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Squalene Production using *Saccharomyces cerevisiae*

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ABSTRACT

Squalene is a valuable natural substance found in nearly all organisms, and can be produced by the yeast *Saccharomyces cerevisiae*. The key regulatory enzyme in squalene biosynthesis is 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase (Hmg1). To overcome further down regulation of Hmg1, a truncated Hmg1 (tHmg1), an enzyme which has the catalytic domain of the Hmg1 only, and is not regulated by intermediates, was overexpressed instead of the original enzyme. Because of its strong and fast glycolysis, *S. cerevisiae* converts substantial portions of glucose into ethanol rather than into the squalene. In order to overcome this problem, the slower glucose assimilating *S. cerevisiae* (SK2) was employed for the overexpression of the tHmg1 enzyme. Accumulated squalene in the SK2 cells were extracted by using the alcohol/KOH method. The SK2 strain with the overexpression of *tHMG1* produced about 30 times more squalene than the strain without the *tHMG1*. Additionally, the Methanol/KOH combination for squalene extraction showed better squalene recovery than ethanol/KOH combination.

INTRODUCTION

Squalene is an oily natural occurring 30-carbon organic compound, found in nearly all organisms (Garaiova *et al*., 2013; Lu *et al*., 2004; Johnson *et al*., 1970). It is a valuable natural substance known for its key role as a biosynthetic precursor, which is converted into more complex products such as cholesterol (Bhattacharjee *et al*., 2001). Squalene is an important ingredient in skin cosmetics because it is an excellent skin protector and safe application for human health. (Ghimire *et al*., 2009; Perma Healthcare, 2012), and can be used as an antioxidant, skin moisturizer, and skin treatment for disorders such as acne, psoriasis, or atopic dermatitis (Wolosik *et al*., 2013). Squalene is a well-known anti-cancer dietary supplement (Tjan *et al*., 2011). Women who consume large quantities of olives and olive oil containing squalene have a lower risk of breast cancer (Kelly, 1999; Perma Healthcare, 2009). Squalene is also found to have chemo-preventive action against colon cancer, and is currently being used as a prophylactic agent after irradiation treatment of cancer patients (Perma healthcare, 2009).

Squalene was originally found in shark liver oil. Shark liver oil is considered to be the richest source of squalene because 80 percent of the shark's liver is squalene (Kelly, 1999; Deprez, 1990; Perma Healthcare, 2009; Kamimura *et al*., 1994). Though shark liver oil contains a high quantity of squalene, it is too dangerous to extract from sharks. Because of the presence of environmental pollutants such as polychlorinated biphenyls, heavy metals, methylmercury residues, and unpleasant fishy odor and taste, squalene purification from shark liver oil is difficult. There is also an international concern for marine animal's protection, limiting squalene production (Ghimire *et al*., 2009; Mantzouridou *et al*., 2009). There are alternative ways to get squalene. Squalene can be obtained from plant sources such as olive oil or amaranth seed oil (Bhattacharjee *et al*., 2001). However, this way cannot produce sufficient quantities at the desired purity level for food and

pharmaceutical applications. The use of microbial production of squalene may be a suitable alternative to address these issues (Ghimire *et al.*, 2009). Yeast is an example of a microbial cell, and can be used for squalene production. In this experiment the yeast *Saccharomyces cerevisiae* was used to produce squalene. The key enzyme in yeast squalene biosynthesis is 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase (Hmg1).

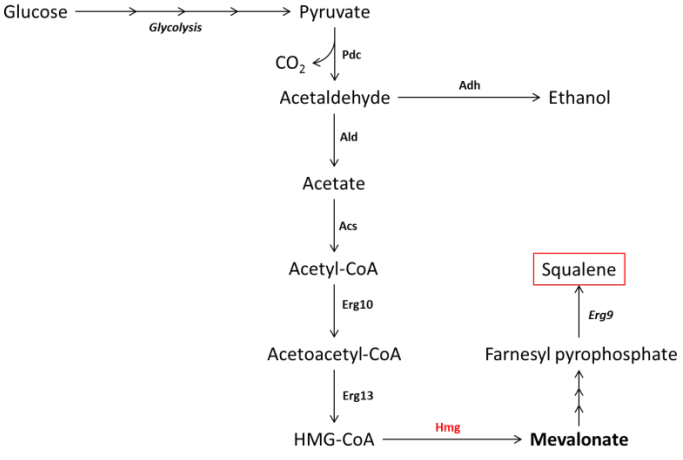
The research goal is to check the effect of the overexpression of the truncated Hmg1 enzyme (tHmg1) in the slower glucose assimilating *S. cerevisiae* strain (SK2). Squalene was extracted from the yeast cell using the alcohol and KOH extraction method. It is hypothesized that the overexpression of tHmg1 gene (*tHMG1*) will improve the squalene content in the SK2 strain, and using a longer carbon chain alcohol during the extraction method will result in more squalene recovery.

