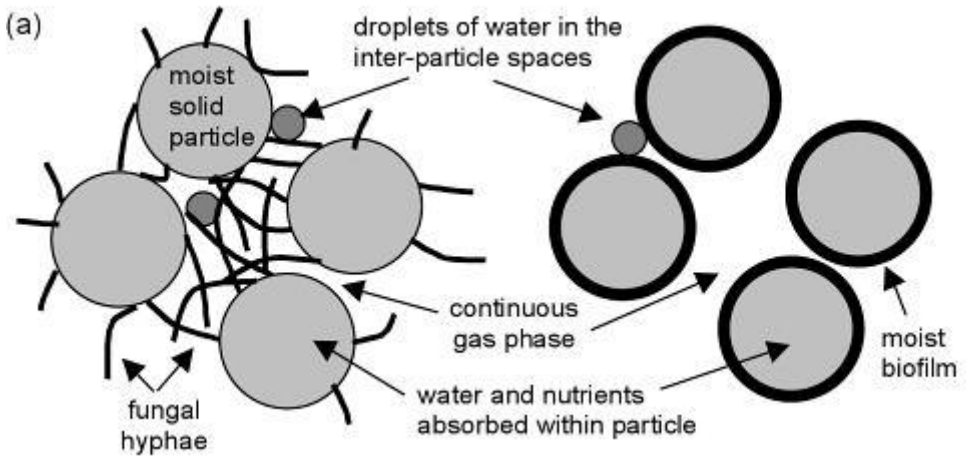

Figure 2.2 The defining features of solid-stage fermentation systems (Mitchell et al., 2006).

2.1.5.2 Deep fermentation

Deep-tank fermentation of *Bacillus sphaericus* has been undertaken in many parts of the world, using techniques well developed for the production of *B. thuringiensis* pesticide for many years. *B. sphaericus* does not use glucose and other carbohydrates for growth and lacks many of the enzymes of sugar metabolism, instead, it grows and develops well with organic acids such as acetate, succinate, arginine and glutamate as sources of carbon and energy although gluconate and glycerol can be used as sole carbon source. This feature of the physiology restricts the use of agricultural products in fermentation media to those rich in protein/aminoacids and prevents the use of surplus, agricultural starchy materials. Industrially, the media mainly composed of proteinaceous substances are used for fermentation of *B. sphaericus*, but the biotin and thiamine are absolutely required for its development. Cations, such as Mn^{2+} and Ca^{2+}, favor sporulation and the associated toxin formation, and can be supplied from local water supplies and media ingredients can be added if sporulation seems poor. In some company, a relatively high-cost peptides were used as carbon and energy resources for *B. sphaericus* fermentation. However, many other low-cost materials can be used for the fermentation of this organism, like agriculture by-product, fishery waste, monosodium glutamate waste, fermented cowpea, cottonseed meal, dextrose, yeast extract, dried cattle blood and so on (Upadhyay, 2003).

2.1.5.3 Submerged fermentation

Submerged fermentation is the most popularly used technique for the production of a large number of products using a wide range of microorganisms. The medium used for submerged fermentation contains relatively highly processed ingredients. The water activity of the medium is high, making it prone to contamination if asepsis is not maintained. Rheological problems can be encountered at high substrate concentrations. Mass transfer from gas to liquid phase is usually a limiting factor. Better bioprocess control of fermentation process is possible with the help of online sensors (Shetty et al., 2006).

2.2 Lycopene

2.2.1 Description and history of lycopene

Lycopene belongs to a group of naturally-occurring pigments known as carotenoids. It is a natural constituent of red fruits and vegetables, and of certain algae and fungi (Olempska-Beer, 2006a). Lycopene was one of the nearly 700 carotenoids that have been characterized. The deep red crystalline pigment produced by lycopene was first isolated from *Tamus communis* berries in 1873. Subsequently, in 1875, a crude mixture containing lycopene was obtained from tomatoes. However, not until 1903, lycopene was different as it was determined that it had a unique absorption from carotenes by spectrum analysis. In the Western diet, lycopene is the most abundant nonprovitamin A carotenoid in the diet and human plasma. Likewise, it can be readily detected in a variety of biological tissues. Lycopene is the strongest singlet oxygen quenching agent and the strongest antioxidant among all carotenoids (Tsao et al., 2010).

2.2.2 Chemical specifications

Lycopene has an acyclic carbon chain with 11 conjugated double bonds ($C_{40}H_{56}$) (Figure 2.3) and a chemical structure similar to tetraterpenes ($C_{40}H_{64}$), since its skeleton contains eight isoprenic groups. In fruits and vegetables, it is found naturally in the all-trans (or E) form, while in processed tomato products and human

serum both trans and mono-cis isomers (5-,9-,13- and 15- cis) of lycopene are present (Oikonomakos, 2002).

The chemical name of lycopene is 2,6,10,14,19,23,27,31-octamethyl-2,6,8,10,12,14,16,18,20,22,24,26,30-dotriacontatridecaene. Common names include Ψ,Ψ-carotene, all-trans-carotene and (all-E)-lycopene (Olempska-Beer, 2006b).

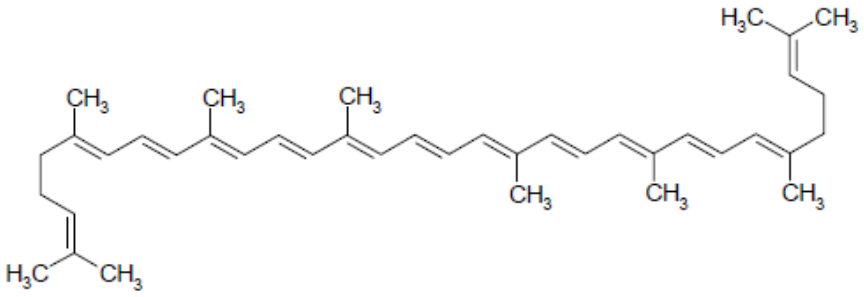

Figure 2.3 Structural formula of all-trans-lycopene (Olempska-Beer, 2006b)

Lycopene is unstable when exposed to light, heat and oxygen. Exposure to light and heat triggers isomerization from the trans to cis configurations. The cis isomers of lycopene have different physical and chemical characteristics than all-trans-lycopene. Some of these differences include lower melting points, lower specific absorption and a shift in the absorption maximum. Lycopene can also undergo oxidation when exposed to oxygen with the formation of epoxides (Olempska-Beer, 2006b).

Tested under illumination (2000-3000 lux for 1-144 h) and heat (50, 100 and 150°C at various time) a standard of trans-lycopene, which contained several cis isomers, such as 5-cis, 9-cis, 13-cis, 15-cis-lycopene, and possibly one more mono-cis and four di-cis isomers. They concluded that during illumination isomerization and degradation of lycopene and its cis- isomers may proceed simultaneously, and that all-trans lycopene might be isomerized to form mono-cis- or di-cis-lycopene. Heating at 50 and 100°C initially showed isomerization of the mono-cis isomers to the di-cis-, however degradation was more prevalent as heating time proceeded. At 150°C, a large decrease was observed for the concentration of all-trans lycopene, and no lycopene was detected after 10 minutes (Oikonomakos, 2002).

2.2.3 Uses of lycopene

2.2.3.1 Main use

Lycopene, one of the most important dietary carotenoids, is synthesized exclusively by plants and microorganisms (Mantzouridou and Tsimidou, 2008).

Carotenoids are important group of natural pigments with specific applications as colorants, feed supplements, nutraceuticals, cosmetic and biotechnological purposes (Lopez-Nieto et al., 2004). It is used as an antioxidant to reduce cellular or tissue damage and as a coloring agent for food products, such as margarine soft drinks, and baked goods (Mantzouridou et al., 2004).

Lycopene from *B. trispora* is intended to use as a color in foods and beverages including orange to red non-alcoholic flavoured drinks, fine bakery wares, such as biscuits, cakes and cookies, dairy products, fruit and flavored yoghurts and dairy desserts, confectioary, fish products, soups and sauces, and certain sweet liquors (Olempska-Beer, 2006b). These are:

In food

- Antioxidants
- Colorants

In medical

- Prevent cardiovascular diseases
- Regulate the immune system
- Anti-carcinogenic agents
- Prevents liver necrosis
- Inhibits the harmful effect of ferric nitrilotriacetate on DNA in rats.

2.2.3.2 Food categories and use levels

The intended use levels of lycopene range from 10 to 50 mg/kg. The intended uses and use levels of lycopene are provided in Table 2.1 (Olempska-Beer, 2006b):

Table 2.1 Use levels of lycopene from *Blakeslea trispora* (Olempska-Beer, 2006b)

Food	**Maximum use level (mg/kg)**
Non-alcoholic flavored drinks	
Bitter	30
Apple	10
Peach	20
Orange	30
Others	20
Fine Bakery Wares	
Biscuits, cakes	20
Cookies	20
Confectionery	20
Edible Ices	
Ice cream, sherbet	25
Desserts, including flavoured milk products	
Fruit, flavoured yoghurts	15
Dairy desserts	15
Fish	
Pre-cooked crustaceans	20
Smoked fish	30
Sauces	
Sauces with reddish coloration	50
Soups	
Soups and creams	30
Spirituous Beverages	
Sweet liquors	20

2.2.3.3 Reactions in food

Lycopene stability in commercial formulations containing lycopene from *B. trispora* was evaluated under various conditions up to 12 months.

Table 2.2 Isomer composition of lycopene from various sources (as % of total lycopene) (Olempska-Beer, 2006b)

Sample	All-trans-lycopene	5-cis-lycopene	9-cis-lycopene	13-cis- and 15-cis-lycopene	Other cis isomers of lycopene
Raw red tomatoes	94-96	3-5	0-1	1	<1
Cooked tomato-based foods	35-96	4-27	<1-14	<1-7	<1-22
Human blood plasma	32-46	20-31	1-4	8-19	11-28
Synthetic lycopene	>70	<23	<1	<1	<3

2.3 Production of lycopene

Lycopene production methods include plant extraction, chemical synthesis and microbial fermentation. Market needs are not met using plant extraction due to the low content of lycopene in plants. Chemical synthesis also presents disadvantages including low yields, product instability, low product quality and high production cost (Wang et al., 2012).

2.3.1 Production of lycopene from tomatoes

Lycopene extract from tomato is produced from a tomato variety with high lycopene content. This particular variety is not generally marketed for direct consumption, but

is used primarily in the production of this lycopene extract. The extract is produced by crushing tomatoes into crude tomato juice that is then separated into serum and pulp. The tomato pulp is then extracted with ethyl acetate. The final product is obtained after solvent removal by evaporation under vacuum at 40-60°C (Rath et al., 71st). Normally, tomatoes contain about 30-50 mg lycopene per kg of fresh raw material (Mantzouridou and Tsimidou, 2008).

2.3.2 Production of lycopene using of microorganisms

Blakeslea trispora, Mucor circinelloides, Candida utilis, Dunalliella salina and *Phycomyces blakesleeanus* have been reported to produce lycopene. The availability of microorganisms with high lycopene yield indicates that microbial sources are attractive alternatives for the industry. As it is pointed out in Table 2.3, *Blakeslea* group is of primary industrial microbial source. The continuous interest in *B. trispora* as a source of β-carotene and, more recently, of lycopene is related to its legal status worldwide and the limited production of other structurally related carotenoids in the end-product. In addition, *B. trispora* is preferred overall in carotenoid production processes because of the increased yield in cells due to the high amount of biomass and intracellular neutral lipids (mainly in the form of triacylglycerols) that might function as a good reservoir for the formed species (Mantzouridou and Tsimidou, 2008).

Table 2.3 Maximum lycopene production for various microorganisms (Mantzouridou and Tsimidou, 2008)

Microorganism	**Lycopene (μg/g biomass dry wt)**
Blakeslea trispora	15,000
Escherichia coli	500
Flavobacterium R1519	1000
Mycobacterium aurum	7000
Mycobacterium marinum	80
Rhodotorula spp.	90
Streptomyces chrestomyceticus	580

2.3.3 Materials used in lycopene fermentation

2.3.3.1 Raw Materials

Lycopene production (0.5 g/liter in 6 days) by a mutant of *Streptomyces chrestomyceticus,* subsp. *rubescens* has been reported in the patent literature. The medium used contained starch, soybean flour and ammonium sulfate (Vandamme, 1989).

2.3.3.2 Glucose

The principal storage forms of glucose are glycogen in vertebrates and many microorganisms, and starch in plants (Pushkar, 2006).

In the production of lycopene *B. trispora* strains were used. *B. trispora* strains NRRL 2895 (+) and 2896 (-) were cultivated together (mated culture) in the fermentation medium (with an initial pH of 4.0) which contained 59.4 g/L glucose, 2.12 g/L L-asparagine, 1.42 g/L yeast extract, 1.26 g/L KH_2PO_4, 0.4 g/L $MgSO_4.7H_2O$, 1.0 g/L vitamin A acetate and 2.0 g/L Span 20. Fermentation medium (45 mL) was prepared in 250-mL flasks and inoculated with 2.5-mL of each mating type from the second preculture (i.e., at the (+) to (-) strain ratio of 1:1 and the inoculation density of the mated fermentation was calculated as 300-350 mg dry cell weight (DW)/L). Fermentation cultures were incubated for 8 days at 28°C on a rotary shaker at 120 rpm. The cell mass was filtered through muslin, washed thoroughly with distilled water, and dried in vacuum freeze dryer at -75°C under 45 Pa for 48 h. The dried cells were weighed to determine biomass. The freeze-dried samples were pulverized and 40.0 mg sample powder was accurately weighed, and then extracted with 6 mL acetone using a ultrasonic cell disruption apparatus for 180 s (400 W power, 2 s ultrasound pulse, 3 s break and 90 repetitions). The supernatant was cleaned through a 0.45 µm filter, and 20.0 µL was directly injected into the LC-UV for analysis. The analysis of lycopene was performed on a Waters 600E system, equipped with an on-line degasser and a Waters 2487 UV detector. The column used for separation was Phenomsil C18 (250x4.6 mm i.d., 5 µm) fitted with a C18 guard column. The optimized mobile phase contained methanol (A), acetonitrile (B) and water (C), and

the separation was carried on by isocratic elution (A:B:C=50:50:3, v/v). The column oven temperature was maintained at 40°C and the flow rate was 1.0 mL/min. The detection wave length was fixed at 470 nm (Wang et al., 2012).

2.3.3.3 Sucrose

The sucrose molecule ($C_{12}H_{22}O_{11}$) consist of glucose and fructose rings. In percentages, the molecule contains 51.5% oxygen, 42.0% carbon and 6.5% hydrogen. The molecular mass (weight) of sucrose is 342.3g (Asadi, 2007). Sucrose is a disaccharide, made from glucose and fructose rings, each with six carbon atoms (Asadi, 2007).

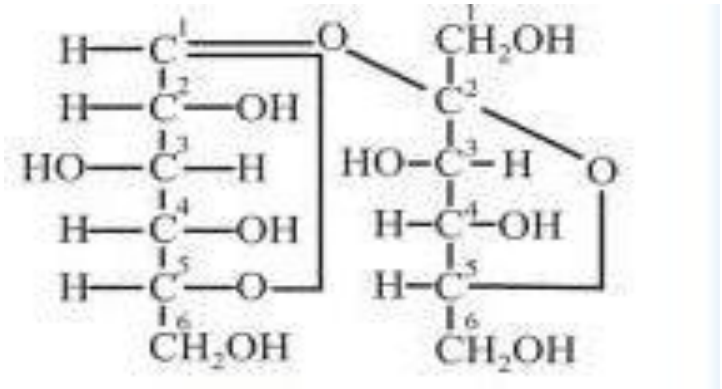

Figure 2.4 Sucrose ($C_{12}H_{22}O_{11}$)

2.3.3.4 Sunflower oil and corn oil

Sunflower oil is obtained from the seed of the plant, *Helianthus annuus* L. Crude sunflower oil is light amber in color; the refined oil is a pale yellow and is similar to other oils (O'Brien, 2009). Sunflower oil usually does not require extensive refining as it contains relatively low levels of free fatty acids, phospholipids, tocopherols, pigments and sterols. The oil is refined by degumming, neutralization, bleaching, deodorization and winterization (Salunkhe et al., 1992).

Corn, *Zea mays* L., a plant belonging to the grass family, is native to both North and South America (O'Brien, 2009). Corn oil belongs to the group of oils with high levels of linoleic and oleic fatty acids. The liquid oils in this group are the most

adaptable of all the fats and oils. They have desirable oxidative stability properties and may be hydrogenated to a varying degrees of saturation from a milky liquid to melting points in excess of 138°F (59°C) (O'Brien, 2009).

The microorganisms were *B. trispora* ATCC 14271, mating type (+) and ATCC 14272, mating type (-). The strains were grown on potato dextrose agar petri dishes at 26°C for 4 days. The spores obtained were suspended in 10 mL sterile water to prepare the inoculums. The concentration of the inoculums was $2.3x10^6$ and $1.5x10^6$ spores/mL for the strains 14271and 14272, respectively. The medium supplemented with 0.1% (w/v) of Tween 80, 1.0% (w/v) of Span 20, and different concentration of olive oil, cotton seed oil, soybean oil, corn oil, sunflower oil, and olive pomace oil indicated. The pH of the solution was adjusted to 7.0 and the substrate sterilized at 121°C for 15 min. The fermentation was carried out in 500 mL conical flasks containing 100 mL of the above medium. The substrate was inoculated with 1 mL of the inoculum and the flasks were incubated at 26°C in a rotary shaker incubator at 200 rpm. At appropriate time intervals, fermentation flasks were removed and the contents were analyzed. Biomass dry weight was determined by filtration of the broth through a Whatman no 541 filter paper. The mycelium was washed with distilled water until the filtrate was colorless. One gram of wet biomass was dried at 105°C overnight. The mobile phase of methanol was eluted at a flow rate of 1.5 mL/min. Under these conditions, β-carotene, γ-carotene and lycopene were eluted within 8.0, 9.5 and 11.0 min, respectively. The detection of β-carotene, γ-carotene and lycopene were done at 429, 450, 478 nm and 444, 468, 501 nm, respectively (Varzakakou and Roukas, 2010).

2.3.4 Lycopene fermentation

Lycopene from *B. trispora* is produced through a co-fermentation process (Winter, 2009). In other words, lycopene from *B. trispora* is manufactured by co-fermentation of two sexual mating types (plus and minus) of the fungus *B. trispora*. Although each strain is capable of producing levels of carotenoids, the co-cultivation of both strains enhances the synthesis of these compounds. Both strains of *B. trispora* are considered to be nonpathogenic and nontoxigenic based on animal feeding studies and immunoassays for several mycotoxins conducted in relation to the production of

β-carotene from *B. trispora*. Lycopene is an intermediate in the biosynthetic pathway of β-carotene. Its production process is nearly identical to that used to manufacture β-carotene from *B. trispora.* The only difference is in the addition of imidazole to the fermentation broth in order to inhibit the formation β- and ɣ-carotene from lycopene. Following the fermentation phase, lycopene is extracted from the biomass and purified by crystallization and filtration. The solvents used in these processes are isopropanol and isobutyl acetate. Lycopene is unstable when exposed to oxygen and light and must be stored under inert gas in light-proof containers. Commercial lycopene preparations intended for use in food are formulated either as suspensions in edible oils or as water-dispersible powders (referred to in the dossier as cold water dispersions or CWDs) and are stabilized with antioxidants such as tocopherol. All processes related to the recovery, formulation, and packaging are carried out under nitrogen to avoid the degradation of lycopene (Olempska-Beer, 2006).

2.4 *Blakeslea trispora*

B. trispora is a commensal mold associated with tropical plants. The fungus exists in (+) and (-) mating types; the (+) type synthesizes trisporic acid, which is both a metabolite of β-carotene and hormonal stimulator of its biosynthesis. When cultures of both sexual forms (+) and (-) strains are mixed, a significant increase in carotene production in the (-) strain is achieved (Rai, 2012). *B. trispora* is an aerobic microorganism and therefore requires oxygen (Mantzouridou et al., 2002).

B. trispora, a heterothallic fungus, the order Mucorales, is a saprophyte that performs its vegetative cycle of spores, filamentous mycelia, fruiting bodies and again spores. There are many studies on the production of all-trans-β-carotene with *B. trispora* and its biosynthetic pathway. *B. trispora* presents the major advantage that it does not need any specific environmental conditions for growth (Papaioannou and Liakopoulou-Kyriakides, 2010).

B. trispora forms multisporous sporangia (with or without a columella), which hang downwards, and also sporangioles lacking a columella, containing usually three spores. Numerous sporangioles arise on the surface of a vesicle on an upright sporangiophore. The mature ellipsoid spores have bristle-like mucilaginous processes

at their ends. In this organism the formation of sporangia and sporangioles also varies with the environmental conditions: in unfavourable nutrient conditions (water agar) sporangia with columellae arise predominantly; but with the addition of nutrients, the size of the sporangia increases and with rich supplies of nutrients sporangioles are dominant (Esser, 1982).

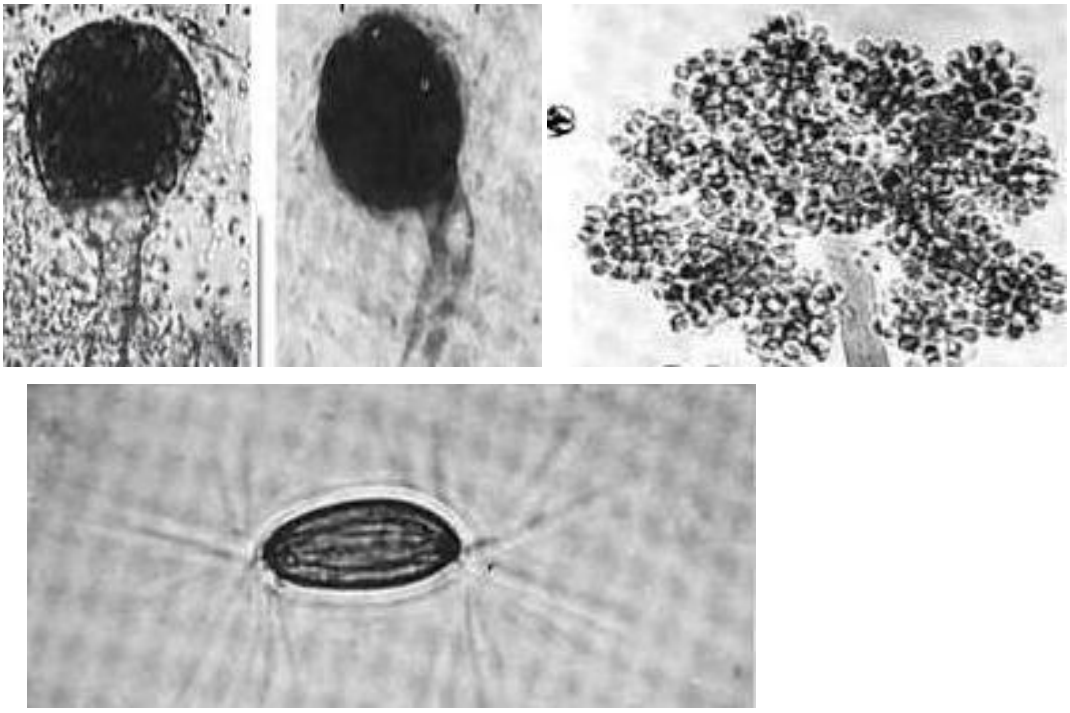

Figure 2.5 Morphological appearance of *B. trispora* (Esser, 1982).

