Rhodosporidium Sp. DR37: A Novel Strain For Production of Squalene in Optimized Cultivation Conditions

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Research

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Abstract

Background: *Rhodosporidium* strain, a well-known oleaginous yeast, has been widely used as a platform for lipid and carotenoid production. However, the production of squalene for application in lipid-based biofuels is not reported in this strain. Here, we isolated and identified newly strain of *Rhodosporidium* sp. DR37 and investigated its potential for production of squalene under various cultivation conditions.

Results: In the present study, *Rhodosporidium* sp. DR37 was isolated from mangrove ecosystem and its potential for squalene production was assessed. When *Rhodosporidium* sp. DR37 was cultivated on non-optimized medium (20 g/L glucose, 5 g/L peptone, 5 g/L YE, 15 g/L agar, seawater (50% v/v), pH 7, 30 °C), 64 mg/L of squalene was produced. Significantly, use of optimized medium (20 g/L sucrose, 5 g/L peptone, seawater (20 % v/v), pH 7, 25 °C) allowed highest squalene accumulation in *Rhodosporidium* sp. DR37 (619 mg/L). The maximum squalene content was obtained as 21.6% of total lipid in comparison to the non-optimized medium (13.9% of total lipid).

Conclusions: This study is the first report to employ marine oleaginous *Rhodosporidium* sp. DR37 for accumulation of squalene in optimized medium. Our findings provide the potential of *Rhodosporidium* sp. DR37 for production of squalene as well as lipid and carotenoids for biofuel applications in large scale.

Background

Squalene (2, 6, 10, 15, 19, 23-hexamethyltetracosa-2, 6, 10, 14, 18, 22-hexaene,), a six double bonded triterpenic hydrocarbon ($C_{30}H_{50}$), is widely produced in microorganisms and the other higher organisms such as plants and animals via mevalonate (MVA) or 2-C-methyl-D-erythritol 4-phosphate (MEP) biosynthetic pathways [1]. It is main precursor of sterols biosynthesis in plants and animals [2]. It has been shown that squalene has antioxidant and anticancer activities with broad applications in food and cosmetics industries [3, 4]. Therefore, demanding for squalene has increased during the last decade because of its wide and diverse applications [5]. Recently, squalene has also successfully been converted to the biofuel and used as a diesel fuel by Mazda Motor Corporation (Fuchu, Japan) in 2011 [6]. Lipid based biofuels have attracted more attention than petroleum based fuels, after growing energy demand and global warming concerns [7]. Microbial production of squalene has been investigated as a promising alternative source for traditional extraction methods from shark liver or plant oils [8]. Microbial strains are capable to produce pollutant-free, low cost, high quality and sustainable squalene source as a major interest to lipid based biofuels industries [8]. Squalene producing microorganisms have been isolated and identified as yeast, fungi, bacteria, microalga and protists [9]. Recently, a protist (*Aurantiochytrium*) and a yeast (*Pseudozyma*) strain have been reported for their potential to produce high amount of squalene [10, 11]. *Aurantiochytrium* is known for its potential for production of high amount of polyunsaturated docosahexaenoic acid (DHA) in large scale [12]. *Pseudozyma* is known as a biosurfactant, biodiesel and enzyme producing yeast [13-15]. In the recent years, a highly squalene producing yeast-like strain of *Pseudozyma* was isolated and identified, which is a potential candidate for commercial production of

squalene [10, 16]. In our study, we focused on oleaginous *Rhodosporidium* species which is basidiomycete, heterotrophic and fast growing yeast. *Rhodosporidium* accumulate high amount of intracellular lipid and carotenoids [17]. Tile now, intensive researches have been done on production of lipid and carotenoids in this strain [18-21]. Wide range carbon and nitrogen substrates utilization, fast growing and high content of lipid (30-70% of dry cell weight) and carotenoids such as β-carotene, make *Rhodosporidium* useful for large scale production of valuable metabolites [22]. Tile now, this biotechnological important yeast strain is not being considered as a possible candidate for squalene production. Since, in carotenoid producing yeasts, MVA pathway for production of carotenoids and squalene is the same; this possibility may exist to find some *Rhodosporidium* species with potential for production of squalene as much as carotenoids or lipids [17]. According to our knowledge, there is no report on squalene producing *Rhodosporidium* species. In this study, we isolated and identified squalene producing *Rhodosporidium* sp. DR37 from mangrove environmental samples. To the best of our knowledge, this study is the first report using *Rhodosporidium* species for production of squalene. Furthermore, the effects of various factors (carbon and nitrogen sources, seawater concentration, pH and temperature) were investigated on cell growth, lipid and squalene production.