LITERATURE REVIEW

Yeast cells can be used for squalene production. A significant amount of squalene can be found in yeast cells grown in specific conditions (Garaiova *et al*., 2014). One type of yeast used to produce squalene is Baker's yeast (*S. cerevisiae*). Growing *S. cerevisiae* is easy, economical, and a rapid process because *S. cerevisiae* cells proliferate themselves approximately every 90 minutes. There are several types of yeast that can be used to produce squalene, but one important strong point about using *S. cerevisiae* is that it can be easily manipulated genetically (Lawrence W., 2001). HMG-CoA reductase is the most important enzyme for biological squalene synthesis. *S. cerevisiae* has two HMG-CoA reductase isoenzymes, Hmg1 and Hmg2. Hmg1 is the stronger and more stable enzyme, while Hmg2 is unstable making Hmg1 the key regulatory enzyme in the squalene production process (Polakowski, *et al.*, 1998). Hmg1 produces mevalonate, which is then easily converted in to squalene. When the amount of intermediates between mevalonate and squalene in the cell reaches a certain level, the intermediates will decrease the intracellular synthesis rate of Hmg1 and the amount of squalene in the cell will be maintained (DimsterDenk, *et al.*, 1994). Because of this down regulation of Hmg1, overexpressing Hmg1 is not effective on increasing the amount of squalene. To eliminate further down regulation of Hmg1, previous researchers used the truncated Hmg1 enzyme instead. Since the truncated Hmg1 has the catalytic domain of the original enzyme only, and is not regulated by intermediates, its overexpression can increase squalene content in *S. cerevisiae* (Polakowski, *et al.*, 1998).

However, *S. cerevisiae* has a hurdle as a host to produce squalene using glucose. *S. cerevisiae* produces ethanol mainly in glucose condition because of its rigid ethanol production pathway. This characteristic has negative effect on squalene yield and productivity because a significant amount of glucose is being converted to ethanol, not into the squalene synthetic pathway (Figure 1) regardless of genetic manipulation for squalene production. Since glycolysis makes reduction-oxidation (redox) status unbalanced, *S. cerevisiae* use ethanol pathway to maintain redox balance. If glucose can be converted more into acetic acid and acetyl-CoA instead of ethanol, the squalene productivity possibly increase because acetyl-CoA is a key metabolic intermediate to produce squalene via mevalonate pathway (Verduyn *et al.*, 1990).. In this research, the engineered yeast strain which utilizes glucose slower than wild type was selected to reduce the risk of the carbon waste which is affecting the amount of squalene produced.

Figure 1
Squalene Pathway in *S. cerevisiae*.



Alcohol and KOH extraction methods can be used to extract squalene from yeast. The KOH is responsible for destroying the cell, so the squalene can be released. The alcohols used in these methods are ethanol and methanol, and they are responsible for absorbing the

squalene from the cell debris. Once the cell is destroyed and the squalene is absorbed to the alcohol, hexane is then added to separate the squalene from the alcohol/KOH solution. A previous researcher used the ethanol and KOH method for extracting squalene. 10 percent KOH and 75 percent ethanol solution was added to yeast cells, and then squalene was extracted after adding hexane (Chen *et al.*, 2010). Another researcher used the alcohol and KOH extraction method, but used methanol instead. 60 percent KOH and methanol solution was added to yeast cells, hexane was then added and squalene was able to be extracted (Mantzouridou, *et al*., 2010). Both of these extraction methods using alcohols were successful at extracting squalene from yeast cells. In the current study, the solution was 40%KOH and 100% alcohol. Both methanol/KOH method and ethanol/KOH method was used to observe which method resulted in more squalene. Previous researches have used each of these methods, but have never used both in the same experiment to compare the amount of squalene extracted when methanol was used to the amount when ethanol was used.