2.4.1 Biosynthesis of lycopene in *B. trispora*

The production process of lycopene is a given in Figure 2.6. Such as sugars and sucrose can be used as carbon sources (Britton et al., 2009). Carotenoids in *B. trispora*, as in most fungi, derive from mevalonate (MVA) with acetyl-CoA as the precursor. MVA is further converted into isopeentenyl pyrophosphate (IPP), which is the crucial C_5 terpene precursor (R 1-3). The isomerization of IPP to dimethylallyl pyrophosphate (DMAPP) is the key step in carotenoid biosynthesis (R 4). This reaction, catalysed by isopentenyl pyrophosphate isomerase, is reversible, the proportions at equilibrium of IPP and DMAPP being approximately 1:9. Successive condensations of C_5 intermediates lead to the production of the ubiquitous halfway metabolite precursor of C_{40} carotenoids that is geranylgeranyl pyrophosphate (GGPP)

(R 5-7). Biosynthesis of the primal carotenoid in the family, phytoene, occurs by a tail to tail condensation of two GGPP molecules by phytoene synthase, encoded by the A domain of the *carRA* gene (R 8). Phytoene lacks the most appealing characteristic of carotenoids, color. The successive introduction of four double bonds in the phytoene skeleton leads to lycopene synthesis through the intermediates γ-carotene and neurosporene (R 9-12). The desaturation process is catalysed by phytoene desaturase, encoded by the *carB* gene. Finally, lycopene cyclase, encoded by the R domain of the *carRA* gene, using lycopene as substrate, forms the rings located at both ends of the β-carotene molecule (R 13, 14) (Mantzouridou and Tsimidou, 2008).

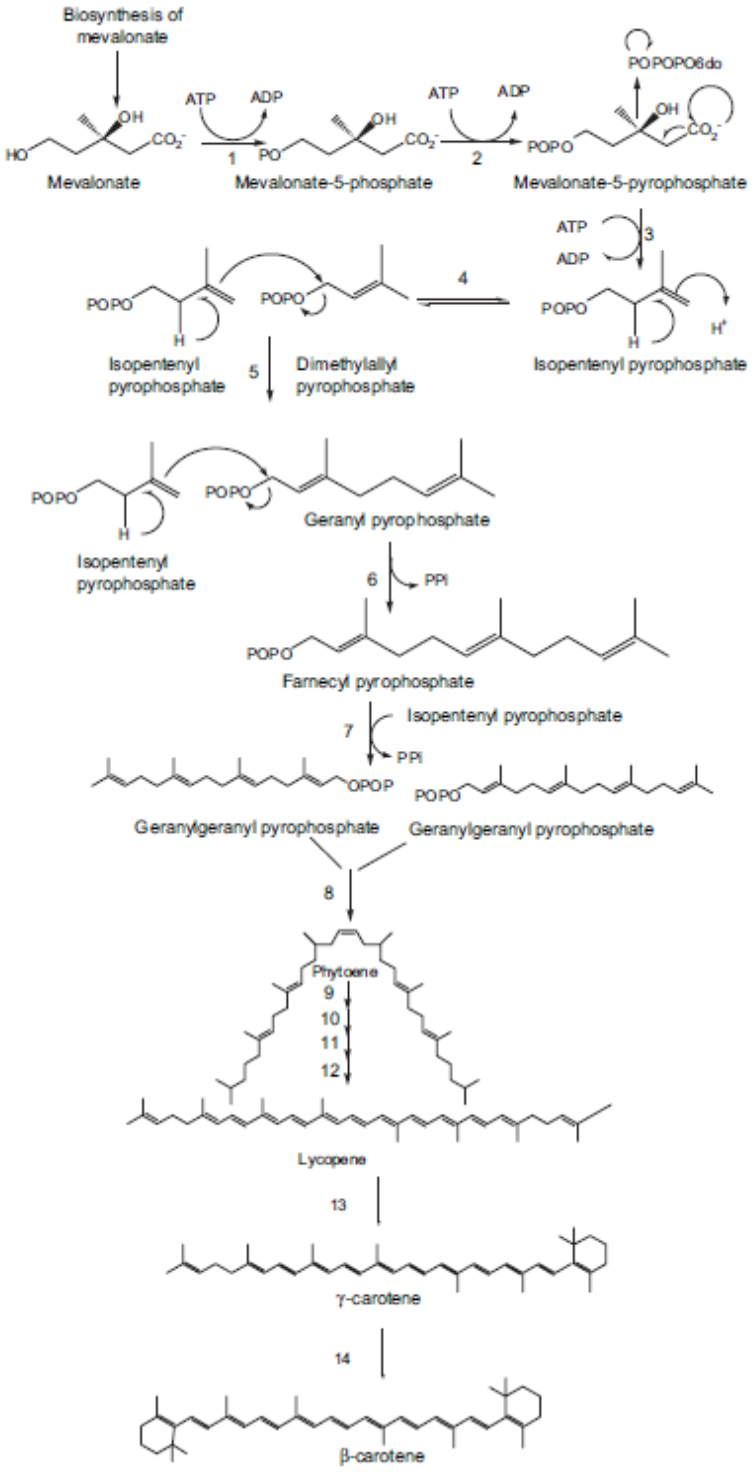

Figure 2.6 Biosynthetic pathway for lycopene and β-carotene formation in *B. trispora* cells.

1-14: reaction (R) numbers (Mantzouridou and Tsimidou, 2008)

2.4.2 Lycopene production from *B. trispora*

Lycopene production from *B. trispora,* was used strain of *B. trispora* ATCC 14271 (+) and ATCC 14272 (-). The (-) strain is the most important in the lycopene production because of more dominant than (+) strain for lycopene production.

Lycopene is an intermediate metabolite along the mevalonate pathway, and it is derived from mevalonate with acetyl-CoA as its precursor. Lycopene can be transformed into β-carotene by lycopene cyclase. Mevalonate synthesis is a key step for the lycopene production. HMG-CoA, the key intermediate metabolite, and mevalonate kinase, the rate-limiting enzymes, are of particular importance within the mevalonate biosynthetic pathway. Chemical agents (such as leucine and penicillin) were reported to stimulate the synthesis of carotenoids by increasing the metabolic activity of HMG-CoA or mevalonate kinase. Lycopene content and production decreased with increasing leucine concentrations, demonstrating that a higher concentration of leucine negatively effects lycopene biosynthesis (Wang et al., 2011). The cyclase inhibitor, (such as nicotine, imidazole, piperidine and creatinine) was decreased lycopene production.

2.5 Selection of culture and fermentation method used

In this study, lycopene was obtained by areobic fermentation using *B. trispora* ATCC 14271 and ATCC 14272. Fungus *B. trispora* has been widely used for the production of lycopene from synthetic media (Mantzouridou et al., 2004). The production of lycopene has been described in shake flask culture by *B. trispora*. Glucose, sucrose, corn oil, sunflower oil was used like substrate in culture under different pH and concentration. Nicotine was added in fermentation medium for increasing lycopene. Nicotine is a cyclization inhibitor that at higher concentrations causes the accumulation of lycopene while at lower concentrations triggers γ-carotene formation (Fazeli et al., 2009).

CHAPTER 3
MATERIALS AND METHODS

3.1 Material and Chemicals

3.1.1 Materials

The materials and equipments used in this study were;

- High Performance Liquid Chromatography (HPLC, DIONEX, California Avenue, Palo Alto, CA 94304 U.S.A) in lycopene analysis components are:
  - Dionex P680 HPLC Pump (isocratic),
  - Dionex ASI-100 Automated Sample Injector,
  - Dionex RF 2000 Fluorescence detector (FLD),
  - Dionex Thermostatted Column Compartment TCC-100,
  - Computer (Packard Bell) and software (Chromeleon) and
  - HPLC column (nucleosil C18 -250 mm-5µm- 4.6 mm).
- Analytical Balance (0.01 g scaled, Sartorious, Goettingen)
- Pure Water System (Elga, Lane End Industrial Park High Wycombe, HP14 3BY, UK)
- Ultra Pure Water System (Elga, Purelab Option Q)
- Filter Paper (Whatman No:4 with pore size 30µm, rough filter papers)
- Glass Microfiber Filter Paper (Whatman, pore size 1.6µm)
- Ultrasound
- pH-meter (WTW pH/mV/Temperature Meters, Models 720; 3150 Commercial Ave Northbrook, IL 60062, USA)

3.1.2 Chemicals

HPLC solvents (grade acetonitrile, methanol, water and dichloromethane) were purchased from Interlab (Adana, Turkey). Ultrapure water was obtained from an Elga Purelab Option Q apparatus from Elga (Lane End Industrial Park High Wycombe Bucks HP14 3BY UK). The other reagents were;

- Lycopene standard (LC grade, Sigma-Aldrich, Steinheim Germany).
- Sodium chloride (J.T Baker Deventer, Nederland)
- Potassium bromide (KBr, Merck, Darmstadt Germany)
- Acetonitrile (LC grade, Sigma-Aldrich, Steinheim Germany)
- Methanol (LC grade, Sigma-Aldrich, Steinheim Germany)
- Mobil phase solvent was prepared by acetonitrile, methanol, water and dichloromethane which were used 7:1.5:0.5:1 in volume.
- Potato Dextrose Agar (PDA, PDA; Darmstadt, Germany)
- Yeast extract (Merck, Darmstadt, Germany)
- K_2HPO_4 (Merck, Darmstadt, Germany)
- $MgSO_4$ (Merck, Darmstadt, Germany)
- Starch (Merck, Darmstadt, Germany)
- Agar (Merck, Darmstadt, Germany)
- Glucose (Merck, Darmstadt, Germany)
- Sucrose (Merck, Darmstadt, Germany)
- Asparagine (Interlab Inc., Adana)
- Sunflower oil and corn oil were obtained from local markets.

3.1.3 Preparation of solutions and reagents

3.1.3.1 Preparation of 0.2 N HCl

1.8 g HCl was weighed and dissolved in 250 ml distilled water in the 500 ml flask.

3.1.3.2 Preparation of 1.0 N NaOH

10 g of NaOH was weighed and dissolved in 250 ml of distilled water. Normality was checked by standardization and adjusted to desired concentration by following formula:

$$V_1 = V_2 \times N_2 / N_1$$

where N_2 and V_2 represent normality and volume of stock solution.

Standardization of NaOH solution: 0.7 g potassium hydrogen phthalate, $KHC_8H_4O_4$, was dissolved in 50 ml of distilled water. 1-2 drops of phenolphthalein indicator were added and it was titrated with NaOH solution being standardized until a pink color persists. Volume of NaOH solution used in the titration was recorded and the normality of solution was calculated by following formula:

$$\text{Normality} = \text{g } KHC_8H_4O_4 \times 1000 / (\text{ml of NaOH} \times 204.229)$$

3.1.3.3 Preparation of nicotine

Nicotine was prepared for 5 mM solution. Nicotine solution was sterilized in autoclave at 121^{o}C for 15 min and was added after 2 days of fermentation.

3.1.3.4 Sterilization of equipments

Supplies in the laboratory were used such as petri dishes, loopful, pipettes, Erlenmeyer flasks, filters, phials, syringes, pers. All materials were sterilized in dry air oven at 180°C for 2 hours.

3.2 Preparation of culture and media

3.2.1 Microorganism

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). Rehydrated cultures were used in the stock culture preparation. Stock cultures of *B. trispora,* were prepared on slant and deep potato dextrose agar (PDA; Darmstadt, Germany) medium in tubes. Agar media were inoculated with rehydrated cultures and incubated at 25^{o}C for 2-3 days. Stock cultures were stored at 4°C and used in lycopene production fermentation.

3.2.2 Media

In the preparation of yeast phosphate soluble starch (YpSS) broth medium, the following ingredients were mixed in distilled water: Yeast extract 4, K_2HPO_4 1, $MgSO_4$ 0.5 and starch 15 g/L. The pH of the medium was adjusted to 6.5 and then broth was dispensed into test tubes (7 ml).

In the preparation of YpSS agar medium, the following ingredients were mixed in distilled water: Yeast extract 4, K_2HPO_4 1, $MgSO_4$ 0.5, starch 15 and agar 20 g/L. The pH of the medium was adjusted to 6.5 before addition of agar. After addition of agar into liquid medium, it was heated up to boiling to solubilize agar in heater. After boiling, agar was dispensed into test tubes to prepare slants (7 ml) and deeps (8 ml).

Test tubes containing broth and agar medium, and remaining agar medium in flask were sterilized in autoclave at 121^{o}C for 15 min. After sterilization: (i) Preparation of agar slant: Tubes of agar media were converted to slants by laying the tubes down in a near-horizontal manner (at a slanted position) as soon as they were removed from the autoclave. A piece of rubber tubing (1.25 cm) was used to support the capped end of the tube. Agar was solidified after cooling. (ii) Preparation of broth and agar deep: Tubes of broth and agar deeps were allowed to cool at room temperature after removal from the autoclave. (iii) Preparation of plates: about 18-22 ml of cooled YpSS agar (cooled to 50^{o}C) was poured into sterile Petri dishes from flask under aseptic conditions (over disinfected desk top and near Bunsen burner flame) and allow to solidify the agar, and then plates were inverted to prevent condensation of vapour from led.

Once they have cooled, test tubes on holder and inverted Petri plates are placed into an incubator at 35^{o}C for overnight to control possible contamination, then store in refrigerator for use. If tubes of media and plates are not to be used immediately, they should be stored in a refrigerator. At refrigerated temperature, media was kept for months.

3.2.3 Preparation of fermentation media

The basal fermentation medium (BFM) had the following composition: Yeast extract 1, asparagine 2, K_2HPO_4 1.5 and $MgSO_4$ 0.5 g/L. The BFM was supplemented with different concentrations of ingredients as given in Table 3.1: natural oils (sunflower and corn oils), glucose, sucrose and orange peel. All medium were prepared at three different pH: 6.5, 5.5 and 4.5. The pH of fermentation medium was adjusted with sterile 0.2 N HCl and 1.0 N NaOH. When the pH of medium was 6.5, it was adjusted before sterilization. When the pH of medium was 4.5 and 5.5, it was adjusted after sterilization using sterile acid and base and cooling the medium to about 50°C. Fermentation media (45 ml) in Erlenmeyer flasks (250 ml) were sterilized at 121°C for 15 min in autoclave. Once they have cooled, flasks were placed into an incubator at 35^{o}C for overnight to control possible contamination, then they were used for fermentation.

Table 3.1 Supplements added into fermentation medium

	Composition (g/L)				
Medium no	**Glucose**	**Sucrose**	**Sunflower oil**	**Corn oil**	**Orange peel**
1	40				
2	60				
3	80				
4		50			
5		60			
6		70			
7	40		10		
8	40		30		
9	40		60		
10	40			10	
11	40			30	
12	40			60	
13		70	10		
14		70	30		
15		70	60		
16		70		10	
17		70		30	
18		70		60	
19					10
20					20
21					40

3.3 Production of Lycopene

3.3.1 Preparation of *B. trispora* for inoculation into fermentation medium

Two strains were inoculated separately on to YpSS agar plates by streaking using sterile cottonwood swab. Inoculated plates were incubated at 25^{o}C for 3 days and used as stock culture for inoculation into fermentation medium. Ten milliliters of sterile distilled water were added to the Petri dish and the spores were collected by scraping off the medium surface. The spore suspension containing 1.0x10^{5} and 1.0-1.85x10^{6} spores/ml was used to inoculate the medium. Strains were inoculated into fermentation medium with 2.5 and 7.5-mL of spore suspension (+) to (-) strains to provide 1:3 ratio inoculation.

3.3.2 Shake flask fermentation process

Fermentation media (45 ml) in Erlenmeyer flasks (250 ml) were inoculated with *B. trispora* strains ATCC 14271 (+) and 14272 (-) by 1:3 ratio from spore stock culture. Then, fermentation media were incubated at 28^{o}C for 7 days on a bench type water bath shaker ST-402 (NÜVE; Sanayi Malzemeleri Imalat ve Ticaret A. Ş., İstanbul, Turkey) wrist shaking at 120 rpm. At every sampling interval, 5 ml of sample was taken from fermentation flasks and the contents were analyzed for lycopene, pH and biomass.

3.3.3 Effect of the addition of ingredients

Each of the glucose and sucrose was added into fermentation medium by 40, 60 and 80 (g/L) and 50, 60 and 70 (g/L) to investigate lycopene production, respectively. Each of the medium containing glucose or sucrose was supplemented with each of sunflower and corn oil by 10, 30 and 60 (ml/L). Fermentation was also studied by adding orange peel into basal fermentation medium with 10, 20 and 40 (ml/L). The lycopene cyclase inhibitor of nicotine (5 m/M) was added on day 2 during the experiments above.

3.3.4 Fermentation process in 7.0 L fermenter

Sterilizable 7 liter fermenter (Bioflow 410,) was used in the fermentation. 4 L of fermentation medium was prepared in fermenter. The initial pH of fermentation was 6.5. The fermentation medium was sterilized at 121°C for 15 min. The base fermentation medium is consist of (g/l):

1. Medium: glucose 60, asparagine 2, yeast extract 1, KH_2PO_4 1.5 and $MgSO_4$ 0.5

Fermentations in this medium studied at different aeration (3 and 12 L/min) and agitation (500 and 750 rpm) rates. The fermenter was incubated at 28^{o}C in controlled conditions (aeration, mixing and temperature). The inoculation ratio of two strains (+/-) into the fermentation medium in 7.0 L fermenter were the same with those in the shake flask. The lycopene cyclase inhibitor of nicotine (5 m/M) was added on

day 2 during the experiments above. Dissolved oxygen tension was not controlled during fermentation but level was recorded.

3.4 Analysis

3.4.1 Sampling

Five ml of fermented samples were aseptically removed from shake flask and fermenter after 2, 3, 4, 6 and 7 days of fermentation time. Two ml of sample was used in lycopene analysis and 3 ml was used in pH and biomass analysis. Two samples were removed and two parallel analyses were performed from each sample. All experiments were repeated three times.

3.4.2 Lycopene Analysis

Samples were analyzed for lycopene by HPLC method as indicated in the Association of Official Analytical Chemists (AOAC Official Method, 2012). Two ml of sample was mixed with 15 ml of petroleum ether. The mixture was subjected to ultrasound for 30 sec and then, sample was centrifuged using table-type centrifuge (Hettich eba III) by 6 rpm for 15 min. The supernatant was taken with Pasteur pipette and passed through 4 µm filter paper. About 2 ml of liquid was added into 3 ml vials. Then the supernatant containing petroleum ether and lycopene was analyzed by HPLC immediately. The presence of lycopene was detected by HPLC using a post-column derivatization electrochemically generated bromine (Kobra cell) and a fluorescence detector. The flow rate was 1 ml/min and the column was nukleosil C18 (250 x 4.6 mm ID). Temperature was 28°C. Lycopene was detected at 450 nm. 20 µm sample was injected to HPLC automatically and the result was read for 40 min. 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 degosed in . The peaks in chromatogram were evaluated according to the standard curve.

3.4.2.1 Validation of the method

The limit of detection (LOD) is the smallest amount or concentration of analyte in the test sample that can be reliably distinguished from zero. Limit of quantification is defined as the concentration at which the entire analytical system must give a recognizable signal and acceptable calibration point. LOD and LOQ were 0.000008 and 0.00002 μg kg^{-1}, respectively, for lycopene. LOD was calculated with a signal-to-noise ratio (S/N) = 3/1 and LOQ with S/N = 10/1. The calibration curve of lycopene was the equations: y = 7E+06x – 13.18. The retention time of lycopene was 29.05 min.

3.4.2.2 Preparation of standard curve

In this study 5 point calibration curve was used. Five different concentrations was prepared from the stock standard (which has a known concentration of (mg/ml) and a curve was drawn (concentration of lycopene (mg/ml) against peak area (mV*min) (Figure 3.1). The peaks of samples were compared with that of lycopene standards.

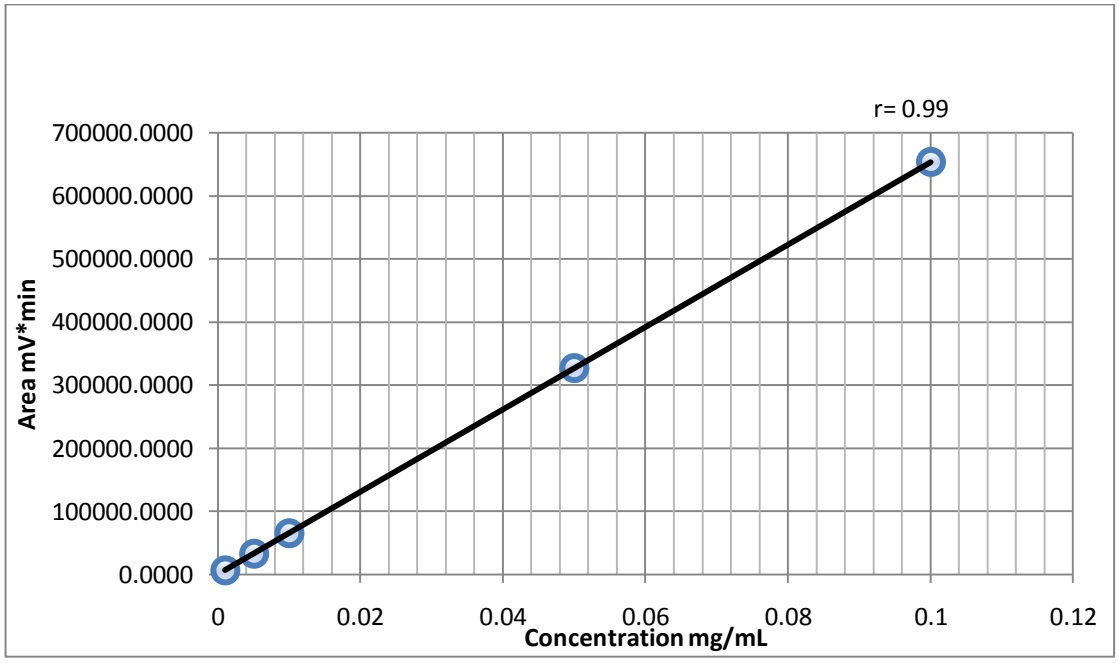

Figure 3.1 Calibration curves of lycopene

3.4.3 pH and biomass analysis

Three ml of remaining fermentation sample was used for pH detection and biomass analysis. Whatman No. 41 filter paper was dried at 105^{o}C to constant weight, cooled in desicator and weighed before use. 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 50^{o}C and dried until constant weight and then weighed to calculate the biomass (g/L).