Methods

Samples collection

Seawater samples, fallen leaves of mangrove trees (*Avicennia marina* and *Rhizophora mucronata*) and sediments were collected from coastal waters and mangrove forest of Persian Gulf and Oman Sea in south of Iran. Samples were kept in sterile bags at 4 °C and sent to the laboratory before use.

Yeast strains isolation

Samples were plated on medium containing 10 g/L glucose, 1 g/L peptone, 0.1 g/L yeast extract (YE), natural seawater (50% v/v), 15 g/L agar and supplemented with 300 mg/L streptomycin and penicillin G. The plates were incubated at 30 °C for 3–6 days with regular observation for yeast growth. The obtained single yeast colonies were picked-up and sub-cultured on fresh YEPD medium (20 g/L glucose, 20 g/L peptone, 10 g/L YE, 15 g/L agar and seawater (50% v/v)) and incubated at 30 °C for 48 h to obtain pure cultures [23].

Light microscopy and morphological characteristics

Yeast strains were grown in YEPD broth medium and incubated at 30°C. Then, morphological characteristics of the isolated yeast strains were determined under light microscope (Zeiss, Germany) during incubation period.

Molecular and phylogenetic analysis

An overnight culture of yeast strain was prepared and used for genomic extraction. Universal primers ITS1 (5´-TCCGTAGGTGAACCTGCG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) were used for

amplification of the internal transcribed spacer (ITS). The polymerase chain reaction (PCR) was performed by gradient thermal cycler (Eppendorf, Westbury, NY, USA). The program was set as denaturation for 4 min at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 55 °C and 90 s at 72 °C. Final extension was 7 min at 72 °C [24]. PCR amplicons were purified and sequenced by Bioneer. Resulting sequences were edited by BioEdit program (BioEdit 7.2) and were searched using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov). ClustalX program was used for sequence alignment and then generation of phylogenetic tree was done using neighbor-joining method and MEGA4 software [25]. The tree reliability was evaluated by bootstrap analysis of 1000 replicates.

Determination of cell growth and biomass

The growth of yeast cells were determined by measuring optical density (OD) of harvested cells at phosphate buffered saline (PBS) and 600 nm. Cell biomass, expressed as cell dry weight (CDW) was harvested by centrifugation (4000 rpm for 5 min) and pellets were dried at 105 °C for 18 h to constant weight and the weight was determined gravimetrically.

Lipid extractionand TLC analysis

A certain amount of freeze dried cells was suspended in 2 mL distilled water in screw cap test tubes. The cell suspension was ultrasonicated and then 2.5 mL chloroform and 5 mL methanol were added. Cell suspension was sonicated again and homogenized with T10 basic IKA homogenizer. 2.5 mL chloroform and 2.5 mL distilled water were added and vortexed for 30 s. Resulting suspension was centrifuged at 4000 rpm for 15 min to separate two phases. The organic bottom layer was transferred to a pre-weighed tube and the solvent was evaporated. The amount of lipid was determined gravimetrically [26]. Thin layer chromatography (TLC) silica gel plates coated with fluorescent indicator F_{254} were used for analysis of extracted lipids. Hydrocarbons such as squalene were separated with developing solvent of hexane/chloroform (9:1). Afterward, the TLC plates were exposed to H_2SO_4 (20%) and then visualized by heating at 70 °C for 60 min [27].