METHODOLOGY

Yeast Culture Condition

Yeast nitrogen base (Table 1, MP Biomedicals, Santa Ana, CA), and amino acids (Table 2, MP Biomedicals, Santa Ana, CA) were used to make Synthetic complete media (SC media). Glucose (Merck, Billerica, MA) was used for carbon source. All yeast cultures were performed in shaking 250 mL baffled flasks with 50 mL medium. YPD medium containing 10 g yeast extract, 20 g peptone, and 20 g glucose per liter was used for SK2 culture. For maintaining plasmids p2LG and p2LGKtH, AT1 and AT2 culture was carried out using synthetic complete (SC) medium containing yeast nitrogen base mix (Table 1) and amino acids (Table 2), with 40 g/L of glucose as a carbon source. For preparation media plates, 20 g bacto agar (BD, Franklin Lakes, NJ) was added into 1 L of media before sterilization. All media and flasks were sterilized at 121˚C for 20 min. Glucose was sterilized separately for evading browning effect. AT1 and AT2 were inoculated with initial absorbance value 0.5 at 600 nm (A_{600nm}) for squalene production experiment. Dry cell weight of yeast culture was calculated using conversion factor 0.3 g dry cell weight/A_{600nm}·L.

Table 1
Yeast nitrogen base composition.

Biotin	2 µg	p-Aminobenzoic acid	0.2 mg
Calcium pantothenate	0.4 mg	Pyridoxine hydrochloride	0.4 mg
Folic acid	2 µg	Riboflavin	0.2 mg
Inositol	2 mg	Thiamine hydrochloride	0.4 mg
Calcium chloride	0.1 mg	Boric acid	0.5 mg
Niacin	0.4 mg	Copper sulfate	40 µg
Magnesium sulfate	0.5 g	Sodium chloride	0.1 g
Potassium iodide	0.1 mg	Ferric chloride	0.2 mg
Manganese sulfate	0.4 mg	Manganese sulfate	0.4 mg
Zinc sulfate	0.4 mg	Potassium phosphate monobasic	1 g

per 1 L of medium

Table 2
Amino acids used for SC medium.

Adenine	10 mg	Histidine HCl	50 mg	Methio-nine	20 mg
Arginine HCl	50 mg	Tyrosine	50 mg	Lysine HCl	50 mg
Aspartic acid	80 mg	Isoleucine	50 mg	Pheny-lalanine	50 mg
Uracil	20 mg	Valine	140 mg	Threo-nine	100 mg
Tryptophan	50 mg	Leucine	0 mg		

per 1 L of medium

Strains and Plasmids Used

Slower glucose utilizing *S. cerevisiae* SK2, and p2LG, p2LGKtH plasmids were provided by Suryung Kwak (Table 3 and 4). p2LG and p2LGKtH plasmids were introduced into SK2 using EZ-YEAST™ transformation kit (MP Biomedicals, Santa Ana, CA). SK2 was cultured and harvested when the absorbance value at 600nm reached 2. 125 µL transformation solution, and 5 µL carrier DNA of transformation kit were added into the harvested cells with 100 ng of plasmids, and were mixed well. Transformation mixtures were subjected to heat-shock at 45˚C for 1 hr, and spread on to SC medium agar plates. AT1 and AT2 colonies were picked from the plates after three days.

Table 3
Plasmids used in this study.

Characteristics	Characteristics	Reference
pRS425	2-micron origin of replication, *LEU2*	(Mumberg, *et al.*, 1995)
p2LG	cloning P_{GPD}-T_{CYC1} cassette into pRS425	(Mumberg, *et al.*, 1995)
p2LGKtH	cloning P_{GPD}-*tHMG1*-T_{CYC1} cassette into pRS425	This study

Table 4
S. cerevisiae strains used in this study.

Strains	Characteristics	Reference
SK2	*MATalpha, leu2, his3, ura3, pfk26, pfk27*	This study
AT1	p2LGKtH was introduced into SK2	This study
AT2	p2LG was introduced into SK2	This study

Squalene Extraction

5 mg of AT1 an AT2 cells were taken from the culture. The cells were centrifuged at 4,000rpm for 10 minutes at 4˚C. The harvested cells were stored in -80˚C to freeze cells until the beginning of the extraction. The solution for squalene extraction, 20% KOH/50% alcohol was prepared by blending 40% KOH and 100% ethanol with 1:1 ratio, just prior to the extraction. 0.75mL of the KOH/ethanol solution and KOH/methanol solution was added into the harvested cell pellets with 0.1g of glass beads. The AT1 and AT2 samples with the KOH/ethanol solution and KOH/methanol solution was mixed well on the vortexer for 1 min. To mix more, the samples were placed in a shaking incubator at 37˚C for 12 hours. 0.75 mL of absolute hexane (Sigma-Aldrich, St. Louis, MO) was added to the samples after incubation. The samples were mixed vigorously for 1 min and then centrifuged at 4000 rpm for 5 min at 4˚C. 0.5 mL of supernatant (hexane phase) was taken from the samples and evaporated using the vacuum dryer for 30 min.