Filtered solution was completed to 10 ml with distilled water and pH of sample was detected using pH meter (EMAF EM78X model) equipped with a glass electrode.

3.5 Statistical analyses

All data were analyzed by SPSS 16 software (SPSS Inc, Chicago, IL). The number of samples with lycopene for sample was compared (between effect of ingredients) using the $\chi 2$ test. Statistical differences among fermentation time (days) were tested by one-way analysis of variance following Duncan's multiple-range test. Statistical differences between regions were tested by independent sample t-test. A probability of 0.05 was used to determine statistical significance.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Production of lycopene from glucose and sucrose

In this study, glucose and sucrose were investigated as carbon sources and three different initial pH (6.5, 5.5 and 4.5) values were studied on the production of lycopene for each carbon source. The initial pH is one of the important parameters in the production of lycopene by *B. trispora*. The pH changes was not adjusted during fermentation.

Figures 4.1, 4. 2 and 4.3 show the effect of different glucose concentrations (4, 6 and 8 %) on lycopene production at initial pH 6.5. At the initial pH 6.5, higher amount of lycopene was produced from 4 % glucose than 6 and 8 %. Lycopene was produced 77.69, 7.70 and 3.72 mg/L with 4, 6 and 8 % glucose in the fermentation medium, respectively. For these glucose concentrations, biomass results were 63.2, 20.6 and 25.3 g/L, respectively. Lycopene content for these values was calculated as 0.12, 0.03 and 0.01 mg/100 mg dry weight (DW), respectively.

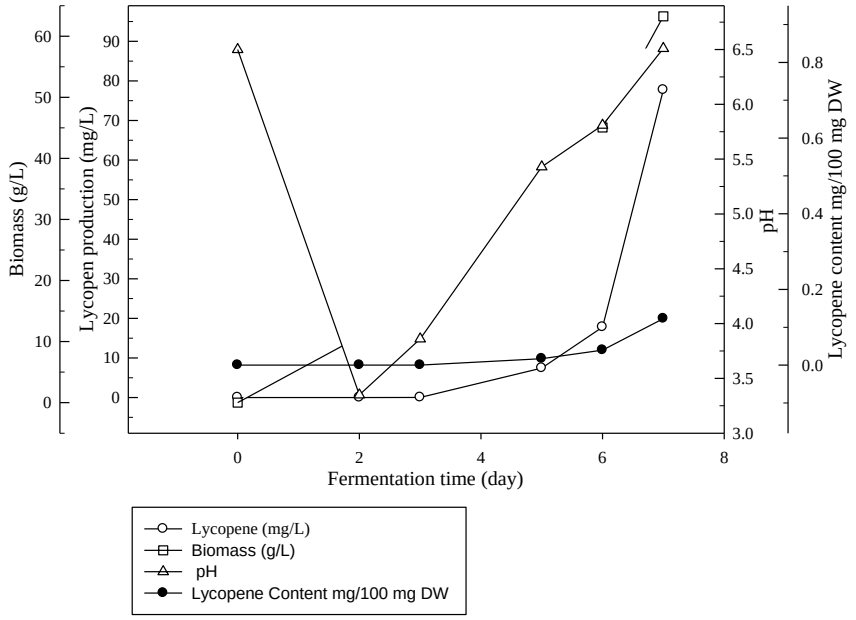

Figure 4.1 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing 4 % glucose

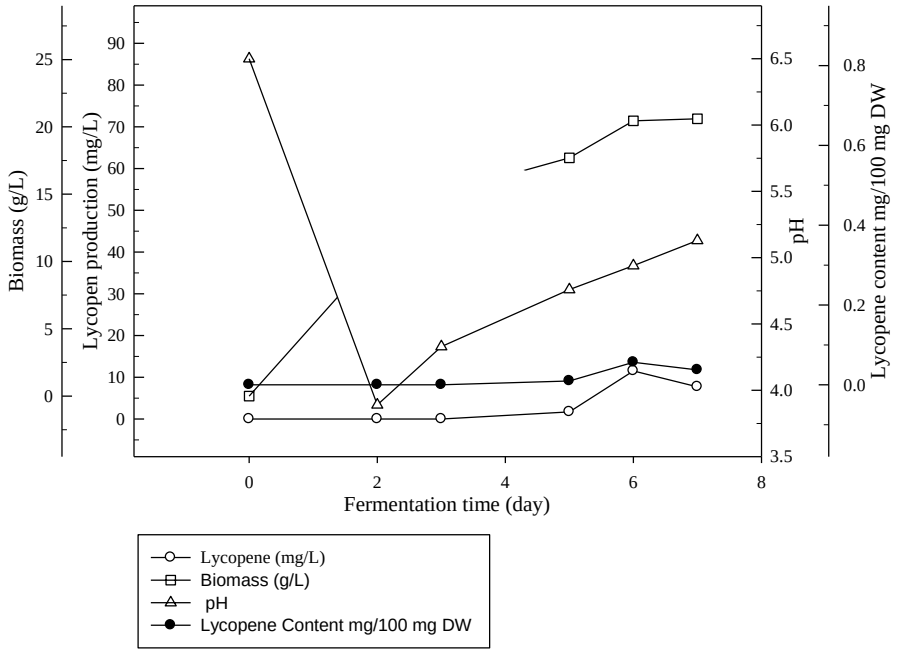

Figure 4.2 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing 6 % glucose

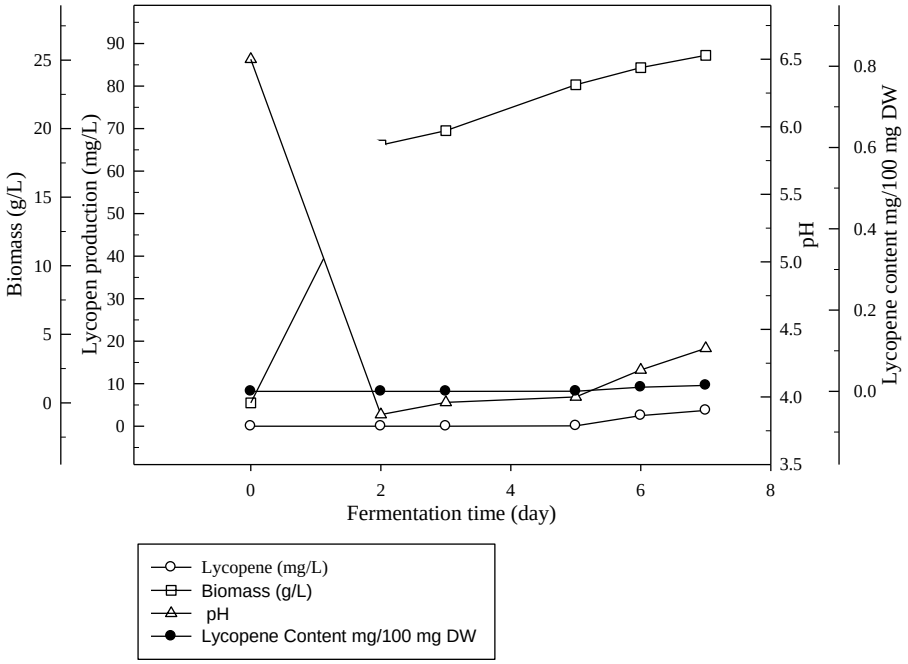

Figure 4.3 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing 8 % glucose

Figures 4.4, 4.5 and 4.6 show the effect of different glucose concentrations (4, 6 and 8 %) at initial pH 5.5 on lycopene production. At the initial pH 5.5, higher amount of lycopene was produced from 4 % glucose than 6 and 8 %. Lycopene was produced 21.4, 5.7 and 0.8 mg/L with 4, 6 and 8 % glucose in the fermentation medium, respectively. For these glucose concentrations, biomass results were 5.0, 18.8 and 6.8 g/L, respectively. Lycopene content was calculated as 0.4, 0.03 and 0.01 mg/100 mg DW, respectively.

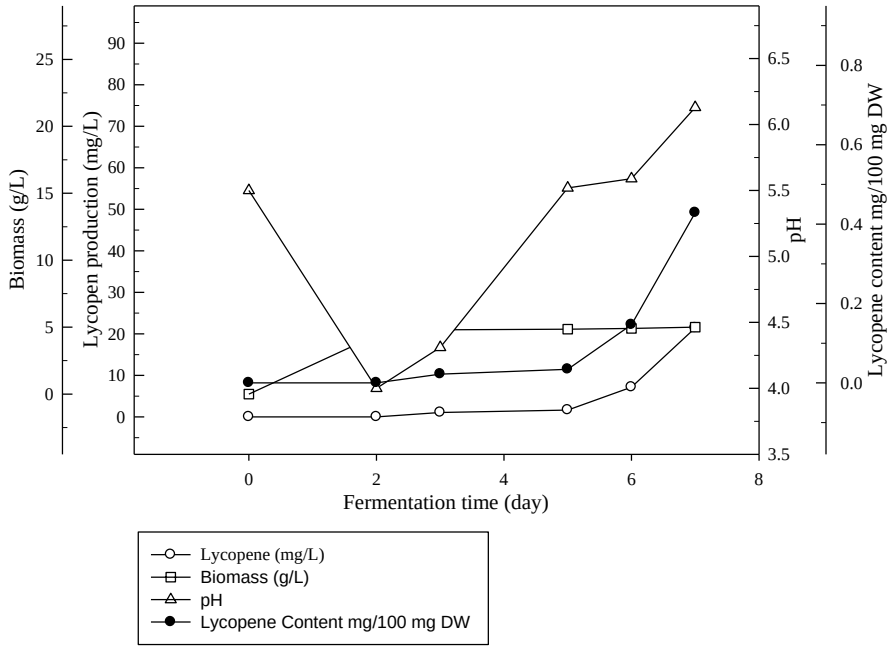

Figure 4.4 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing 4 % glucose

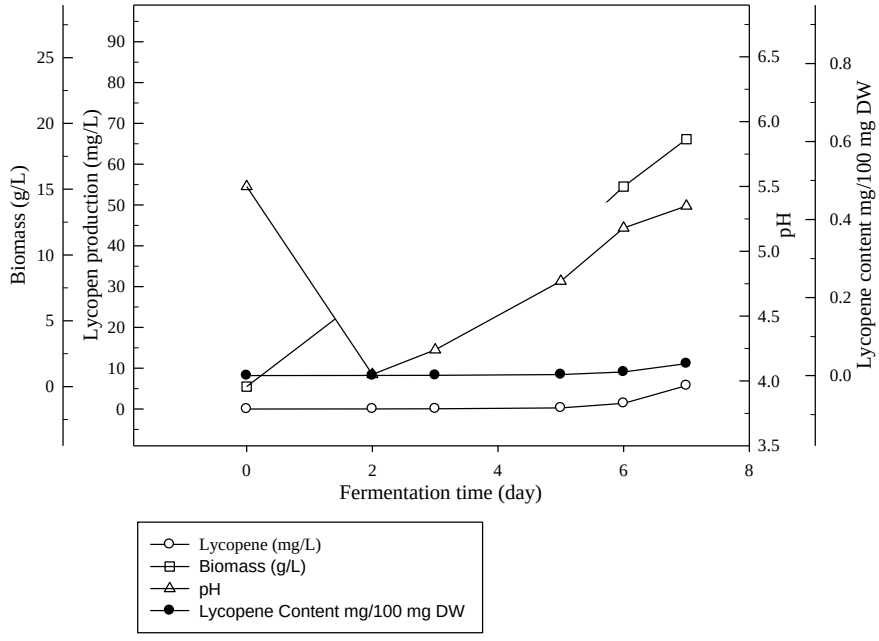

Figure 4.5 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing 6 % glucose

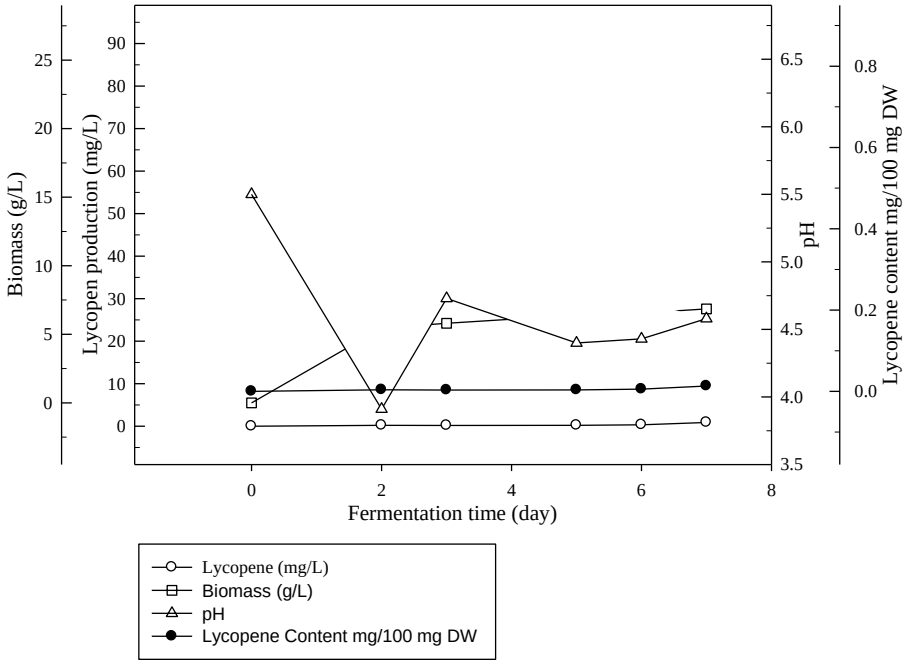

Figure 4.6 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing 8 % glucose

Figures 4.7, 4.8 and 4.9 show the effect of different glucose concentrations (4, 6 and 8 %) at initial pH 4.5 on the lycopene production. Lycopene was produced 18.3, 2.6 and 2.0 mg/L with 4, 6 and 8 % glucose, respectively. Maximum lycopene was produced with 4 % glucose. Biomasses were 14.6, 45.9 and 30.6 g/L with 4, 6 and 8 % glucose, respectively. Lycopene contents were 0.12, 0.005 and 0.006 mg/100 mg DW with 4, 6 and 8 % glucose respectively.

There are no significant differences ($p>0.05$) in lycopene production between pH 6.5. There are significant different differences ($p<0.05$) in lycopene production during fermentation periods.

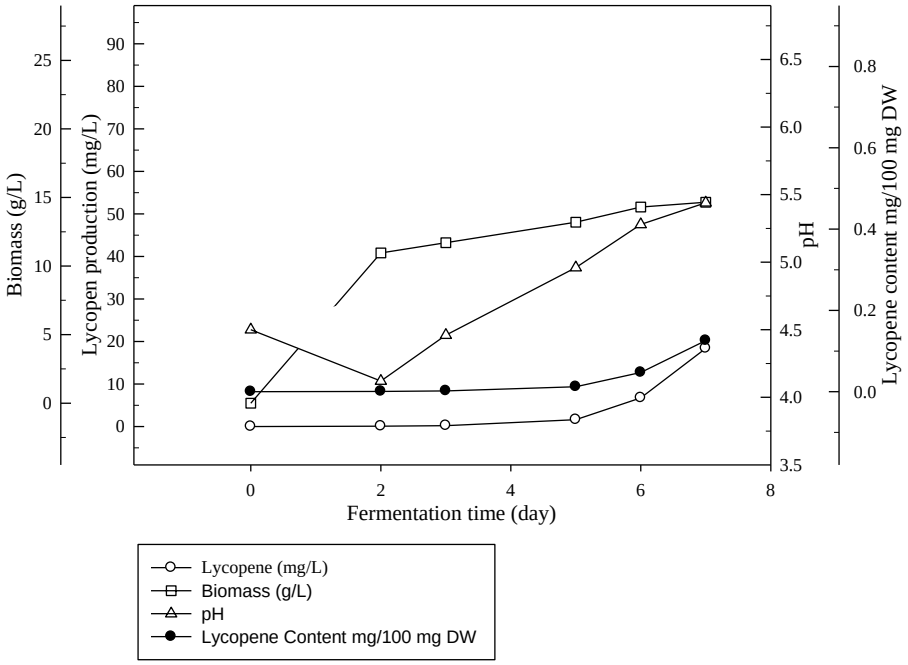

Figure 4.7 Changes biomass and pH in fermentation with initial pH 4.5 in medium containing 4 % glucose

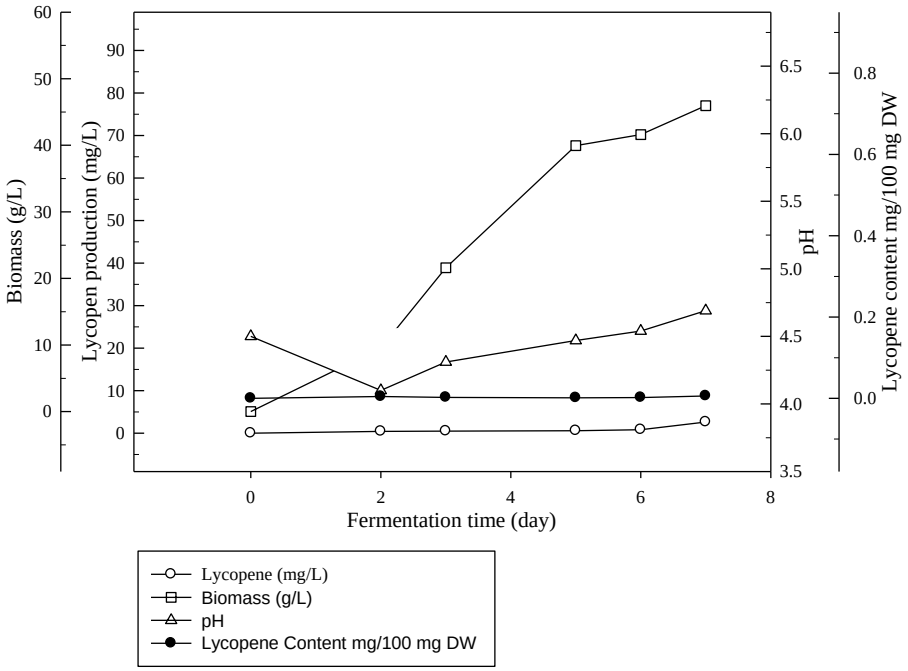

Figure 4.8 Changes biomass and pH in fermentation with initial pH 4.5 in medium containing 6 % glucose

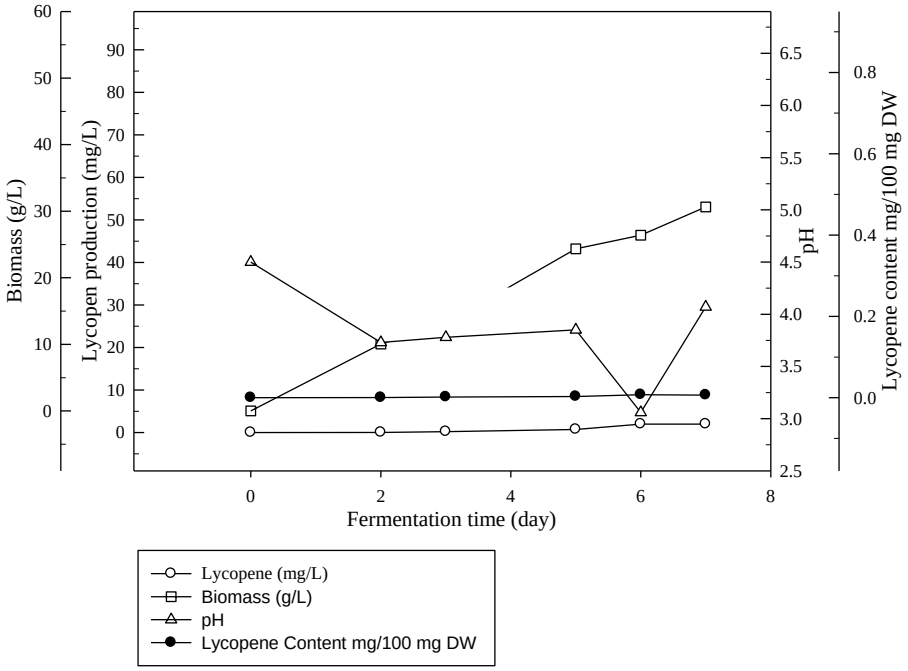

Figure 4.9 Changes biomass and pH in fermentation with initial pH 4.5 in medium containing 8 % glucose

Figures 4.10, 4.11 and 4.12 show the effect of different sucrose concentrations (5, 6 and 7 %) at initial pH 6.5 on lycopene production. Lycopene was produced 12.4, 57.0 and 57.2 mg/L with 5, 6 and 7 % sucrose in the fermentation medium, respectively. The amount of biomass formation was 13.8, 16.9 and 15.9 g/L with 5, 6 and 7 % sucrose, respectively. Lycopene content was produced 0.09, 0.33 and 0.36 mg/100 mg DW in three respective sucrose concentration.