Fatty acid methyl esters analysis

Total lipid was converted into fatty acid methyl esters (FAMEs) by methanolic sulfuric acid (4% v/v) at 80 °C for 90 min in sealed vials. Then 1 mL of H_2O was added and FAMEs were extracted by several hexane extraction (3×2 mL). FAMEs were dried over anhydrous Na_2SO_4 and solvent was removed by evaporation. The samples were stored at 4 °C prior analysis. Gas chromatography (GC) analysis was performed using Agilent 6890 equipped with a flame-ionization detector (FID) and DB-23 (30 m×0.32 mm, 0.25 μm; Agilent Technologies) capillary column. 0.5 μL of FAMEs sample was injected under splitless injection mode. Nitrogen was used as carrier gas and temperature of injector and detector was set at 300 °C. Column temperature program were 50 °C; 2 min, 10 °C/min to 180 °C; 5 min, 5 °C/min to 240 °C; 7 min. C19:0-FAME (Sigma, USA) was used as an internal standard [28].

Quantitative determination of squalene by HPLC analysis

High performance liquid chromatography (HPLC) was used for identification and quantification of squalene. Lipids were saponified using 0.5 M potassium hydroxide containing ethanol (0.5 M KOH/EtOH) at 90 °C for 1 h. Then non-saponifiable lipids were extracted with hexane. Afterward, solvent was evaporated and squalene redissolved with 1 mL of acetonitrile/tetrahydrofuran (THF) (9:1, v/v) [29]. Squalene was identified and quantified by HPLC (Agilent, 1100 Series) equipped with a Zorbax, sb-C18 (4.6×250 mm, 5 micron) column. Acetonitrile/THF (80:20, v/v) was considered as mobile phase at a flow rate of 1 mL/min and ran under isocratic conditions. The sample injection volume was 10 µL and the column temperature was set at 30 °C and identification and quantification were done at 210 nm. Squalene (St. Louis, Mo., U.S.A.) was used as external standard for squalene quantification. A standard calibration curve was established by plotting peak area against concentration, by using different concentrations of squalene [30].

Fourier-transform infrared spectroscopy (FT-IR) analysis

IR spectrum of purified squalene was determined between 4000–400 cm^{-1} using a Bruker ALPHA FT-IR spectrometer. Three spectral replicates were determined for purified squalene sample.

Strain selection and cultivation

Strain DR37 was isolated based on its characteristic for squalene production. This strain was cultivated in modified YEPD medium (non-optimized medium) (20 g/L glucose, 5 g/L peptone, 5 g/L YE, 15 g/L agar and seawater (50% v/v)) and incubated at 30 °C and 150 rpm.

Optimization of culture conditions

For investigation the effect of various chemical and physical factors on cell growth, lipid and squalene production by *Rhodosporidium* sp. DR37, five carbon sources (20 g/L) (glucose, sucrose, glycerol, starch and olive oil) and five nitrogen sources (5 g/L) (YE, malt extract (ME), peptone, ammonium chloride and sodium nitrate) were tested. Seawater concentration (50% v/v) was constant in all above experiments. During carbon source experiments, YE and peptone (5 g/L each) were used as nitrogen source. Also, 20 g/L sucrose was used as the carbon source during nitrogen source experiments. Afterward, the effect of different concentrations of seawater (0, 20, 50, 70 and 100% v/v) were assessed along with 20 g/L sucrose and 5 g/L peptone as selected carbon and nitrogen sources, respectively. Also, different concentrations of selected carbon source (sucrose, g/L) (20, 40, 60, 80 and 100) were investigated on cell growth, lipid and squalene production. The effects of various growth parameters, including temperature (25, 30 and 37 °C) and pH (5.0, 7.0 and 9.0) were investigated in medium containing 20 g/L sucrose, 5 g/L peptone and seawater (20% v/v). The experiments were incubated for 3 days at 150 rpm.

Statistical analysis

Analysis of variance (ANOVA) was used for data analysis with GraphPad Prism 8.0.1 ($P<0.05$). The results are presented as the mean ± standard deviation of three replicates.