Analysis of Squalene and Other Chemicals

50 µL of 100% absolute ethanol was added to the extracted and dried squalene samples, mixed well, and centrifuged. 450 µL of 100% acentonitrile was added to the samples and mixed well. The extracted squalene was analyzed by HPLC with an ultra violet detector (Beckman Coulter, Brea, CA) and C18 column (Shisheido, Tokyo, Japan). HPLC grade acetonitrile (Merck, Billerica, MA) was used as mobile phase at a flow rate of 1 ml/min at room temperature. Squalene was detected at 190nm wavelength. The glucose, glycerol, acetate, and ethanol concentrations of the culture samples were analyzed by high performance liquid chromatography (HPLC) with a refractive index detector (1200 Series, Agilent Technologies, Santa Clara, CA) and a Rezex ROA Organic Acid H+ column (Phenomenex Inc., Torrance, CA). 0.005N of H_2SO_4 was used as mobile phase at a flow rate of 0.6 ml/min at 50'C.

FINDINGS

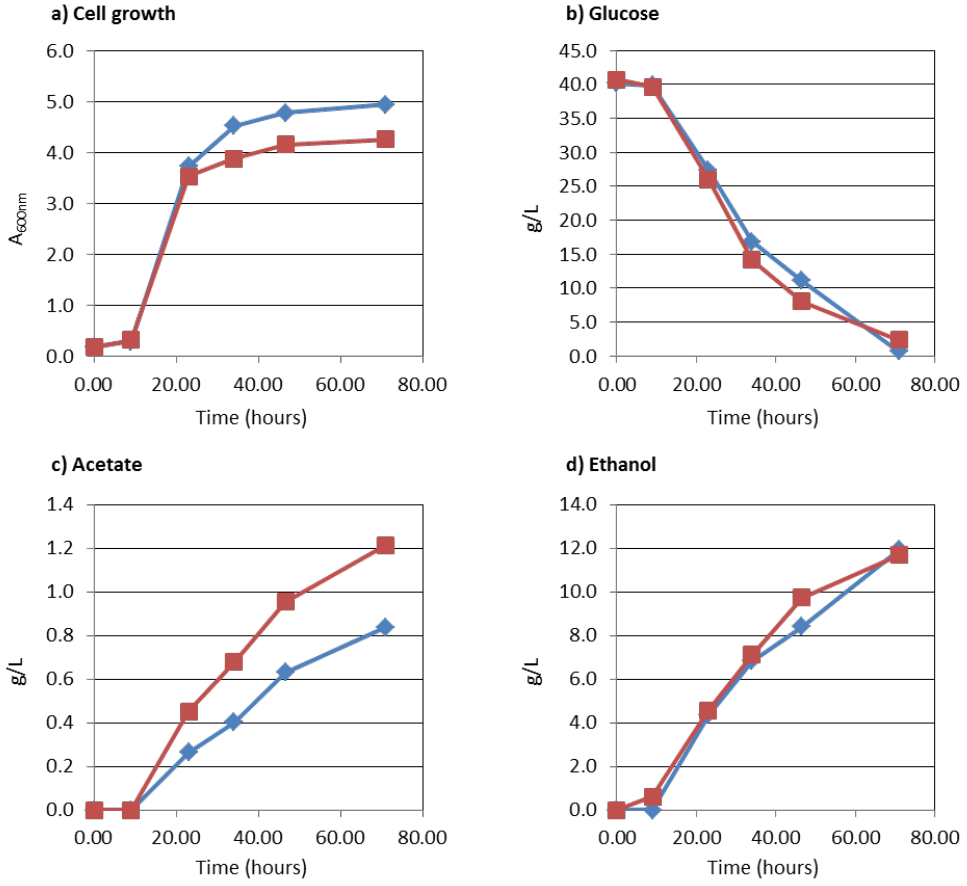
Yeast Culture

AT1 and AT2 strains were grown in SC media with glucose. The strains were both constructed with the SK2 strain, and tHmg1 enzyme was overexpressed in AT1, but not in AT2. The cell growth, glucose, acetate, and ethanol concentration in the media was measured (Figure 2). Though the AT1 and AT2 cells consumed the same amount of glucose, the better cell growth and lower acetate level in AT1 is the result of the overexpression of the tHmg1 enzyme. Hmg1 enzyme is the key metabolic pathway to produce sterols from glucose, via squalene. Since tHmg1 overexpression could increase the sterol synthetic pathway, and sterols are important materials for cells to proliferate themselves, we could assume that the overexpressed tHmg1 was working in AT1 and changed its growth rate. The acetate level in the culture media of AT2 was higher than AT1. AT1 cells could utilize more acetate, and the acetate could be converted in to squalene because the strengthened Hmg1 reaction was able to pull acetate. The ethanol level was the same for both AT1 and AT2.