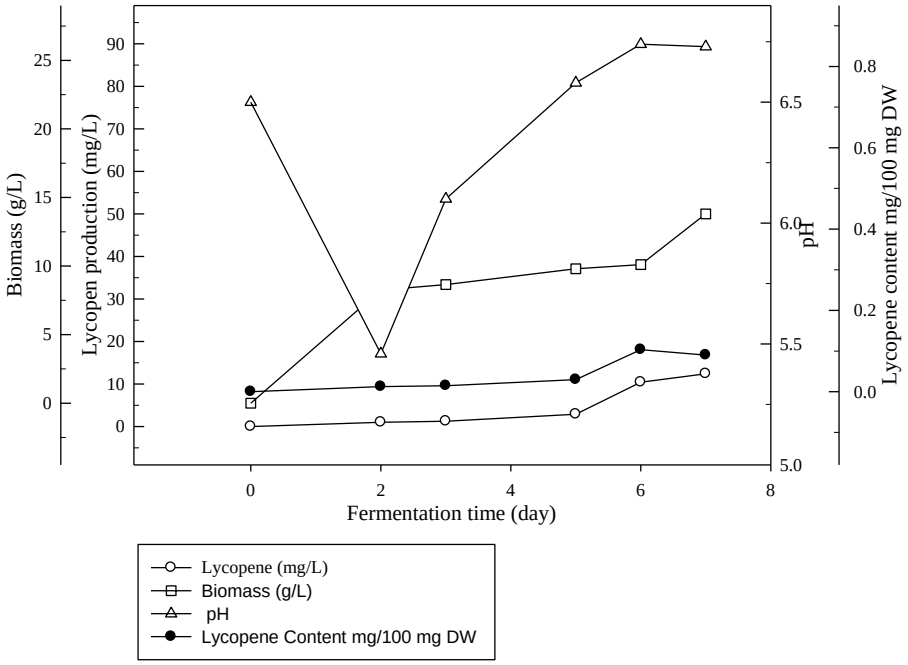

Figure 4.10 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing 5 % sucrose

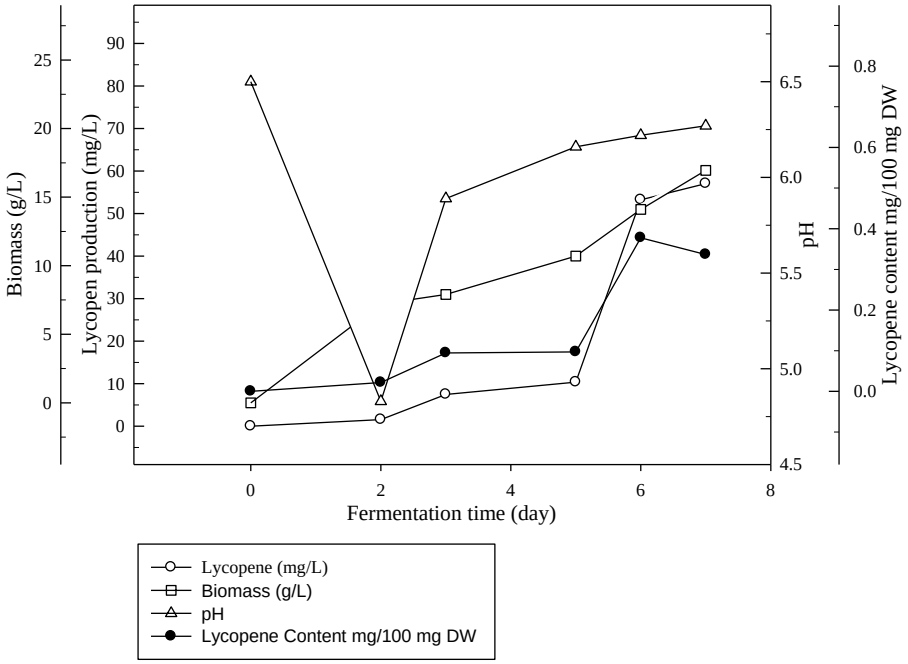

Figure 4.11 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing 6 % sucrose

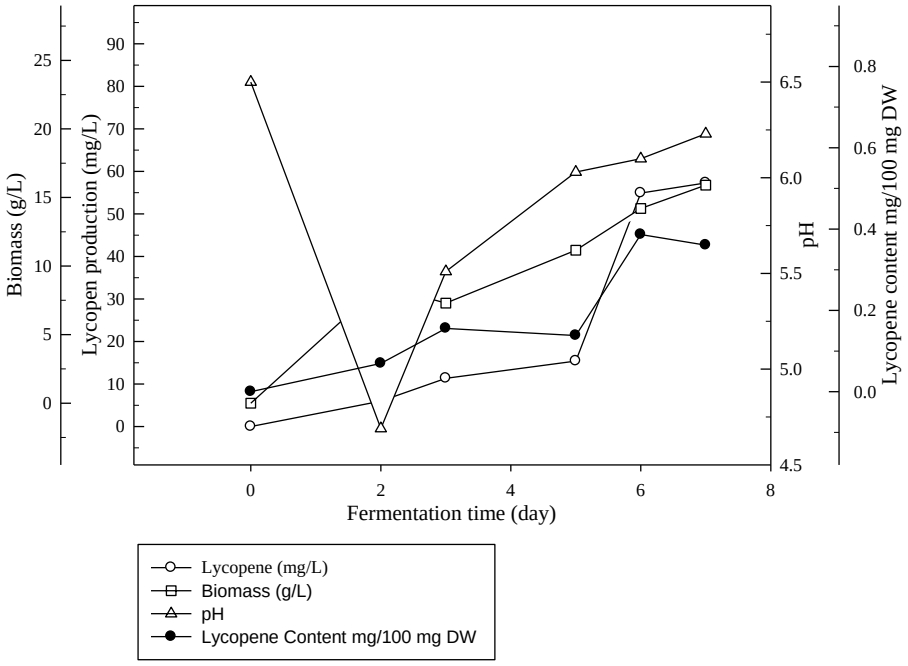

Figure 4.12 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing 7 % sucrose

Figures 4.13, 4.14 and 4.15 show the effect of different sucrose concentrations (5, 6 and 7 %) at initial pH 5.5 on the production of lycopene. Lycopene was produced 7.4, 12.0 and 16.6 mg/L with 5, 6 and 7 % sucrose, respectively, at the end of the fermentation. Biomass was 7.0, 40.1 and 10.4 g/L with 5, 6 and 7 % sucrose, respectively, at the end of fermentation. Lycopene content was 0.10, 0.03 and 0.15 mg/100 mg DW with 5, 6 and 7 % sucrose, respectively.

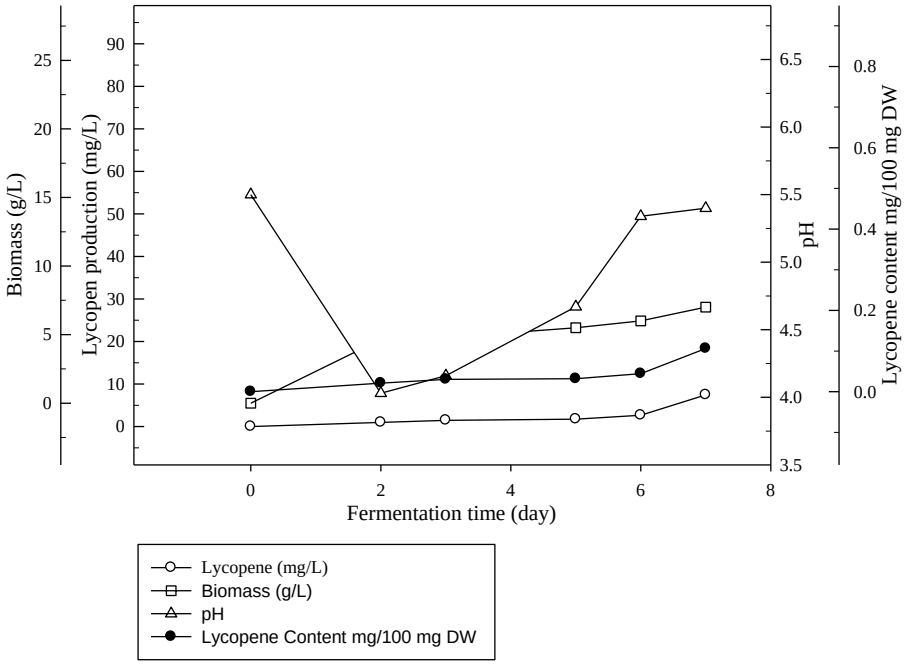

Figure 4.13 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing 5 % sucrose

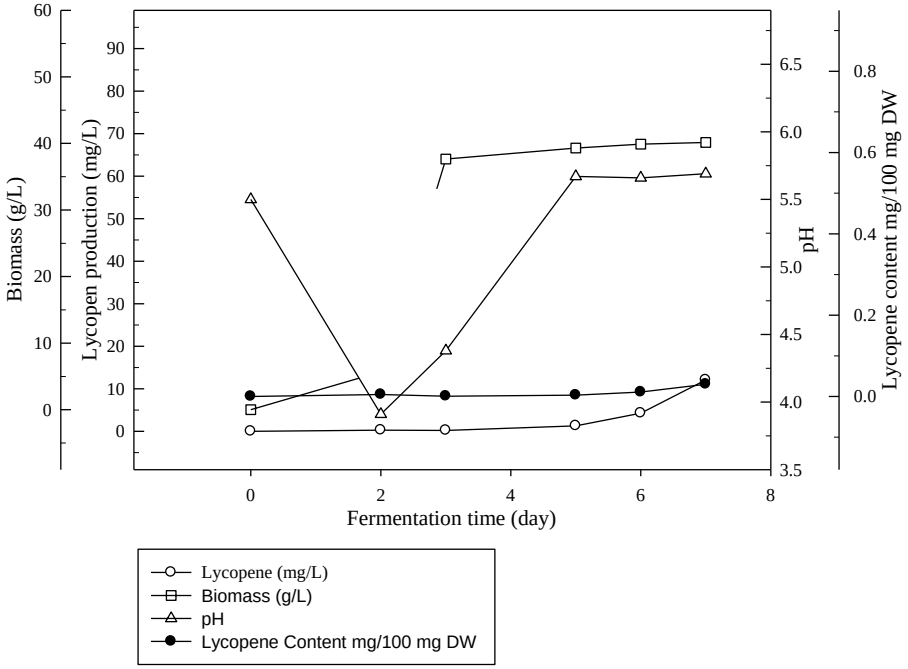

Figure 4.14 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing 6 % sucrose

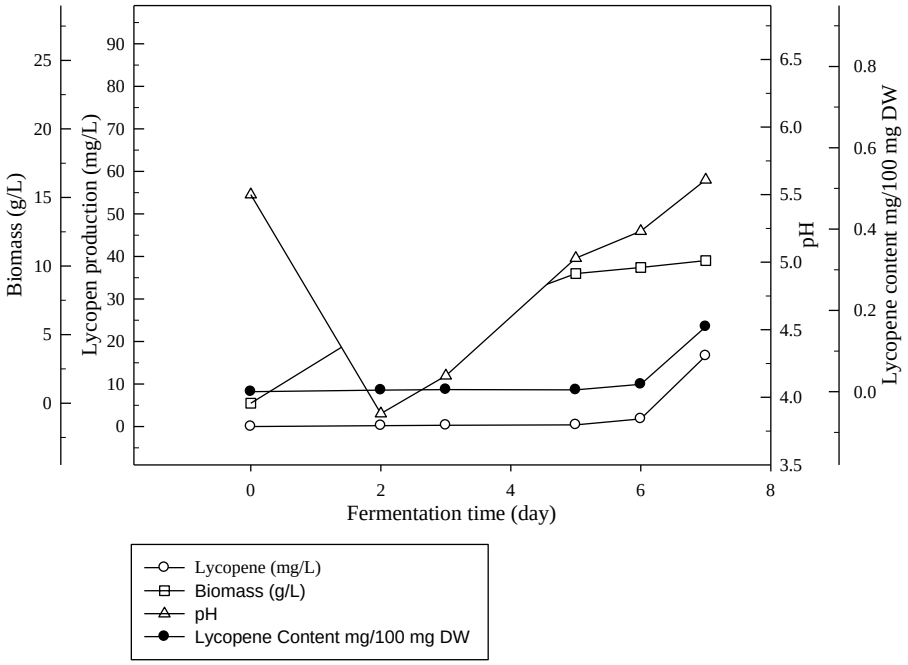

Figure 4.15 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing 7 % sucrose

Figures 4.16, 4.17 and 4.18 show the effect of different sucrose concentrations (5, 6 and 7 %) at initial pH 4.5 on the production of lycopene. Lycopene was produced 2.5, 6.0 and 8.2 mg/L with 5, 6 and 7 % sucrose, respectively. Maximum lycopene was produced with 5 % sucrose. Biomasses were 11.5, 10.8 and 10.6 g/L with 5, 6 and 7 % sucrose, respectively. Lycopene contents were 0.02, 0.05 and 0.07 mg/100 mg DW for 5, 6 and 7 % sucrose respectively.

There are no significant differences ($p>0.05$) in lycopene production between pH 6.5. There are significant different differences ($p<0.05$) in lycopene production during fermentation periods.

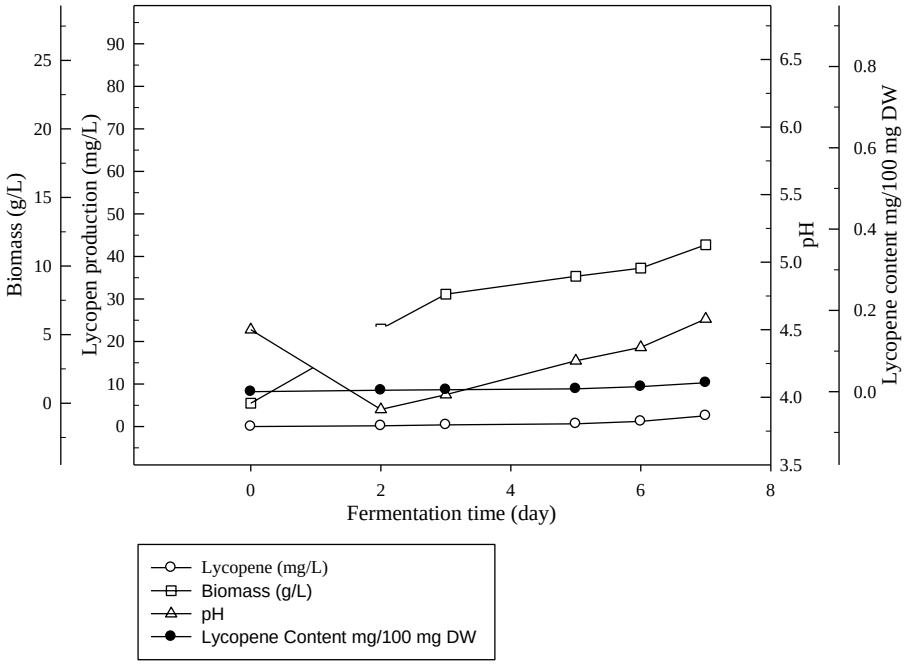

Figure 4.16 Changes biomass and pH in fermentation with initial pH 4.5 in medium containing 5 % sucrose

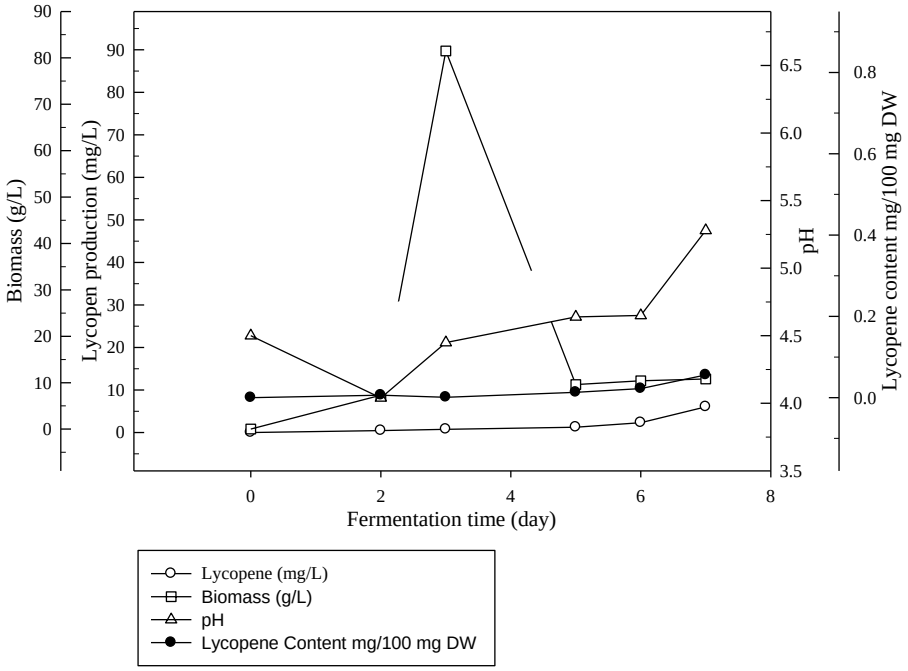

Figure 4.17 Changes biomass and pH in fermentation with initial pH 4.5 in medium containing 6 % sucrose

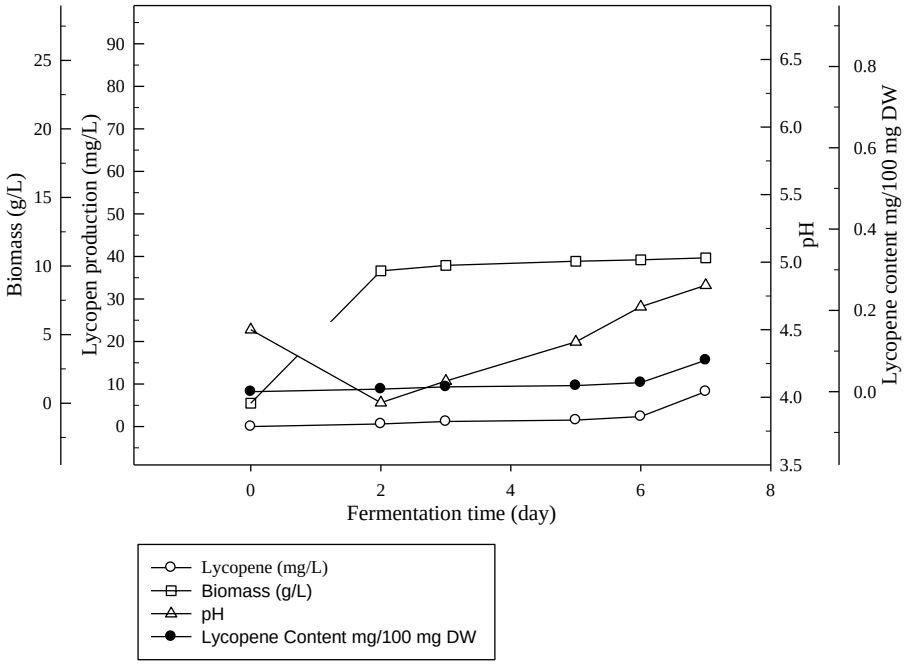

Figure 4.18 Changes biomass and pH in fermentation with initial pH 4.5 in medium containing 7 % sucrose

pH was measured during fermentation periods from medium. pH was changed during fermentation time in medium. pH was decreased and increased during 7 days fermentation. Higher amount of lycopene was produced with initial pH 6.5 in all glucose and sucrose concentrations.

4.1.2 Production of lycopene from orange peel

Orange peel was studied with 1, 2 and 4 % amount in fermentation medium in the shaker studier. Figures 4.19, 4.20 and 4.21 show the effect of three concentrations of orange peel on the production of lycopene. Lycopene productions from were studied with pH 6.5 and fermentation time 7 days, nicotine (5 m/M) was added second day of fermentation. The maximum amount of lycopene was produced with 1 % orange peel. Lycopene was produced 2.9, 1.2 and 1.4 mg/L with 1, 2 and 4 % orange peel, respectively. Biomasses were 8.5, 11.6 and 41.6 g/L for 1, 2 and 4 % orange peel, respectively. Lycopene was produced 0.03, 0.01 and 0.003 mg/100 mg DW in 1, 2 and 4 % orange peel, respectively. There are significant different differences ($p<0.05$) in lycopene production during fermentation periods.

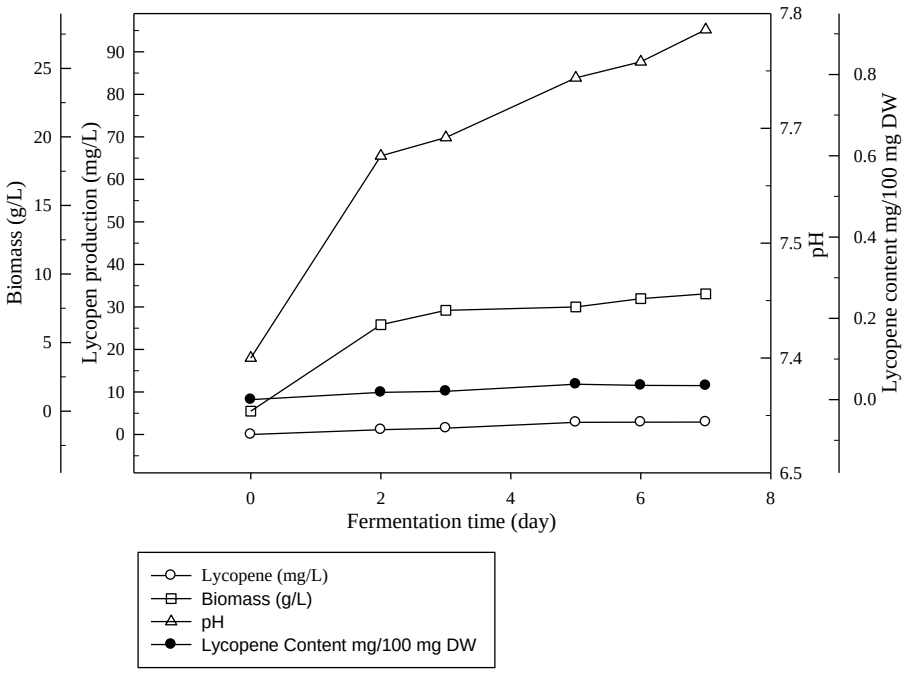

Figure 4.19 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing 1 % orange peel

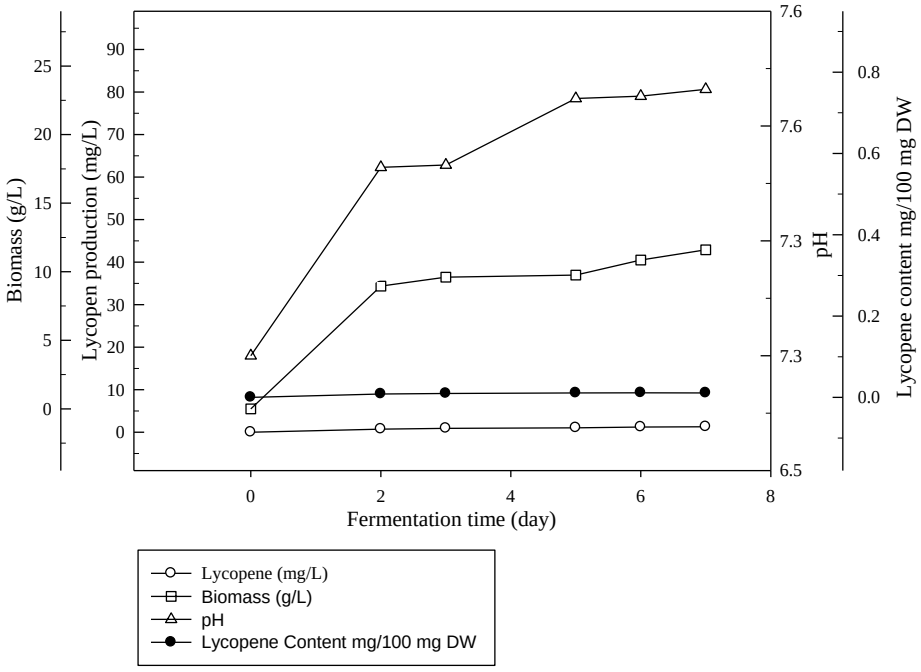

Figure 4.20 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing orange 2 % peel

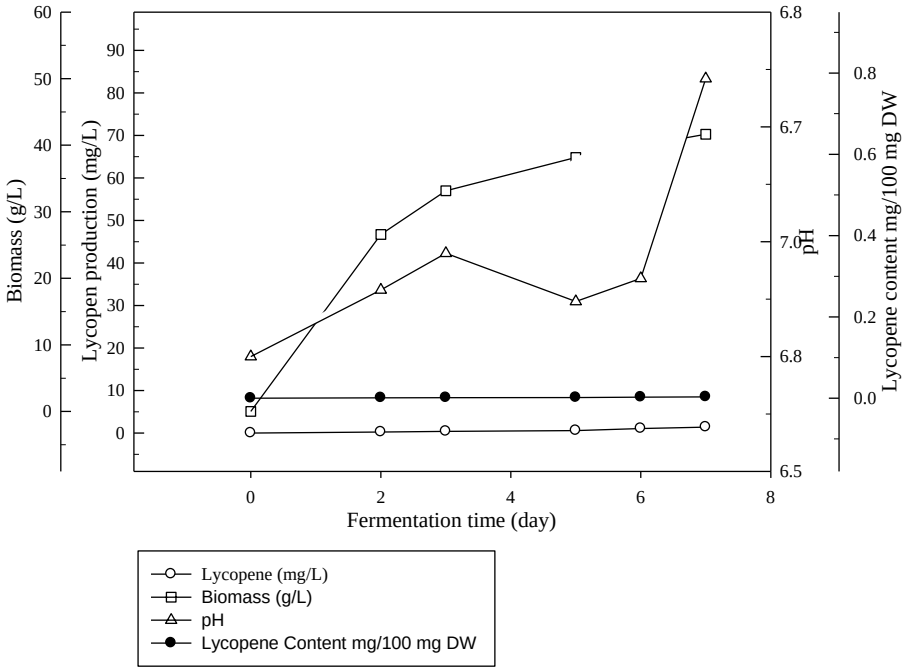

Figure 4.21 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing 4 % orange peel

4.1.3 Fermenter Studies

Lycopene production was studied from different concentrations of glucose and orange peel in fermenter. Orange peel was studied with 4 L fermentation volume in 7 L in-place sterilizable fermenter and agitation rate 500 rpm (Figure 4.22). Lycopene was produced 4.8 mg/L with 1 % orange peel. Biomass was 9.7 g/L and lycopene content was 0.05 mg/100 mg DW at the end of fermentation time.