Results And Discussion

Isolation and identification of *Rhodosporidium* sp. DR37

Squalene is known for wide range applications as antioxidant, dietary food supplement and vaccine adjuvant [31, 32]. Also, it has been accepted for production of oil-based biofuels [6]. In the past, squalene was mainly extracted and provided from deep sea shark liver oil. Also plant sources such as olive oil have been used as minor sources of squalene [5]. At present, significant amount of squalene can be produced in large scale by microbial strains [8]. *Rhodosporidium* sp. is a very well-known yeast strain for its ability to produce lipids for biofuels [20]. In this study, we isolate and identified a novel strain of *Rhodosporidium* sp. which is able to produce and accumulate large amount of intracellular lipid granules rich in squalene. DR37 strain was isolated from water samples obtained from mangrove ecosystem of Qeshm, Iran. Morphology of DR37 colonies was observed as smooth, red, convex and round on the YEPD agar medium (Fig. 1a). Light microscopy showed single cells of DR37 containing red pigment and possess size ranging from 2 to 8 µm (Fig. 1b-arrow). Two phases of yeast like and dikaryotic filamentous were observed after 20 days of incubation at 30 °C (Fig. 1c-arrow). As shown, arrow indicated an elongated basidium formed from germination of teliospore. Haploid basidiospores will be emerged, germinate and form yeast phase of *Rhodosporidium* species [33]. The teleomorphic reproduction involves a transition between yeast phase and dikaryotic filamentous phase. These two phases are common features of *Rhodosporidium* species in sexual reproduction cycle [34].

Phylogenetic analysis of strain DR37

Phylogenetic tree of strain DR37 was generated by Mega X based on ITS1, 5.8S rRNA and ITS2 regions. Phylogenetic analysis confirmed *Rhodotorula* and *Rhodosporidium* species were the closest relatives of strain DR37 and indicated strain DR37 belongs to the genus of *Rhodotorula* (Fig. 2). These results were in agreement with data obtained after major revision on subphylum *Pucciniomycotina* published by Wang et al. [35]. It is shown that *Rhodosporidium* species is categorized under the revised *Rhodotorula* genus [36]. Our results showed nucleotide sequences of ITS1, 5.8S rRNA and ITS2 from DR37 had 98% similarity to the species of *Rhodosporidium* JN383899. However, the main difference between *Rhodosporidium* and *Rhodotorula* species is the presence or absence of sexual reproduction, respectively. As a result, formation of basidium was observed by strain DR37 which is a part of sexual reproduction of *Rhodosporidium* species. Subsequently, based on morphological and phylogenetic characteristics, strain DR37 was named *Rhodosporidium* sp. DR37 with GenBank accession number MG022778.

Determination of fatty acid profile of *Rhodosporidium* sp. DR37

Rhodosporidium sp. DR37 was cultivated in modified YEPD broth medium (non-optimized medium) for 72 h. Lipid was extracted and subsequently esterified and analyzed by GC. *Rhodosporidium* sp. DR37 produced 5.2 g/L CDW and 0.46 g/L lipid. GC results showed that profile of fatty acids produced by *Rhodosporidium* sp. DR37 was similar to the other strain of *Rhodosporidium* TJUWZ4 [19]. The major

fatty acids (% of TFA) were identified as C14:0 (myristic acid, 1.84%), C16:0 (palmitic acid, 26.22%), C16:1 (palmitoleic acid, 1.87%), C18:0 (stearic acid, 6.49%), C18:1 (oleic acid, 47.16%) and C18:2 (linoleic acid, 12.91%) (Table 1). Total content of saturated and unsaturated fatty acids was 34.55% and 61.94% of TFA, respectively. Oleic acid, palmitic acid and linoleic acid were predominant fatty acids with 86.29% of TFA. Higher content of these fatty acids among other fatty acids makes *Rhodosporidium* oil as a good feed-stock for production of biodiesel with desirable properties [37]. The obtained profile of fatty acids from *Rhodosporidium* showed a similar composition to fatty acids obtained from plant oils and can be considered as a microbial alternative for production of biodiesel [38, 39]. Similar to our strain, oleic acid was produced with high content among other fatty acids in total lipid (53.34%) by *Rhodosporidium toruloides* 21167 in medium containing of cassava starch hydrolysate at shake flask. This is notable that long-chain fatty acids containing of 16 and 18 carbon atoms are very desirable for biodiesel production [40].