Effect of tHmg1 Overexpression on the Squalene Content in Slower Glucose Assimilating Strain

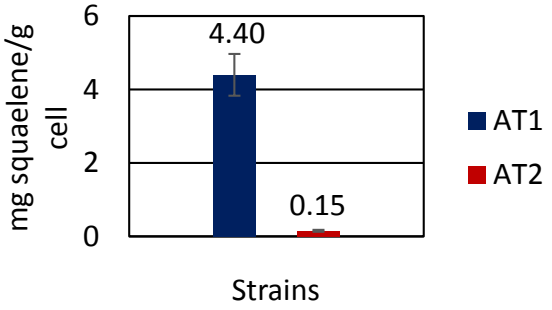
The ethanol/KOH method was used for the comparison of squalene in AT1 and AT2 cells. It was hypothesized the overexpression of tHmg1 will

Figure 2
Culture Profile of AT1 (blue diamond) and AT2 (red square).



improve the squalene content in the SK2 strain. The amount of squalene in AT1 cells was 30 times more than the amount present in AT2 cells (Figure 3). The overexpression of tHmg1 in a regular *S.cerevisiae* improves squalene production (Polakowski, *et al.*, 1998). In the current research it was proved that the overexpression of *tHMG1* in a slower glucose assimilating strain, also improves the amount of squalene in the cell.

Figure 3
Comparison of Squalene Content in AT1 and AT2.

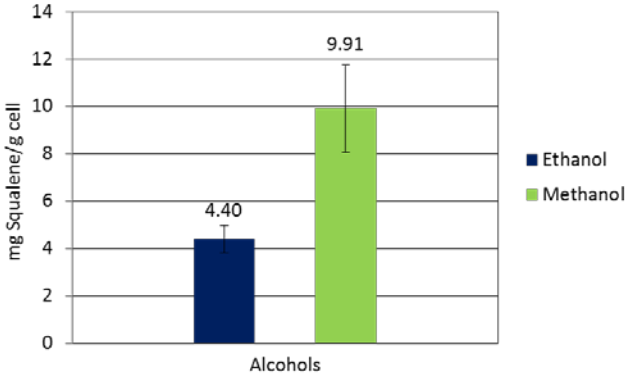


Squalene Recovery Variance between Ethanol and Methanol Methods

Because squalene is hydrophobic, and the carbon chains of alcohols are hydrophobic as well, it was hypothesized the longer the carbon chain, the more squalene it could absorb and recover. Different alcohols such as butanol, propanol, ethanol, and methanol were going to be used to test the hypothesis. However, we found that longer carbon chain alcohols like butanol and propanol did not mix well with 40% KOH solution, so they could not be used in the alcohol/KOH extraction method, and only ethanol and methanol was used to compare the amount of squalene extracted when methanol was used to the amount when ethanol was used. Contrary to the original hypothesis, methanol/KOH resulted in more squalene recovery than the ethanol/KOH extraction (Figure 4). The alternative hypothesis why methanol is better is, squalene can be dissolved into methanol/KOH

solution, but methanol and squalene interaction is lower than ethanol and squalene interaction, so when hexane is added to absorb the squalene from the alcohol/KOH solution, it is harder to absorb from ethanol than it is from methanol. This results in less squalene recovery when ethanol is used in the alcohol/KOH extraction method.

Figure 4
Comparison of Ethanol and Methanol Extraction Methods.



CONCLUSIONS

In conclusion, this study showed the overexpression of tHmg1 in a slower glucose assimilating *S. cerevisiae* does improve the squalene content in the cell. It also showed using a shorter carbon chain alcohol in the alcohol/KOH extraction method to extract squalene, results in more squalene recovery. In the future, a comparison of the overexpression of tHmg1 in a regular *S. cerevisiae* to the overexpression of tHmg1 in a slower sugar metabolizing yeast will be made to see the difference of the squalene production.

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