For glucose, two different fermentation conditions were used: aeration 3 L/min with 500 rpm agitation and aeration 12 L/min with 750 rpm agitation (Figure 4.23 and 4.24, respectively), the end of fermentation, lycopene production was 92.2 and 0.6 mg/L, respectively. The higher amount of lycopene was produced with 3 L/min aeration and 500 agitation. Biomass formations were 154.9 and 196.7 g/L for 3

L/min and 12 L/min for aeration, respectively. Lycopene content was 0.05 and 0.0003 mg/100 mg DW from glucose for 3 L/min and 12 L/min aeration, respectively. There are significant different differences ($p<0.05$) in lycopene production during fermentation periods.

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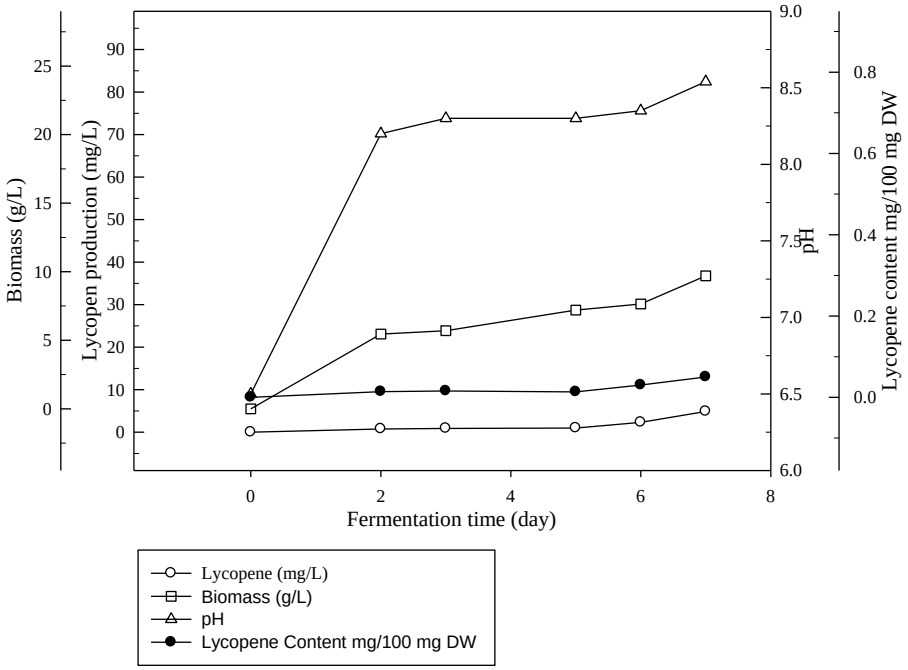

Figure 4.22 Changes biomass and pH in fermentor with initial pH 6.5 in fermentation medium containing 1 % orange peel

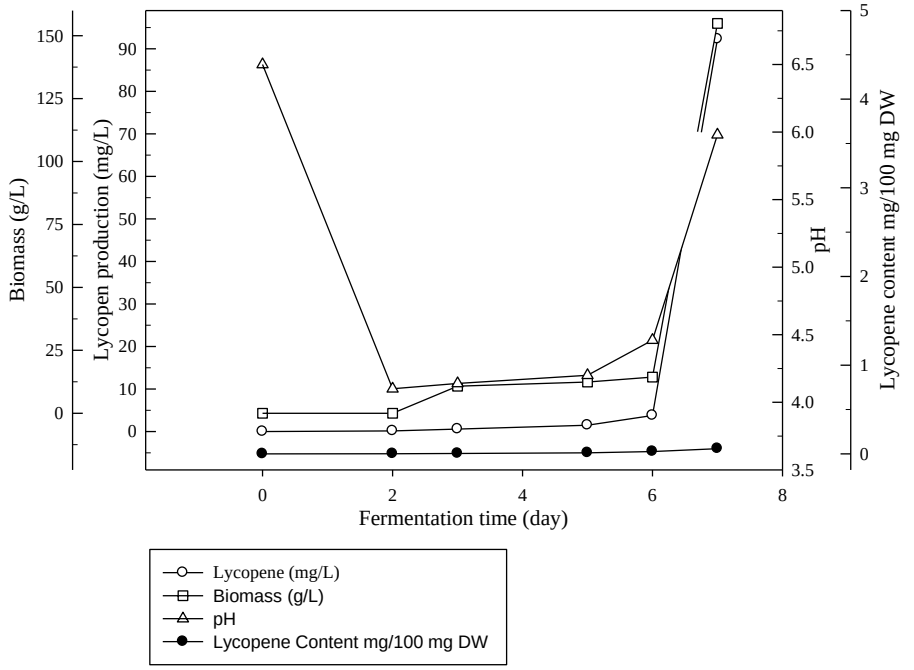

Figure 4.23 Changes biomass and pH in fermentor with initial pH 6.5 in fermentation medium containing 4 % glucose (aeration 3 L/min with 500 rpm agitation)

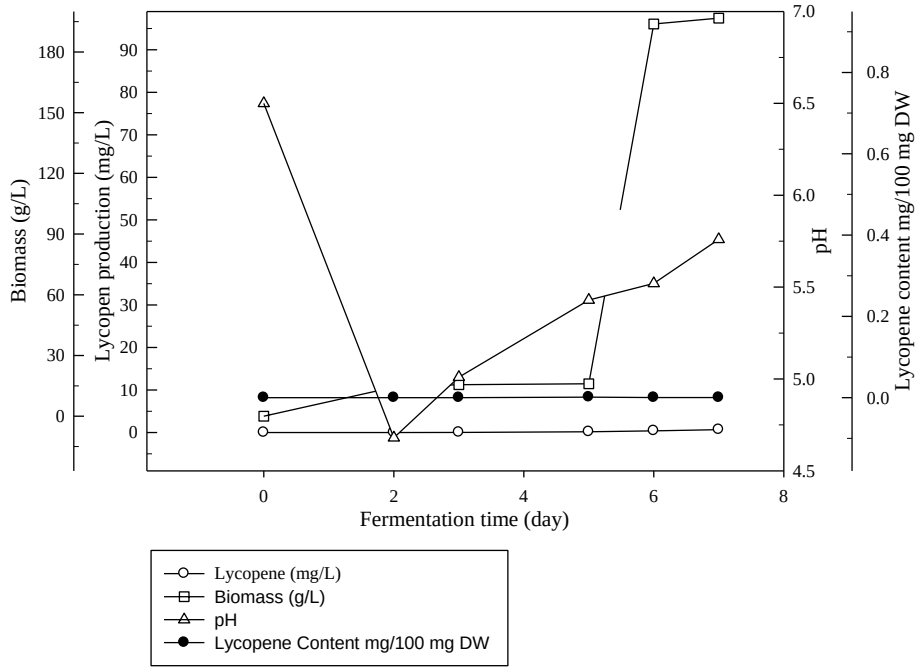

Figure 4.24 Changes biomass and pH in fermentor with initial pH 6.5 in fermentation medium containing 4% glucose (aeration 12 L/min with 750 rpm agitation)

Figure 4.25 and 4.26 show effect of sunflower and corn oil added into 4% glucose containing medium. Lycopene production was 944.8 and 859.8 mg/L, respectively. Biomass formations were 17.6 and 9.6 g/L. Lycopene content was 5.3 and 8.9 mg/100 mg DW. There are significant differences ($p<0.05$) in lycopene production between with and without oil. There are significant different differences ($p<0.05$) in lycopene production during fermentation periods.

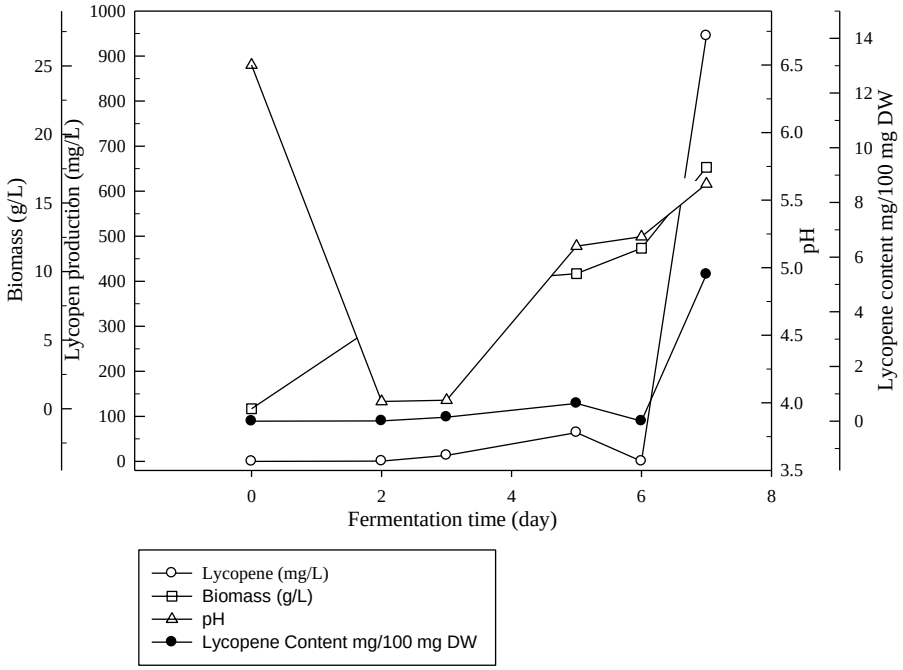

Figure 4.25 Changes biomass and pH in fermentor with initial pH 6.5 in fermentation medium containing 4 % glucose added 1 % sunflower oil (aeration 3 L/min with 500 rpm agitation)

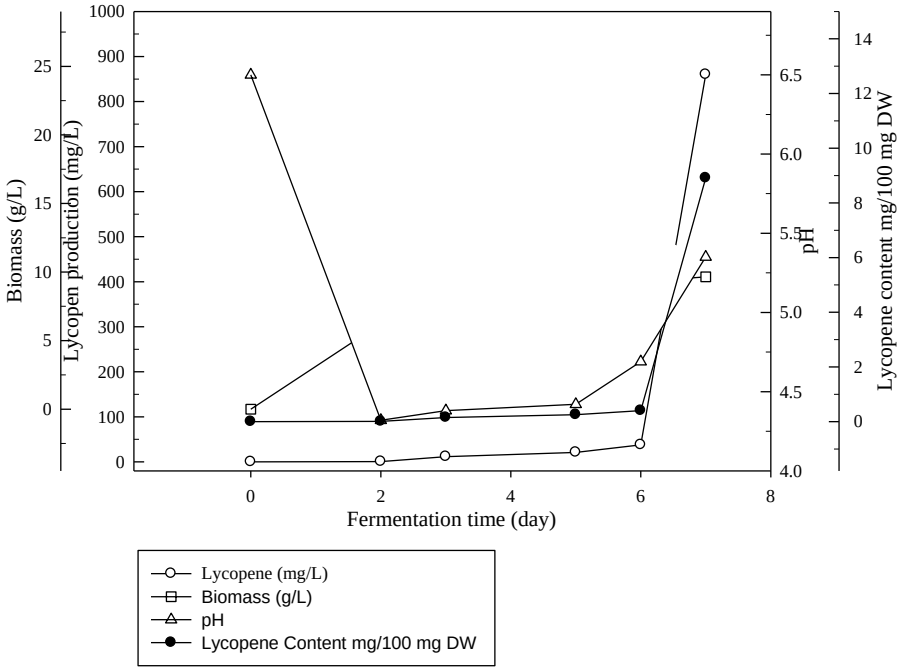

Figure 4.26 Changes biomass and pH in fermentor with initial pH 6.5 in fermentation medium containing 4 % glucose added 1 % corn oil (aeration 3 L/min with 500 rpm agitation)

4.1.4 Effect of natural oils in shake flasks

In this study, effect of natural oil (natural sunflower oil and corn oil) on the production of lycopene was studied in glucose and sucrose medium at different initial pH. Figures 4.27, 4.28 and 4.29 show the effect of sunflower oil (1, 3 and 6 %) on the production of lycopene in 4 % glucose medium with initial pH 6.5. The maximum lycopene was produced 795.1 mg/L with 1 % sunflower oil. Biomass content was 24.1 g/L and lycopene content was 3.3 mg/100 mg DW with 1 % sunflower oil at the end of fermentation. Lycopene was produced 275.3 mg/L with 3 % sunflower oil (Figure 4.28). Biomass content was 31.3 g/L and lycopene content was 0.8 mg/100 mg DW at the end of fermentation. Lycopene was produced 54.3 mg/L and biomass content was 19.4 g/L in 3 % sunflower oil and lycopene content was 0.3 mg/100 mg DW (Figure 4.29). There are significant differences ($p<0.05$) in lycopene production between with and without oil. There are significant different differences ($p<0.05$) in lycopene production during fermentation periods.

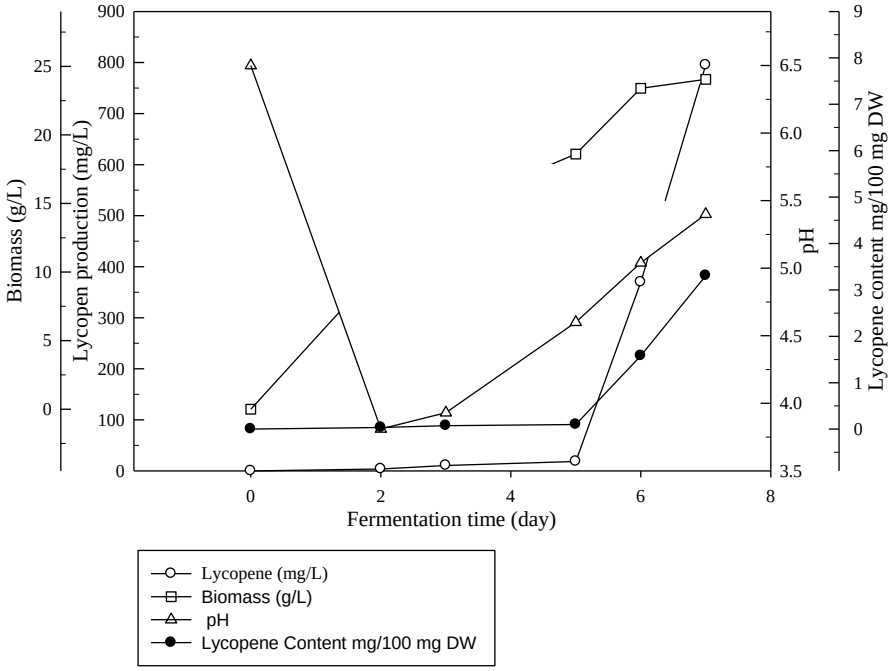

Figure 4.27 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 4 % glucose and 1 % sunflower oil

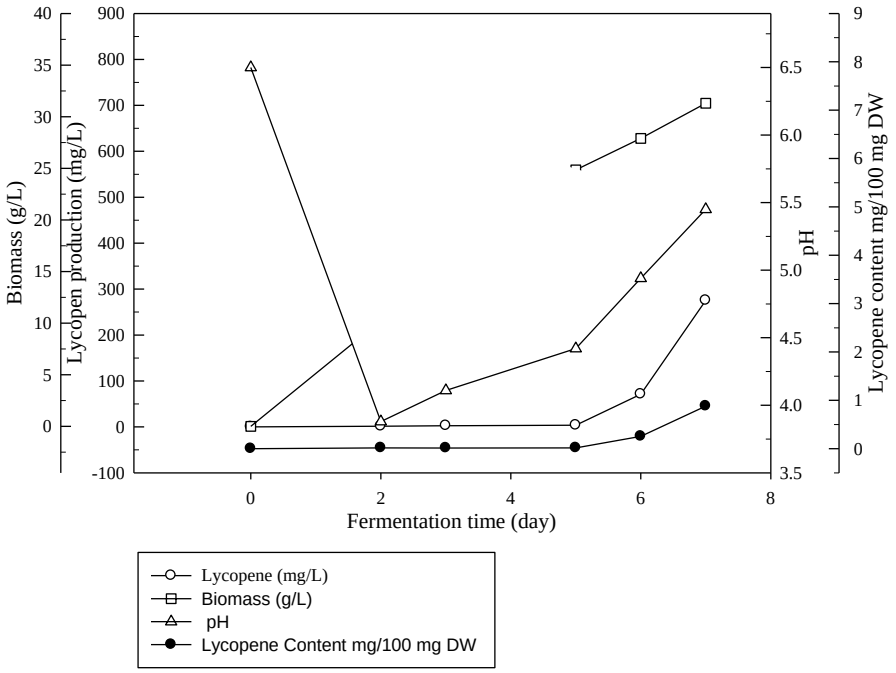

Figure 4.28 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 4 % glucose and 3 % sunflower oil

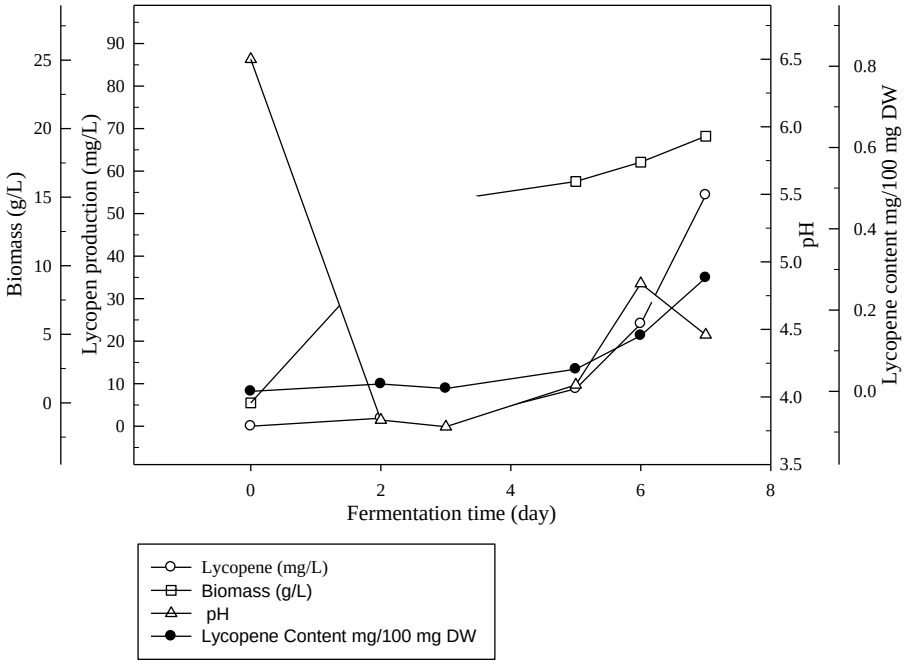

Figure 4.29 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 4 % glucose and 6 % sunflower oil

Lycopene was produced 35.4 mg/L with 1 % sunflower oil in glucose medium with pH 5.5 (Figure 4.30). Biomass content was 29.5 g/L and lycopene was 0.1 mg/100 mg DW at the end of fermentation. Lycopene was produced 13.0 mg/L with 3 % sunflower oil in glucose medium with pH 5.5 (Figure 4.31). Biomass content was 13.8 g/L and lycopene content was 0.09 mg/100 mg DW at the end of fermentation. Lycopene was produced 8.8 mg/L with 6 % sunflower oil (Figure 4.32). Biomass content was 49.3 g/L and lycopene content was 0.01 mg/100 mg DW at the end of fermentation time.