Table 1 fatty acid profile of *Rhodosporidium* sp. DR37

Fatty acids	(% of TFA)
C14:0	1.84
C16:0	26.22
C16:1	1.87
C18:0	6.49
C18:1	47.16
C18:2	12.91
Others	3.51
Total	100

TLC analysis of total lipids

Fig. 3a shows TLC of total lipid extracted from biomass of *Rhodosporidium* sp. DR37. Total lipids were extracted from *Rhodosporidium* and subjected to TLC and then, squalene and triacylglycerol (TAG) were separated by developing solvent. After visualization step, squalene spot was identified based on standard squalene. The RF value of visualized squalene spot from *Rhodosporidium* sp. DR37 was the same as standard squalene. TLC screening of squalene production among microbial strains is a simple and fast method in comparison to chromatography methods such as GC and HPLC [29]. As shown in Fig. 3a, TAG and steryl sters (SE) are recognized together squalene which is in accordance with results of Milla et al [41]. In another study, Patel et al analyzed total lipid extract from *Aurantiochytrium* sp. T66 (ATCC PRA-276) by TLC. The two spots of SE and TAG were detected and recognize together squalene spot [42].

Quantitative determination of squalene by HPLC

Further purification of squalene was performed by saponification of total lipid and then squalene extraction was carried out by *n*-hexane from unsaponifiable fraction. Detection and quantitative evaluation of purified squalene was performed by HPLC. Chromatographic peak of squalene was identified based on the retention time of standard squalene. As shown in Fig. 3b, the elution time for extracted squalene was 12.37 min. Then, squalene concentration was determined based on peak area and using a calibration curve. Squalene was produced at concentration of 64 mg/L and content of 13.9 % of CDW after 72 h of *Rhodosporidium* sp. DR37 cultivation in modified YEPD medium. *Rhodosporidium* strains are well-known for their potential for production of carotenoids and lipids in their CDW. However, squalene content has been reported very low in *Rhodosporidium* strains in comparison to genetically modified yeasts or naturally squalene producing microorganisms such as *Schizochytrium*, *Aurantiochytrium* and *Pseudozyma*. A genetically modified *Saccharomyces cerevisiae* (strain YUG37-*ERG1*) was reported to accumulate 18.0 mg/L squalene [43] which is much lower than naturally produced squalene (64 mg/L) by *Rhodosporidium* sp. DR37. Kaya et al reported an *Aurantiochytrium* strain which accumulates high amount of squalene with concentration of 1.29 g/L after 4 days of cultivation [27]. Also, another extended screening program was performed for isolation of squalene producing thraustochytrid strains in Okinava mangrove forest. 14 novel squalene producing thraustochytrid strains were isolated and identified. The amount of produced squalene was determined between 7.54 to 13.9 g/g CDW [44].

FT-IR analysis of squalene

The IR spectrum of squalene is shown in Fig. 3c. Three intense bands corresponding to C-H stretching were observed at 2968, 2920 and 2853 cm^{-1}. Furthermore, four intense skeletal vibration modes were observed at 1444, 1379, 1103 and 837 cm^{-1}. More importantly, one low intensity band corresponding to C=C stretching was observed at 1668 cm^{-1}. These IR results are completely in agreement with Hall et al [45].

The effect of carbon sources on cell growth, lipid and squalene production

Optimum condition for squalene production was investigated by one-factor at the time method. The cell growth, lipid and squalene production were assessed on various carbon and nitrogen sources and seawater concentrations under various temperatures and pH. Glucose, sucrose, glycerol, olive oil and starch were used to investigate their effect on cell growth, lipid and squalene production in shake flask. As shown in Fig. 4. *Rhodosporidium* sp. DR37 was able to grow on all carbon sources. The maximum CDW was found in medium with glucose as sole carbon source (5.2 g/L). *Rhodosporidium* sp. DR37 produced lipid in almost all carbon sources except in medium containing starch. In fact, starch was not an efficient carbon source for lipid and squalene production. This result was in agreement with previous data obtained for *Rhodosporidium* TJUWZ4 by Wang et al [19]. Their results showed that *Rhodosporidium* TJUWZ4 produced very low lipid content (0.039 g/L) in medium containing starch as