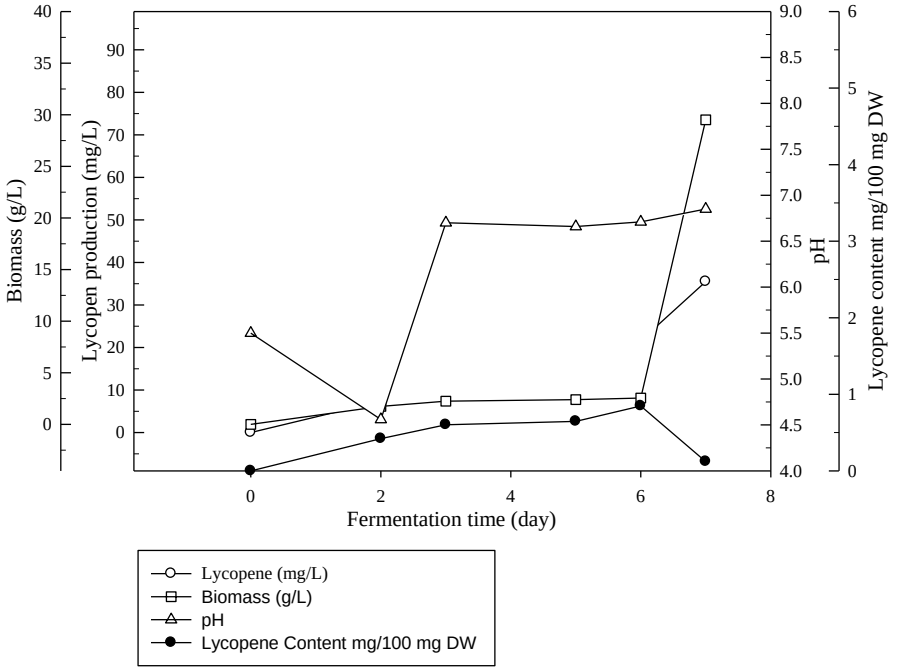

Figure 4.30 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 4 % glucose and 1 % sunflower oil

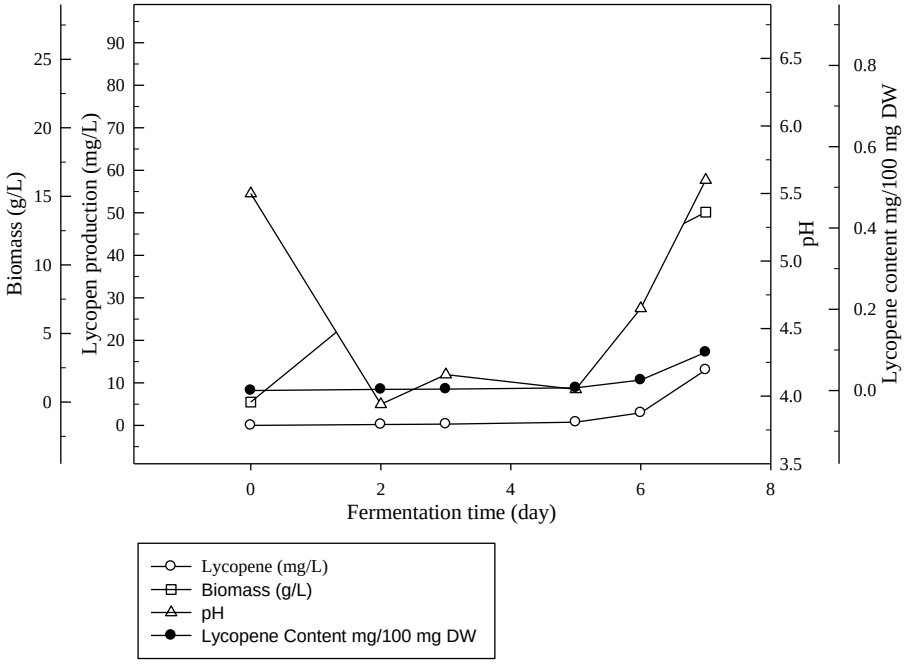

Figure 4.31 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 4 % glucose and 3 % sunflower oil

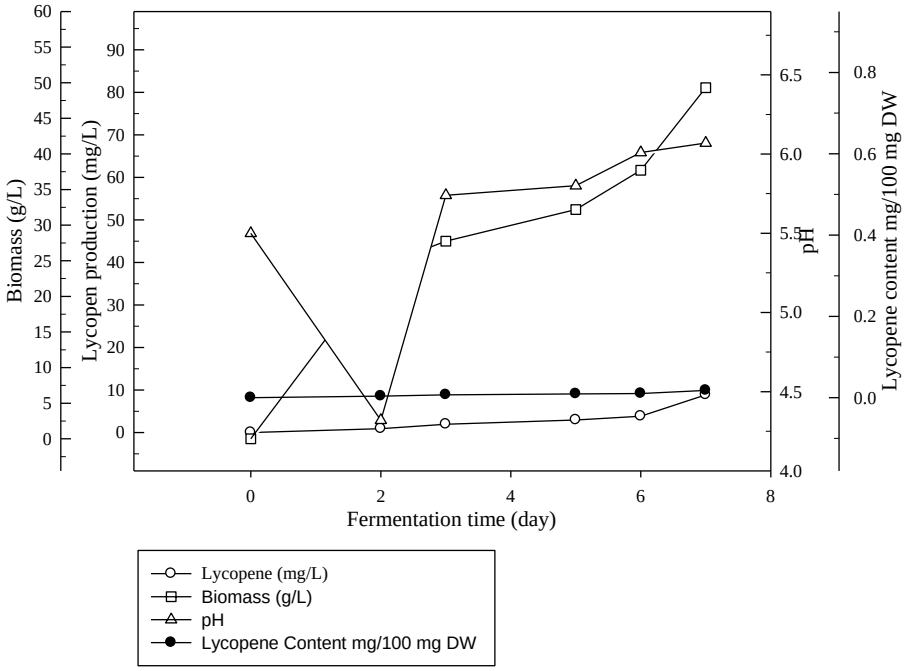

Figure 4.32 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 4 % glucose and 6 % sunflower oil

Lycopene was produced 714.2 mg/L with 1 % corn oil in glucose medium with pH 6.5 (Figure 4.33). Biomass content was 16.3 g/L and lycopene content was 4.3 mg/100 mg DW at the end of fermentation. Lycopene was produced 244.4 mg/L with 3 % corn oil in glucose medium with pH 5.5 (Figure 4.34). Biomass content was 19.6 g/L and lycopene content was 1.2 mg/100 mg DW at the end of fermentation. Lycopene was produced 48.7 mg/L with 6 % corn oil (Figure 4.35). Biomass content was 29.7 g/L and lycopene content was 0.2 mg/100 mg DW at the end of fermentation.

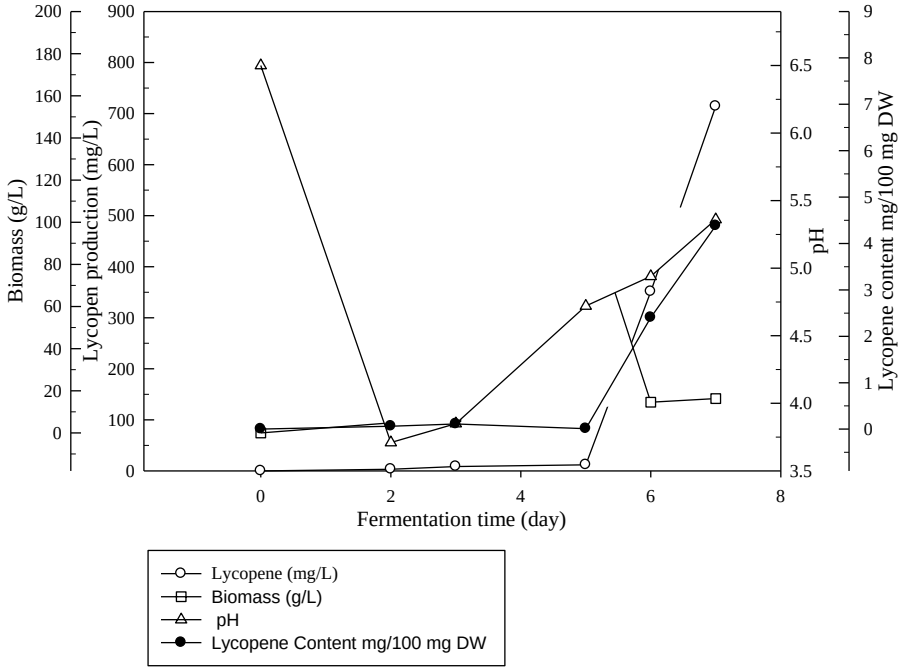

Figure 4.33 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 4 % glucose and 1 % corn oil

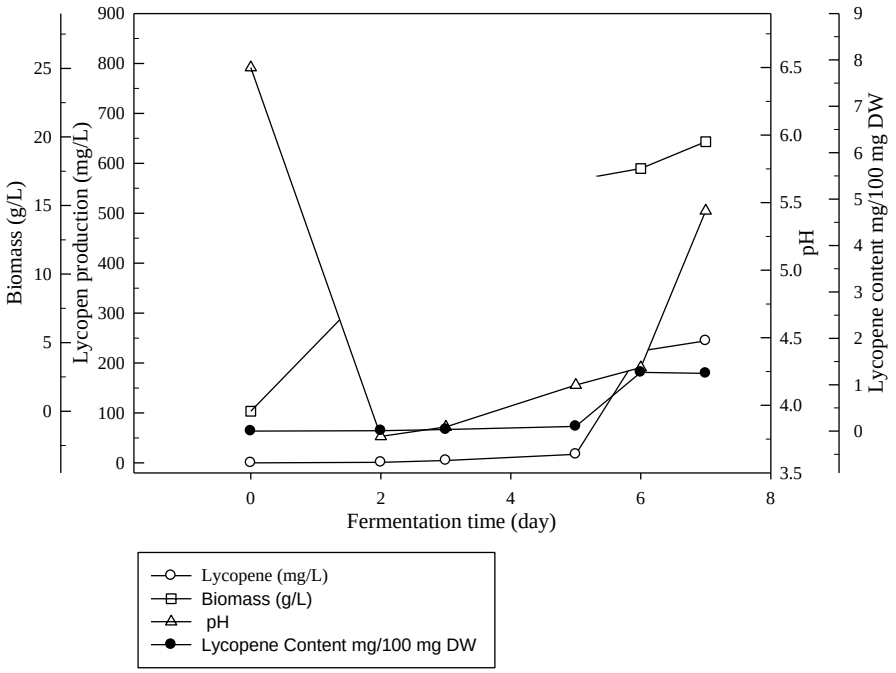

Figure 4.34 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 4 % glucose and 3 % corn oil

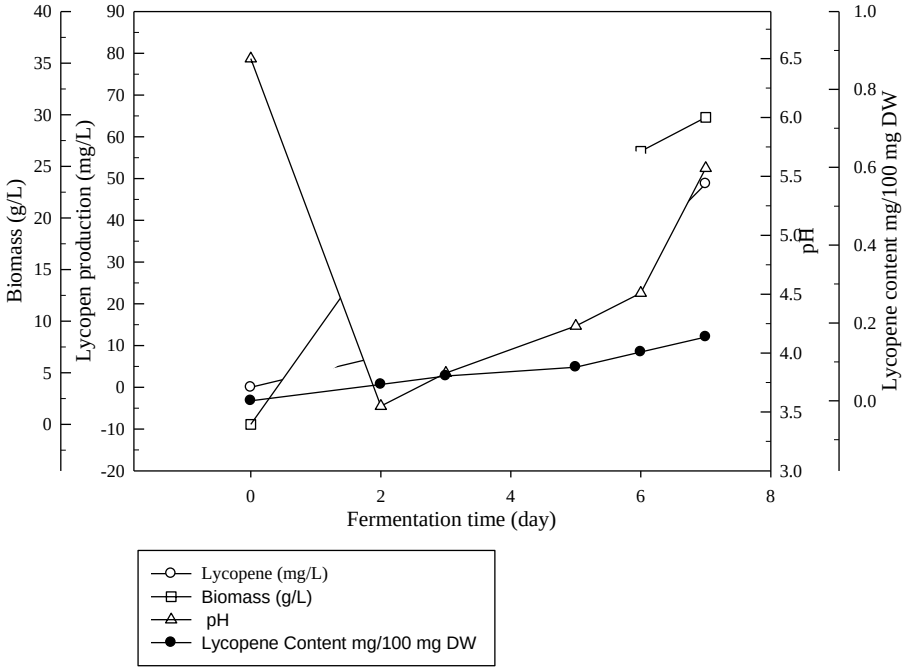

Figure 4.35 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 4 % glucose and 6 % corn oil

Lycopene was produced 0.33 mg/L with 1 % corn oil in glucose medium with pH 5.5 (Figure 4.36). Biomass content was 6.8 g/L and lycopene was 0.004 mg/100 mg DW at the end of fermentation. Lycopene was produced 0.32 mg/L with 3 % corn oil in glucose medium with pH 5.5 (Figure 4.37). Biomass content was 25.3 g/L and lycopene content was 0.001 mg/100 mg DW at the end of fermentation. Lycopene was produced 0.18 mg/L with 6 % corn oil (Figure 4.38). Biomass content was 24.2 g/L and lycopene content was 0.0007 mg/100 mg DW at the end of fermentation time.

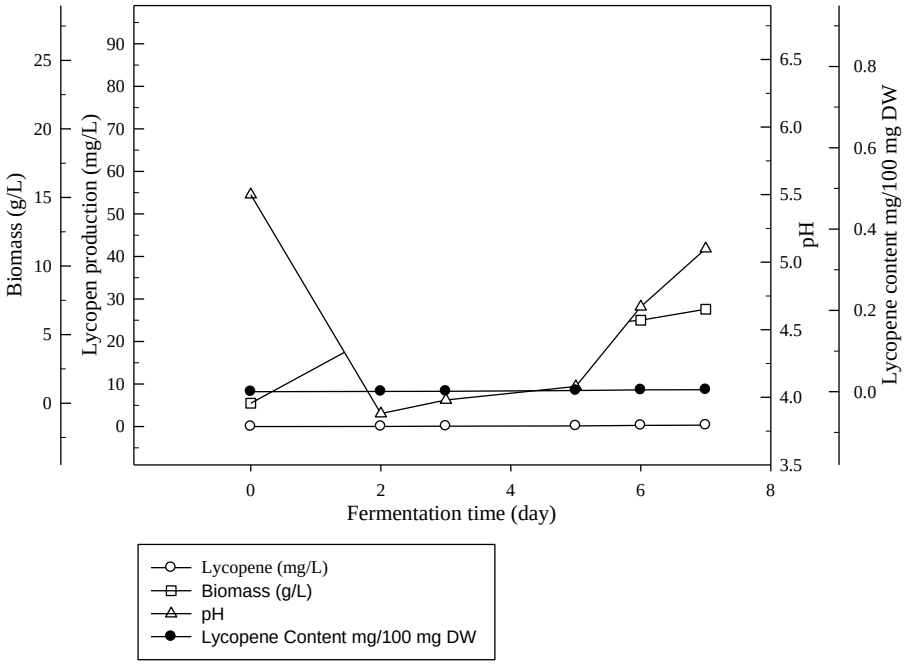

Figure 4.36 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 4 % glucose and 1 % corn oil

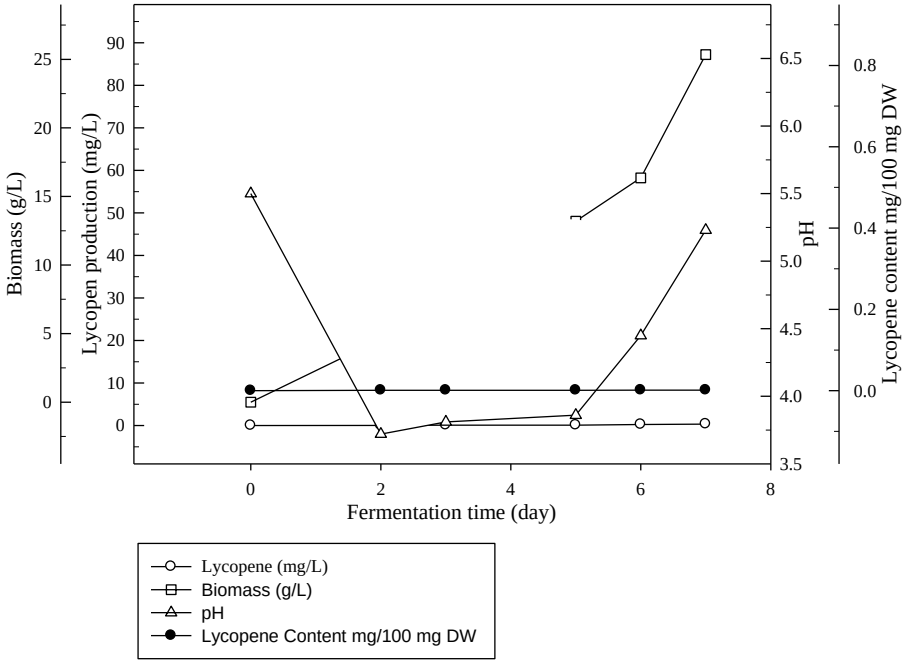

Figure 4.37 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 4 % glucose and 3 % corn oil

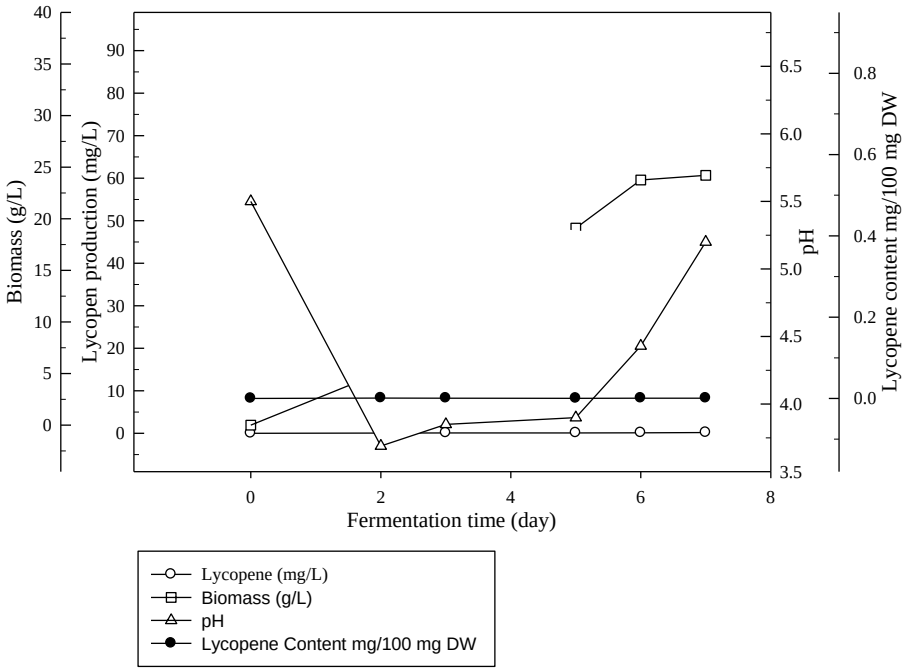

Figure 4.38 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 4 % glucose and 6 % corn oil

Lycopene was produced 53.1 mg/L with 1 % sunflower oil in sucrose medium with pH 6.5 (Figure 4.39). Biomass content was 21.9 g/L and lycopene was 0.2 mg/100 mg DW at the end of fermentation. Lycopene was produced 30.1 mg/L with 3 % sunflower oil in sucrose medium with pH 6.5 (Figure 4.40). Biomass content was 22.2 g/L and lycopene content was 0.1 mg/100 mg DW at the end of fermentation. Lycopene was produced 19.1 mg/L with 6 % sunflower oil (Figure 4.41). Biomass content was 20.7 g/L and lycopene content was 0.09 mg/100 mg DW at the end of fermentation time.

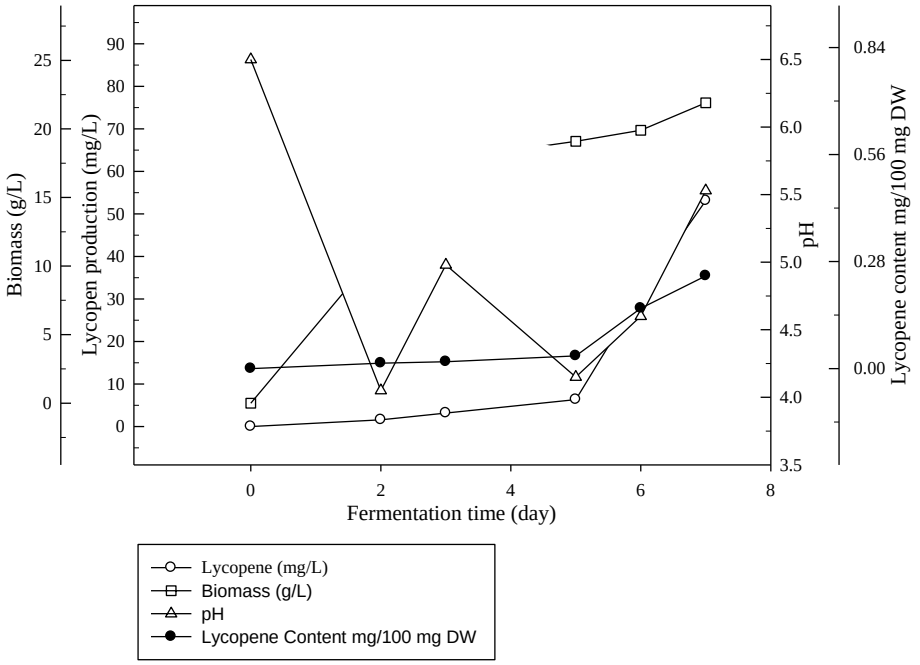

Figure 4.39 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 7 % sucrose and 1 % sunflower oil

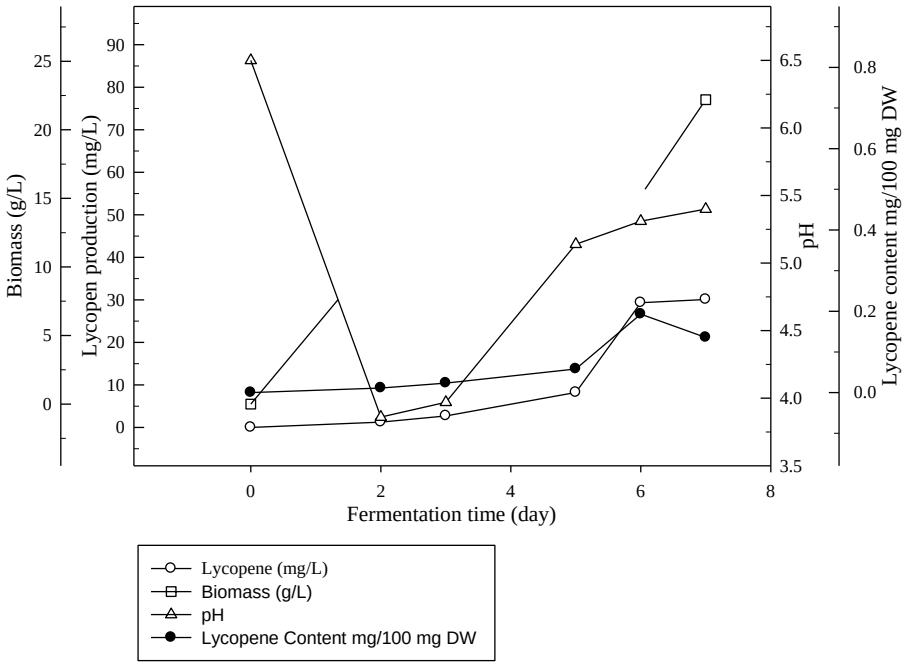

Figure 4.40 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 7 % sucrose and 3 % sunflower oil

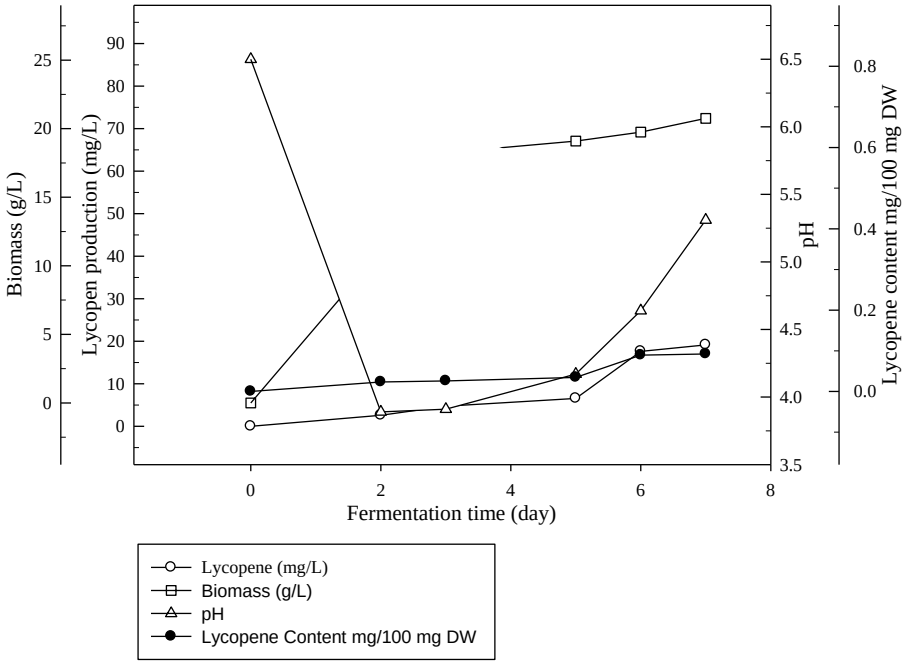

Figure 4.41 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 7 % sucrose and 6 % sunflower oil

Lycopene was produced 47.8 mg/L with 1 % corn oil in sucrose medium with pH 6.5 (Figure 4.42). Biomass content was 15.4 g/L and lycopene was 0.3 mg/100 mg DW at the end of fermentation. Lycopene was produced 29.3 mg/L with 3 % corn oil in sucrose medium with pH 6.5 (Figure 4.43). Biomass content was 28.7 g/L and lycopene content was 0.1 mg/100 mg DW at the end of fermentation. Lycopene was produced 15.9 mg/L with 6 % corn oil (Figure 4.44). Biomass content was 11.8 g/L and lycopene content was 0.1 mg/100 mg DW at the end of fermentation time.