sole carbon source. Their results on optimization of culture conditions for biomass and lipid production by *Rhodosporidium* TJUWZ4 showed that biomass was produced on starch as sole carbon source but accumulation of lipid was not noticeable. Maximum lipid concentration of 1.2 g/L was achieved in medium containing olive oil as carbon source. However, sucrose was shown to be the best carbon source for squalene production (204 mg/L). The squalene concentration was slightly higher in *Rhodosporidium* grown on sucrose in comparison to olive oil (194 mg/L). Therefore, sucrose was selected as the best carbon source for further optimization experiments. Sucrose is one of the main compositions of sugarcane molasses as a low-cost carbon source for production of high-added value bioproducts by microorganisms [46]. Furthermore, *Rhodosporidium* species is capable to accumulate high amount of lipid from 5 carbon carbohydrates such as xylose, which is presented in plant biomass hydrolysates [47].

The effect of nitrogen sources on cell growth, lipid and squalene production

Five different nitrogen sources including organic (YE, peptone and ME) and inorganic (ammonium chloride, and sodium nitrate) were used to investigate their effect on biomass, lipid and squalene production. As shown in Fig. 5, results indicated YE, peptone and ME supported noticeable cell growth in comparison to inorganic nitrogen sources. On the other hand, inorganic nitrogen sources were not able to support lipid and squalene production. Lipid concentration was 0.55 g/L, 1.11 g/L and 0.69 g/L, when YE, peptone and ME were used as sole nitrogen source in culture medium, respectively. The highest squalene concentration (386 mg/L) was obtained in culture medium containing peptone as sole nitrogen source. Squalene concentration was 199 mg/L and 110 mg/L, when YE and ME were used as sole nitrogen source in culture medium, respectively. Maximum amounts of lipid and squalene were obtained in culture medium with peptone as sole nitrogen source. These results showed that organic nitrogen sources such as peptone are effective in production of squalene by marine strain *Rhodosporidium* sp. DR37. Nitrogen sources are necessary for cell growth and maintenance of *Rhodosporidium* during exponential growth phase in the initial fermentation stage with balanced C/N ratio [22]. Although, nitrogen limitation is recommended for high lipid production in *Rhodosporidium*, lipid accumulation could be achieved by a proper carbon to phosphorus (C/P) ratio, regardless of high amount of nitrogen source in fermentation medium [48]. Peptones (containing amino acides, polypeptides and inorganic salts) are derived by enzymatic digestion or acid hydrolysis of animal and plant tissues [49]. Peptones are usually used for enrichment of culture medium to promote cell growth and multiplication. As mentioned above, maximum levels of CDW, lipid and squalene were produced by *Rhodosporidium* sp. DR37, when peptone was used as sole nitrogen source in its culture medium.

The effect of salinity on cell growth, lipid and squalene production

The effect of different concentrations of seawater (0, 20, 50, 70 and 100% v/v) was assessed on cell growth, lipid and squalene production by *Rhodosporidium* sp. DR37, in culture medium containing 20 g/L sucrose and 5 g/L peptone as selected carbon and nitrogen sources, respectively. As shown in Fig. 6, *Rhodosporidium* sp. DR37 was capable to grow and produce biomass between 1.6-3.9 g/L in medium containing 0-100% seawater concentration. Addition of 20% v/v seawater to the culture medium, resulted

in production of lipid at its maximum level (1.86 g/L) in comparison to other media. Lowest amount of lipid production was observed in medium with 100% seawater strength. In fact increasing of seawater concentration showed a negative effect on lipid production by *Rhodosporidium* sp. DR37. The maximum level of squalene (520 mg/L) was obtained in medium with 20% v/v seawater and then the amount of squalene was decreased to its minimum level in medium with 70% v/v seawater. Neither lipid nor squalene, produced in 100% seawater concentration. These results are in accordance with Tchakouteu et al [50]. Their results showed that *Rhodosporidium toruloides* DSM 4444 was able to tolerate and grow well in medium containing 6% w/v NaCl. The presence of high level of salt in culture medium was reported as a good factor for prevention of microbial contaminations in fermentation broth. Interestingly, their results showed that lipid accumulation was stimulated in medium with 4% w/v NaCl. In our study, maximum levels of lipid and squalene were produced in medium containing 20% v/v seawater.

The effect of different concentrations of sucrose on cell growth, lipid and squalene production

As mentioned before, glucose was a good substrate for production of high amount of biomass, not for squalene accumulation. Therefore, sucrose was selected as proper carbon source for production of higher amount of squalene. So, the effect of various concentrations of sucrose as selected carbon source was investigated on cell growth, lipid and squalene production. As shown in Fig. 7, increase in concentration of sucrose from 20 to 100 g/L significantly dropped production of biomass, lipid and squalene. A sharp decrease in the lipid and squalene production was observed in medium with 60 g/L sucrose. These results showed that growth and lipid production of *Rhodosporidium* sp. DR37 were decreasing while the concentration of sucrose was increasing more than 40 g/L in the culture medium. Jiru et al. showed that optimum glucose concentration for maximum biomass and lipid production by *Rhodosporidium kratochvilovae* SY89 was 50 g/L [51]. Patel et al. showed that the content of polyunsaturated fatty acids such as linoleic acid was significantly higher in *Rhodosporidium kratochvilovae* HIMPA1 while cultivated on medium containing sucrose than medium containing glucose [52]. It could be concluded that sucrose was a favorable carbon source for production of fatty acids and compounds which have more than one double bond in their structure.

The effect of temperature on cell growth, lipid and squalene production

After determination of proper carbon and nitrogen sources and seawater concentration, temperature and pH were used to test their effects on cell growth, lipid and squalene production by *Rhodosporidium* sp. DR37. The effect of the temperature was tested from 25 to 37 °C. As shown in Fig. 8, a decrease in cell growth, lipid and squalene production was observed when the temperature was increased in culture medium. At 25 °C, biomass, lipid and squalene were 4.9 g/L, 2.85 g/L and 614 mg/L, respectively. *Rhodosporidium* sp. DR37 showed its maximum growth and squalene production at 25 °C. Biomass and squalene were decreased to 4.2 g/L and 501 mg/L, when temperature was increased to 30 °C. The minimum level of squalene (33 mg/L) was obtained at 37 °C. Raising temperature showed a negative effect on cell growth and squalene production and the maximum amount of lipid and squalene were produced at 25 °C. So, culture medium that was incubated at colder temperature showed best condition

for growth and squalene production by *Rhodosporidium* sp. DR37. Actually, a shift to a colder temperature probably can regulate biosynthesis pathways towards squalene and sterol production and accumulation [53]. Also, Wang et al reported that an increase in temperature from 30 to 35 °C significantly decreased biomass and lipid production by *Rhodosporidium* TJUWZ4 [19]. They reported that temperature range of 20-25 °C is favorable for lipid production by *Rhodosporidium* TJUWZ4.

The effect of pH on cell growth, lipid and squalene production

As shown in Fig. 9, it was found that the pH 5 and 7 had similar effects on cell growth, lipid and squalene production. The amounts of biomass, lipid and squalene were 4.28 g/L, 2.61 g/L and 570 mg/L at pH 5, and 4.88 g/L, 2.7 g/L and 604 mg/L at pH 7 respectively. But at pH 9, a significant decrease in cell growth, lipid and squalene was observed to 2.59 g/L, 0.8 g/L and 144 mg/L, respectively. *Rhodosporidium* sp. DR37 showed good adaptation to pH 5 similar to pH 7. Increasing of pH to 9, had a negative effect on its growth and squalene production in comparison to pH 5 and 7. These results are in accordance with Wang et al [19]. Their results showed that two strains of *Rhodosporidium* TJUWZ4 and *Rhodosporidium* TJUWZA11 were able to accumulate lipid in media with pH range of 3-7. They concluded that this acid tolerance property makes both strains as good candidates for high lipid production in media with low pH. The presence of low pH in culture medium is a good factor for prevention of microbial contaminations in fermentation broth.