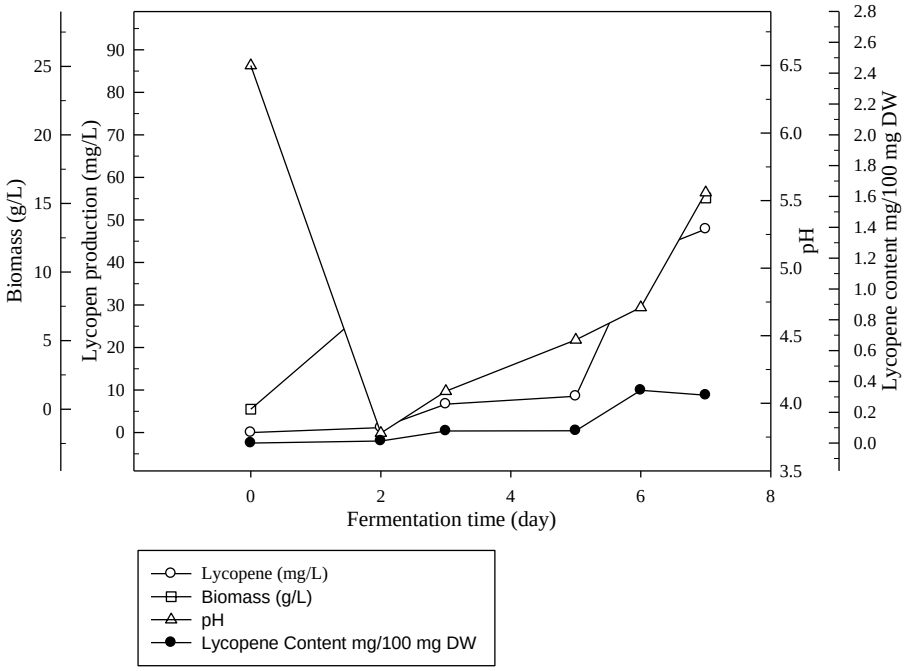

Figure 4.42 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 7 % sucrose and 1 % corn oil

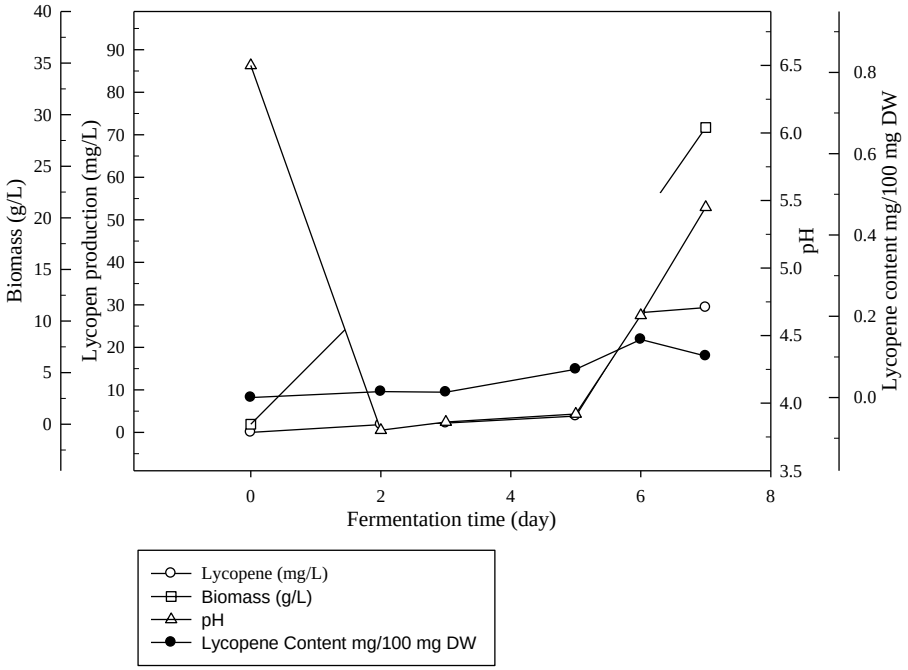

Figure 4.43 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 7 % sucrose and 3 % corn oil

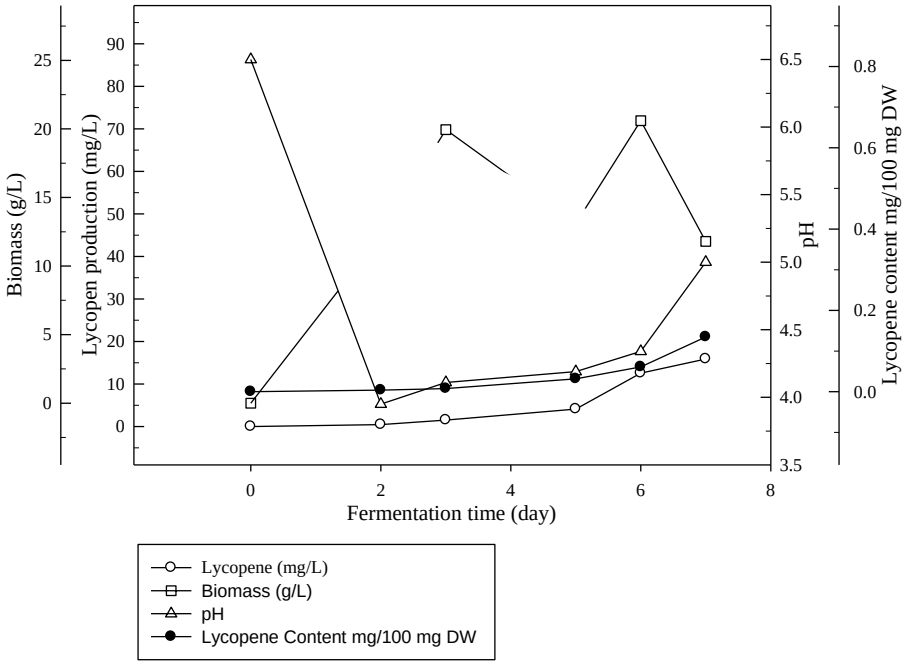

Figure 4.44 Changes biomass and pH in fermentation with initial pH 6.5 in medium containing mix of 7 % sucrose and 6 % corn oil

Lycopene was produced 6.7 mg/L with 1 % sunflower oil in sucrose medium at pH 5.5 (Figure 4.45). Biomass content was 49.9 g/L and lycopene was 0.01 mg/100 mg DW at the end of fermentation. Lycopene was produced 1.6 mg/L with 3 % sunflower oil in sucrose medium with pH 5.5 (Figure 4.46). Biomass content was 24.1 g/L and lycopene content was 0.006 mg/100 mg DW at the end of fermentation. Lycopene was produced 0.7 mg/L with 6 % sunflower oil (Figure 4.47). Biomass content was 40.7 g/L and lycopene content was 0.001 mg/100 mg DW at the end of fermentation time.

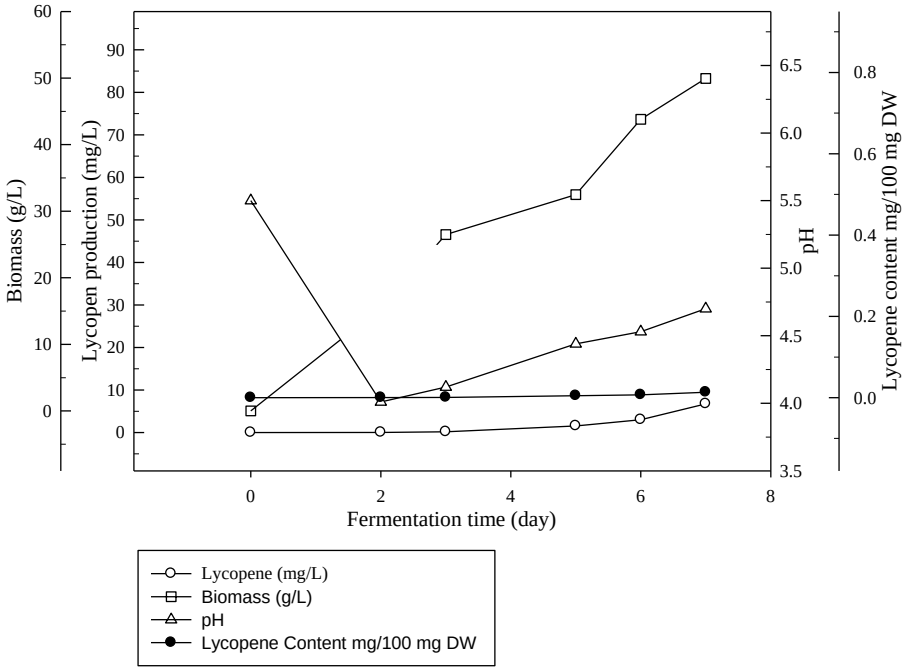

Figure 4.45 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 7 % sucrose and 1 % sunflower oil

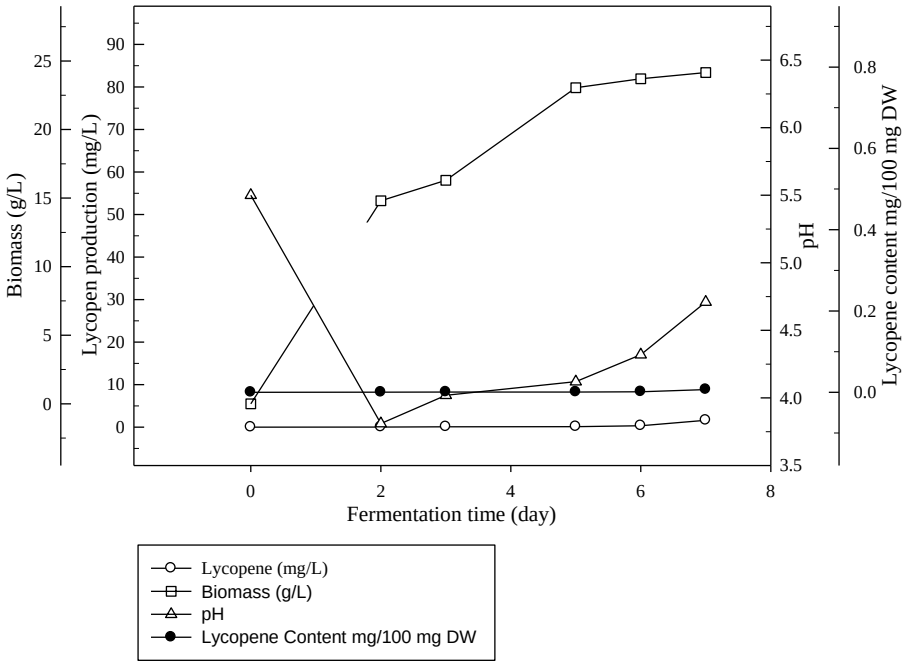

Figure 4.46 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 7 % sucrose and 3 % sunflower oil

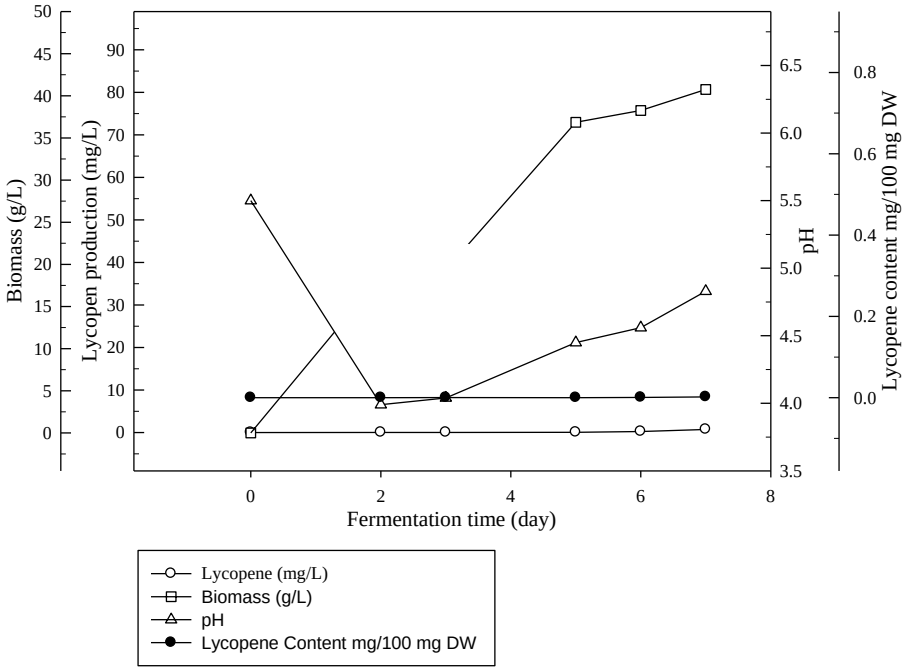

Figure 4.47 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 7 % sucrose and 6 % sunflower oil

Lycopene was produced 2.1 mg/L with 1 % corn oil in sucrose medium at pH 5.5 (Figure 4.48). Biomass content was 9.9 g/L and lycopene content was 0.02 mg/100 mg DW at the end of fermentation. Lycopene was produced 0.6 mg/L with 3 % corn oil in sucrose medium with pH 5.5 (Figure 4.49). Biomass content was 18.1 g/L and lycopene content was 0.003 mg/100 mg DW at the end of fermentation. Lycopene was produced 0.3 mg/L with 6 % sunflower oil (Figure 4.50). Biomass content was 21.8 g/L and lycopene content was 0.001 mg/100 mg DW at the end of fermentation time.

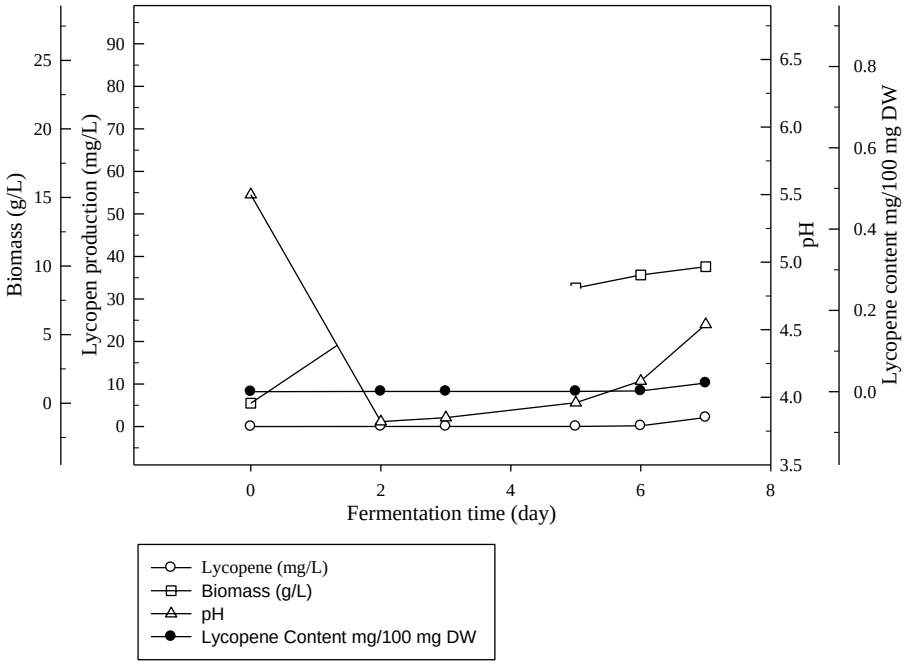

Figure 4.48 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 7 % sucrose and 1 % corn oil

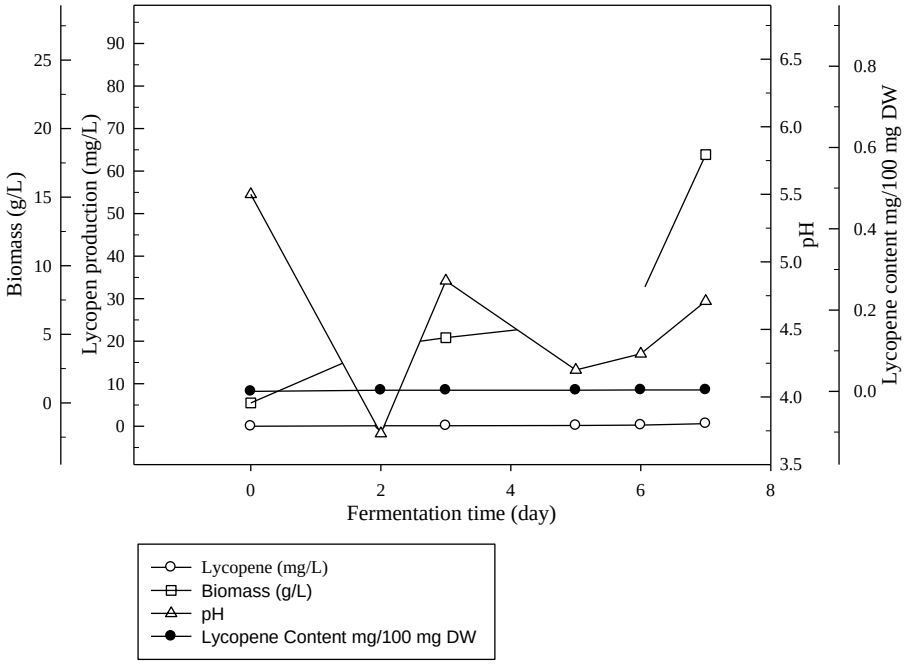

Figure 4.49 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 7 % sucrose and 3 % corn oil

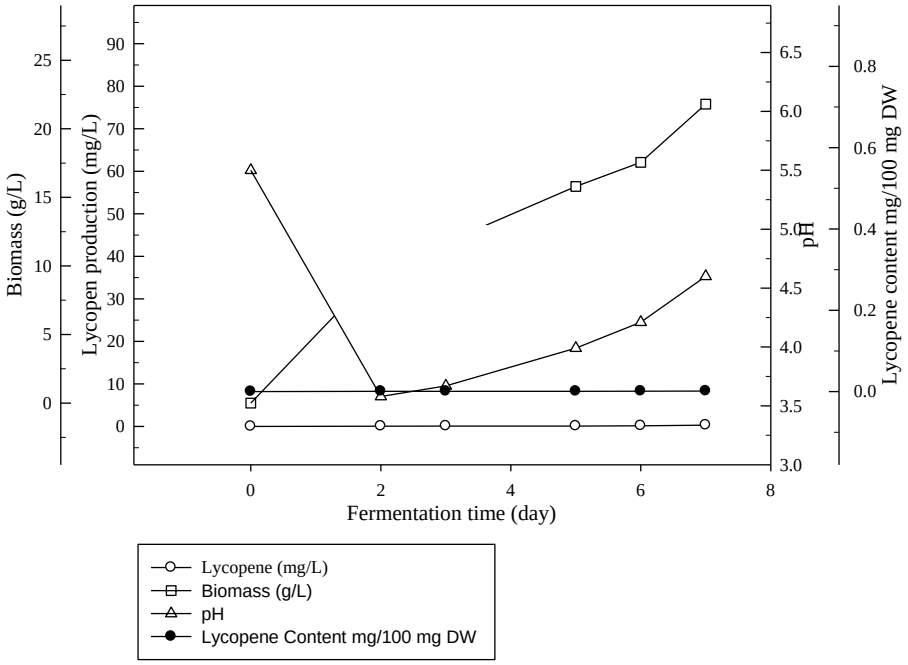

Figure 4.50 Changes biomass and pH in fermentation with initial pH 5.5 in medium containing mix of 7 % sucrose and 6 % corn oil

4.2 Discussion

The fermentations were carried out at 28°C for 7 days using different carbon sources and ingredients. The fermentation was performed in shake flasks and fermenter. In that study, approximately 10^6 spores of each strain type were inoculated from 7 day-old agar slants into 250 mL Erlenmeyer flasks containing a 50 mL sterile medium.