Finally, the optimum culture condition was included 20 g/L sucrose, 5 g/L peptone, 20% v/v seawater and pH 7 at 25 °C. Cell growth, lipid and squalene production were monitored in optimized shake flask culture condition during 120 h. As shown in Fig. 10, the amount of squalene was increasing during 72 h of cultivation. The maximum amount of lipid (2.86 g/L) and squalene (619 mg/L) was achieved at 72 h of incubation. Both lipid and squalene production were decreased after 72 h and reached to 1.8 g/L and 181 mg/L at 120h, respectively.

As shown in Fig. 11, lipid content (% of CDW) and squalene content (% of total lipid) in *Rhodosporidium* sp. DR37 cultivated in optimized medium were investigated by time course analysis. The lipid content was 38.7 % and 53.3 % at 24 and 48 h, respectively and reached to its maximum level (58.2 %) at 72. A similar pattern was observed for squalene content. The maximum content of squalene (21.6 %) was achieved at 72 h and then decreased to 10.1 % at 120h.

It has been reported that two strains of *Pseudozyma* and three strains of *Aurantiochytrium* are able to produce significant amount of squalene (Table 2). The final yield of 1290 mg/L with content of 33.07% was achieved for production of squalene by *Aurantiochytrium* 18W-3a [27]. Another research work on this strain has reported yield and maximum content of squalene as 900 mg/L and 68.99%, respectively [11]. It has been reported the highest content of squalene was achieved by *Aurantiochytrium* Yonez5-1 as 74.05% with yield of 1073.66 mg/L [29]. In our study, *Rhodosporidium* sp. DR37 showed squalene yield of 619 mg/L and content of 21.6 %. Also, two strains of *Pseudozyma* were reported to able for production of 340-2445 mg/L squalene. The yield of produced squalene by *Rhodosporidium* sp. DR37 was 1.8 times more than produced squalene by *Pseudozyma* sp. JCC 207 [10] and 3.9 times less than produced

squalene by *Pseudozyma* sp. SD301 [16]. It must be considered that 2445 mg/L produced squalene by *Pseudozyma* sp. SD301 was achieved in fed-batch fermentation in a 5 L bioreactor, while 619 mg/L produced squalene by *Rhodosporidium* sp. DR37 was obtained in a shake flask cultivation medium.

Table 2 Comparison of the squalene yield and content by *Rhodosporidium* sp. DR37 mentioned in this study

		Shake flask studies		
Strain	**Squalene yield (mg/L)**	**Squalene content (% of lipid)**	**Cultivation mode**	**Reference**
Aurantiochtrium 18W-3a	1290	33.07 %	Shake flask	[27]
Aurantiochtrium Yonez5-1	1073.66	74.05 %	Shake flask	[29]
Aurantiochtrium 18W-3a	900	68.99 %	Shake flask	[11]
Pseudozyma sp. JCC 207	340.52	NR	Shake flask	[16]
Pseudozyma sp. SD301	2445	NR	Fed-batch fermentation	[10]
Rhodosporidium sp. DR37	619	21.6 %	Shake flask	This study

NR: Not reported

Conclusion

In this study, it is shown that *Rhodosporidium* sp. DR37 is a new and favorable candidate for production of squalene. *Rhodosporidium* strains are currently being used for production of lipids but *Rhodosporidium* sp. DR37 showed ability to accumulate high amount of squalene, which is desirable for biofuel applications. Interestingly, cultivation and growth of *Rhodosporidium* sp. DR37 on medium containing sucrose as sole carbon source indicated that squalene can be co-produced with other high value metabolites such as carotenoids from consumption of cost-effective agro-industrial feedstocks. The acceptable squalene concentration (619 mg/L) and content (21.6 %) were achieved in shake flask cultivation system. Therefore these results prove further investigations on squalene production in fed-batch fermentation in bioreactor which is the subject of our forthcoming research work.

Declarations

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Authors' contributions

S. Shakeri and M. Maleki designed the study and interpreted the results. S. Shakeri and F. Khoshbasirat carried out the experimental works. Also, the manuscript was prepared by S. Shakeri and M. Maleki.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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