4.2.1 Effect of substrates on lycopene production

ATCC 14271 (+) and 14272 (-) were inoculated with the ratio 1:3. When compared different concentrations of substrates, 4 % glucose was produced with the highest lycopene 77.6 mg/L. This means that high concentrations of glucose may cause inhibiton of the activity of mevalonate kinase (MVA) (Mantzouridou et al., 2004), which is one of the key enzymes of the biosynthesis pathway. During the microbial fermentations, the carbon source can not only acts as a major constituent for building of cellular material, but also as an important energy source (Choudhari and Singhal, 2008). Papaioannou et al., (2008) studied 50.0 g/L of glucose medium with containing ethyl acetate and reported 45 % lycopene in extract. Papaioannou and

Liakopoulou-Kyriakides (2010) indicated that lactose and starch are more efficient than glucose-containing medium, since *B. trispora* possess the necessary enzyme system to utilize more complex carbohydrates. This may be related to improve in a way the fungus growth and carotenoid production, probably due to slower monosaccharide release into culture medium, limiting the product inhibition observed in high carbohydrate concentration (Papaioannou and Liakopoulou-Kyriakides, 2010). Lopez-Nieto et al., (2004) was reported 68 % of lycopene production according to carbon sources in a medium containing 19 g/L corn meal. Shi et al. (2012) was studied with 40 g/L corn starch and 45 g/L soybean in fermentation media. They reported the highest lycopene production (578 mg/L) in mated cultures of *B. trispora*. They reported 62 and 47 % lycopene production according to carbon source with adding isopentenyl alcohol and dimethyl allyl alcohol respectively, after 36 h.

In this study, the maximum lycopene (57.2 mg/L) was obtained in 7 % sucrose medium. 12.4 and 57.0 mg/L lycopene were produced from 5 and 6 % sucrose, respectively. Monosaccharides are produced in the medium by the break down of complex sugars and are well-known as the food of first choice for fungi (Choudhari and Singhal, 2008). The poor yield of the present species on sucrose may be due to the lack of intervase and not to the inability of the organisms to utilize the component sugars (Goksungur et al., 2002). Shi et al. (2012) was obtained the maximum lycopene (578 mg/L) with adding geraniol into mix of corn starch and soybean meal medium. The addition of geraniol may probably increase the carbon flux towards lycopene and later enhance lycopene accumulation. The structure of geraniol is similar to the isoprene sides of geranyl pyrophosphate (GPP) and geranylgeranyl pyropohosphate (GGPP) (Shi et al., 2012). The isoprene side chains of lycopene are GPP and GGPP in biosynthetic pathway (Shi et al., 2012).

Papaioannou and Liakopoulou-Kyriakides (2010) reported lycopene production in glucose medium containing of olive oil, soybean oil and sunflower oil, about 7, 11 and 6 mg/g DW were obtained from these oils, respectively. The use of refined vegetable oils was crucial in the stimulation of different biosynthetic pathways in *B. trispora*. In this study, about 944.7 mg/L of lycopene was produced in fermenter studies from 4 % glucose supplemented with sunflower oil 1 % (initial pH 6.5). With 1 % corn oil, about 859.8 mg/L of lycopene was produced in glucose medium (initial

pH 6.5). These results indicate that sunflower oil has a greater influence on lycopene production than corn oil. Due to sunflower oil is high in vitamin E and linoleic acid. On the other hand, sunflower oil predominantly containing linoleic and oleic acid are similar to the fungus cellu fatty acid composition (Vereschagina et al., 2010). Besides, triacylglycerols, the main component of oils can be used in the biosynthesis (involving acetyl-CoA formation resulting from β-oxidation of fatty acids) lipids of the fungal mycelium. Oils can be hydraysed to fatty acids and glycerol (resulting from oil hydrolysis by fungal exolipases) (Vereschagina et al., 2010). When the concentration of natural oils increases, the production of the pigment decreases while the biomass dry weight increases significantly (Mantzouridou et al., 2002). This means that this may be related to the consumed amount of the oils converted to biomass instead of lycopene. Varzakakou and Roukas (2010) studied with 'telemes' cheese contained 5 % (w/v) lactose with a pH of 6.3. Supplementation with 0.1 % (w/v) of Tween 80, 1% (w/v) of Span 20, and different concentration of olive oil, cotton seed oil, soybean oil, corn oil, sunflower oil and olive pomace oil without lycopene cyclase inhibitor. The proportion of β- and ɣ-carotene (as percent of total carotenoids) increased during fermentation while the proportion of lycopene decreased, this means that lycopene is an intermediate compound of the carotenoid pathway in *B. trispora* and the maximum proportion of lycopene was 7.3 %. They compared the production of lycopene with and without oils in medium, the production of lycopene without oils in medium was not as well as added oils medium. This may be one to formation of exdened amount of biomass without formation of lycopene. According to Papaioannou and Liakopoulou-Kyriakides (2010) in medium, where oils are not present, fungal growth is delayed.

Orange peel was studied in this work as a raw material for production of lycopene in shake flask. The highest lycopene (2.9 mg/L) was produced from 1 % orange peel when it was compared with 2 and 4 % orange peel. When dried citrus peels are rich in cellulose, hemicelluloses, proteins and pectin, the fat content is low (Ylitervo, 2008). Pourbafrani et al. (2007) reported one of the main obstacles for using orange peel waste for fermentation is its content of peel oil. More than 95 % is of the peel oil is D-limonene which is extremely toxic to fermenting microorganisms (Pourbafrani et al., 2007). Radu et al. (2012) studied two bakery products (short pasta) enriched

with carotenoids from tomato sauce. The addition of tomato sauce enhanced the production of lycopene. Lycopene production was 3.52 mg/100 g cell dry weight.

In this study, two different aerations (3 and 12 v/min) and agitations (500 and 750 rpm) were studied in 7 L fermenter (pH=6.5). The highest amount of lycopene was 944.8 mg/L with 3 vol/min aeration and 500 rpm agitation. Nanou et al. (2012) studied with 50 g/L glucose in a fermenter and lycopene was produced 2.15 % with respect to sugar content. Agitation of 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 (Mantzouridou et al. 2002). *B. trispora* is an aerobic microorganism and requires the oxygen in the production of lycopene (Mantzouridou et al., 2002). In this study, when shake flask and fermenter are compared with respect to lycopene production, lycopene productions were higher in fermenter than shake flask. Aeration could be beneficial to the growth and performance of microbial cells by improving the mass transfer characteristics with respect to substrate, product/by-product and oxygen (Mantzouridou et al., 2002). Using low agitation rates (around 150 rpm), lycopene production was lower due to the failure of the fermenter to maintain the required level of dissolved oxygen in the culture broth; and the viscosity of the culture was higher than is pointing to a great filamentous development of the biomass (Lopez-Nieto et al., 2004). When the agitation rate was increased to 300 rpm, hyphae were shorter and lycopene production improved, but above 300 rpm carotenoid production and biomass decreased due to shear stress (Lopez-Nieto et al., 2004). Lycopene biosynthesis was also improved by increasing the air flow rate, showing a positive effect of high dissolved oxygen and low carbon dioxide concentrations on lycopene production (Lopez-Nieto et al., 2004). The enrichment of oxygen concentration (up to 25 %) in the air stream increased the accumulation of lycopene and lycopene production decreased (Lopez-Nieto et al., 2004).

4.2.2 Effect of cell growth on lycopene formation

Figure 4.51 shows formation of biomass from glucose and supplements as given in Table 4.1. Figure 4.52 shows the formation of biomass from sucrose and supplements as given in Table 4.2. When lycopene production was increased,

biomass formation was decreased. Papaioannou and Liakopoulou-Kyriakides (2010) reported that the maximum biomass dry weight (21g/L) occurred at 120 h of fermentation, while the highest carotenoid production (45 mg/g biomass dry weight) was achieved later at 192 h. They studied with the medium contained 50 g/L glucose supplemented with 0.67 % (v/v) each of olive oil, soybean oil and sunflower oil. Biomass reduced gradually after 168 h fermentation. This indicate that lycopene and biomass is inversely proportional. Nanou et al., (2012) reported the concentration of residual sugars fell rapidly during the first 4 days of the fermentation, after which it decreased slowly and this was accompanied with the rapid increase of biomass concentration. Filotheou et al. (2012) was reported maximum amount of biomass (24.6 mg/g) in the basal medium containing 50 g/L glucose was observed after 8 days of incubation.

Table 4.1 Supplements added into glucose fermentation medium

Medium number	Glucose (g/L)	Sunflower oil (%v/v)	Corn oil (%v/v)
1	*40*		
2	60		
3	80		
4	40	10	
5	40	30	
6	40	60	
7	*40*		10
8	40		30
9	40		60

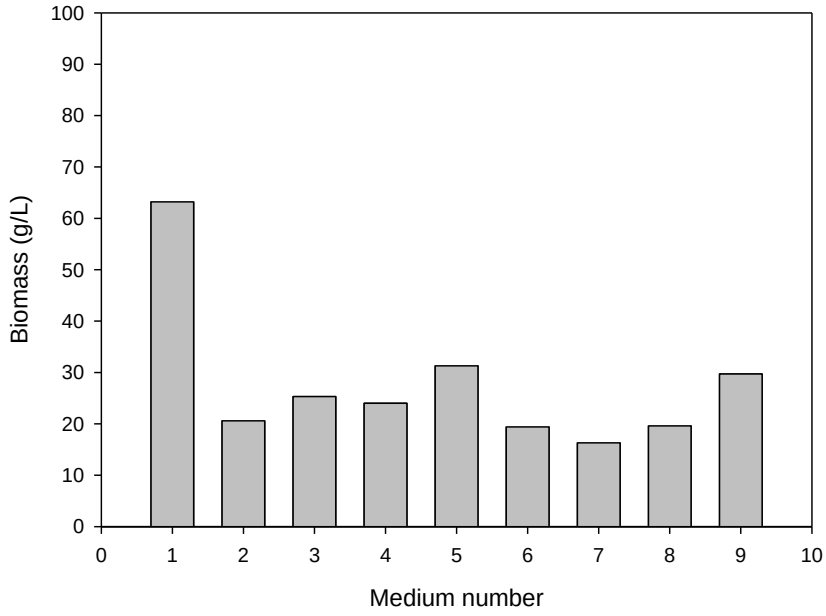

Figure 4.51 Biomass g/L after fermentation time at initial pH 6.5 according to substrate given in Table 4.1

Table 4.2 Supplements added into sucrose fermentation medium

Medium number	Sucrose (g/L)	Sunflower oil (%v/v)	Corn oil (%v/v)
1	*50*		
2	60		
3	70		
4	70	10	
5	70	30	
6	70	60	
7	*70*		10
8	70		30
9	70		60

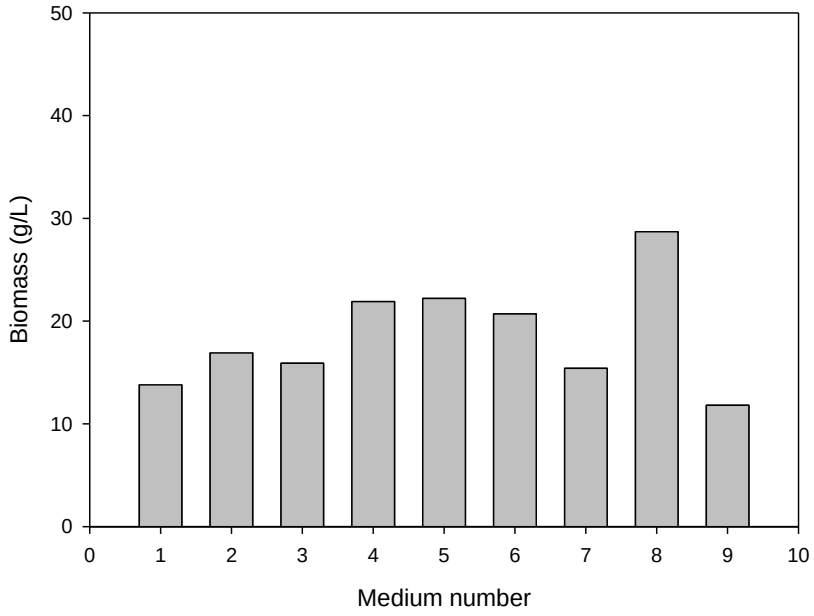

Figure 4.52 Biomass g/L after fermentation time at initial pH 6.5 according to substrate given in Table 4.2

4.2.3 pH changes during fermentation

In this study, pH of fermentation media were decreased during first 48 h then increased until 72 h (Figure 4.53). During fermentation, pH was not controlled. At 48 h, pH was approximately 3.80 ± 1, and then pH was increased to 4.33 ± 1. At the end of fermentation, pH was almost close the initial pH as given in Table 4.1. Papaioannou and Liakopoulou-Kyriakides (2010) was measured pH of fermentation medium during fermentations period in different media. The drop of pH after 24-48 h was observed in all cases due to trisporic acid or/and other unidentified acidic formations. According to Nanou et al. (2012), the pH of the 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. This was probably increased by ammonia liberated during degradation of proteins of the medium by the fungus. The increase of pH after 120 h of fermentation was observed in all media, this would be indicative of amine metabolic product release as a consequence of cell death and subsequent lysis occurring after that time (Papaioannou and Liakopoulou-Kyriakides, 2010).

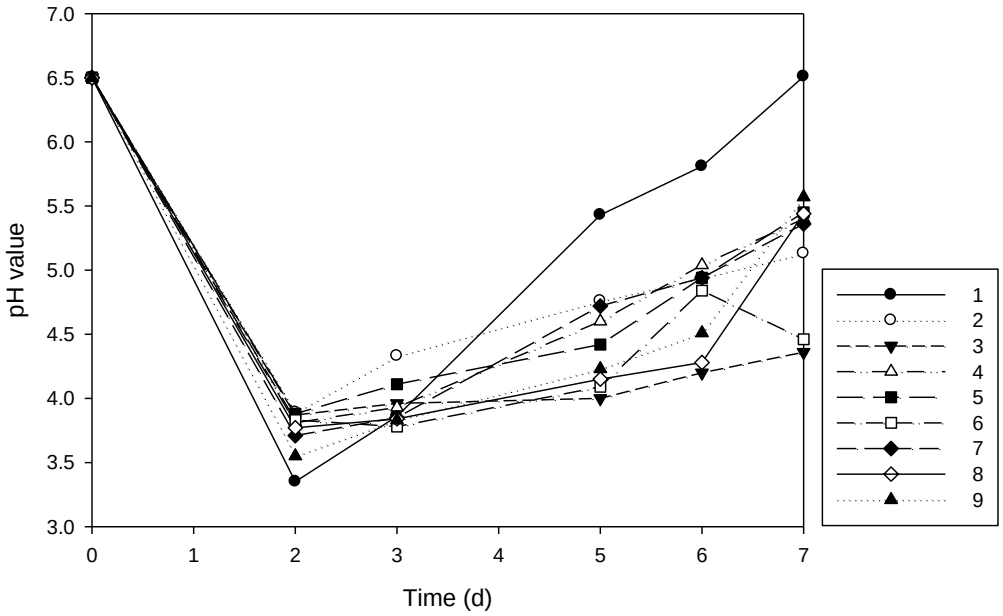

Figure 4.53 pH changes during fermentation time with different substrates initial pH 6.5 (Table 4.1)

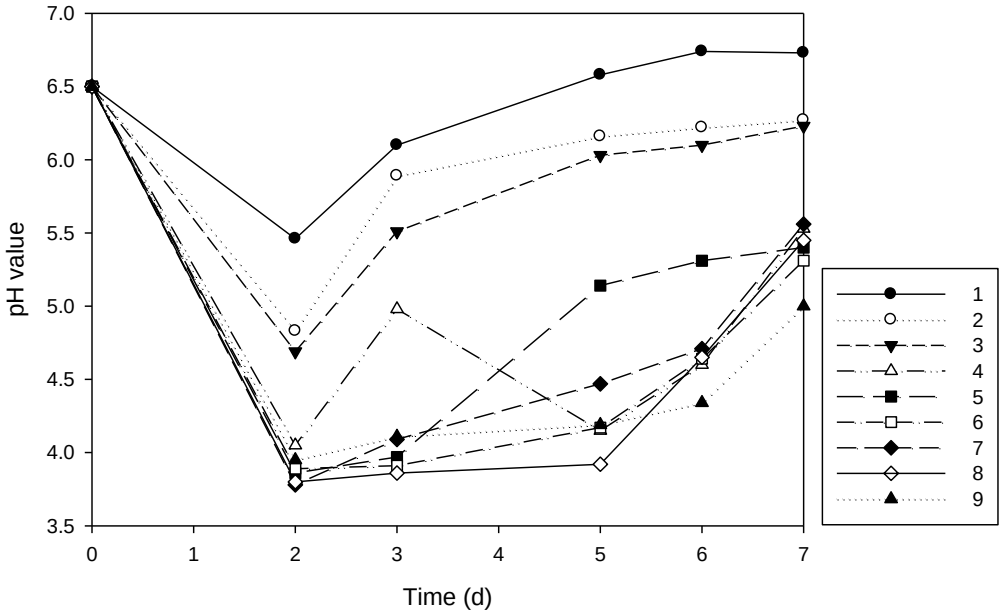

Figure 4.54 pH changes during fermentation time with different substrates initial pH 6.5 (Table 4.2)

4.2.4 Effect of inhibitors on lycopene formation

In this study, nicotine (5 m/M) was used to inhibit carotene production and stimulate lycopene formation. It was added after 2 days of fermentation into fermentation media. Lycopene was not produced without the use of nicotine during fermentation period. Several chemical agents affect lycopene formation, such as imidazole or pyridine (Lopez-Nieto et al., 2004), piperidine and creatinine (Wang et al., 2012), nicotine (Shi et al., 2012). The biosynthetic pathway can was not completely inhibited at the lycopene cyclase step, which allowed the formation of β-carotene and γ-carotene. A notable drop in β-carotene and γ-carotene leads to improvement of lycopene formation (113 %). The concentrations of inhibitor higher than 0.8 g/L affected biomass growth and drastically reduced lycopene production (Lopez-Nieto et al., 2004). Fazeli et al. (2009) reported that nicotine is a cyclization inhibitor that at higher concentrations causes the accumulation of lycopene while at lower concentrations triggers γ- carotene formation. Different concentrations of nicotine (20, 50, 100 and 200 µm) were studied (Fazeli et al., 2009). The highest amount of lycopene (0.68 mg/L) was obtained in the medium containing 20 µm of nicotine and higher the concentrations of nicotine suppressed lycopene production. Wang et al. (2012) reported 98.6 mg/L lycopene was produced with the use of piperidine as inhibitor and 98.1 mg/L lycopene was produced with the use of creatinine in medium containing 59.4 g/L glucose. Debieu et al. (2000) demonstrated that piperidine inhibited the synthesis of ergosterol, which was the main component of the cell membrane of *B. trispora*, and this possibly damaged the cell growth. Due to piperidine is toxic effect but creatinine is a safe and nontoxic food additive (Wang et al., 2012).

CONCLUSION

In this study various substrates ere compared with respect to lycopene production in shake flask and fermenter. The results showed some important aspects of lycopene production from *B. trispora.* In order to enhancing lycopene production, nicotine was used as cyclase inhibitor in all media. The amount of lycopene production is based on carbon sources that using in the fermentation. There were poor fungal growth than in media containing sucrose than potential glucose. Orange peel has in the production of lycopene carbon sources strongly effected formation of lycopene.

The use of natural oils is important for stimulation of biosynthetic pathway in *B. trispora*. The medium with natural oils lead to more lycopene than only carbon sources in medium. The linoleic acid are degraded into acetyl-CoA, which is the precursor of lycopene derived from mevalonic acid (MVA).

This study indicated that production of lycopene from glucose, sucrose and oils on large scale. The mated fermentation process for lycopene production may be useful and a reference to the other fermentation process. Also, lycopene production from microorganism is improved as an alternative plant extract which is one of the method in the lycopene production. In the future, this study is provided to contribute development of synthetic lycopene production of microorganism.

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APPENDICE

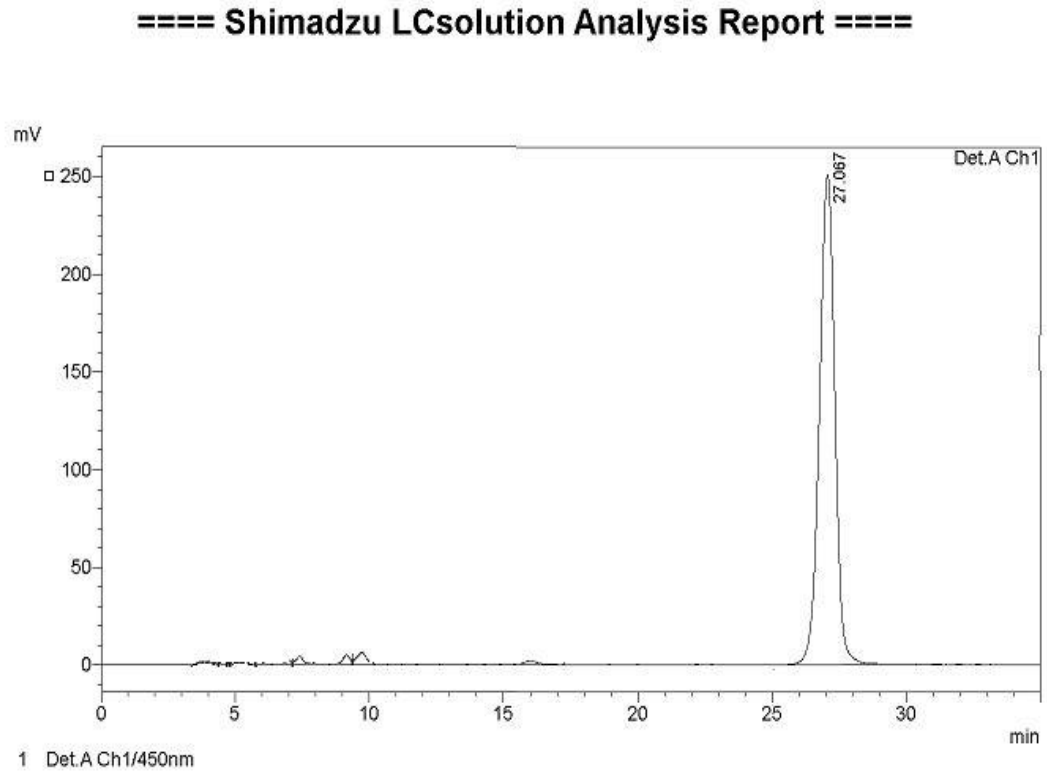

Figure 1 The lycopene formation in HPLC

Peak	Ret. Time	Area	Height	Area %	Height %
1	27.067	10028334	250968	92.608	85.591