DOI: http://doi.org/10.21698/simi.2018.fp04

GROWTH ACTIVATION OF *SACCHAROMYCOPSIS (ENDOMYCOPSIS) FIBULIGERA Y-436* STRAIN BY SQUALENE AND ANOLYTE WATER IN LABORATORY EXPERIMENTS

Irina Senicovscaia, Tatiana Grozova, Georgiy Poleshchuk

"Moldova Verde Project" Association, 12 Meshterul Manole, MD 2044, Kishinev, irina_sen@mail.ru , tanya.grozova87@gmail.com, Republic of Moldova

Abstract

The phenomenon of growth activation of the industrial strain of mycelial fungus *S. fibuligera Y-436* under the influence of squalene and anolyte water has been investigated. Squalene had a stimulating effect on the growth of the strain on YEPD Broth in all doses. The stimulating effect of squalene was maintained for 54 hours, in a dose of 0.0005% - up to 78 hours of the strain cultivation under aerobic conditions. The growth rate of the strain in the exponential phase between 4 and 6 hours on medium with a squalene of 0.0005 % is 0.30, 0.001 % - 0.44, 0.002 % - 0.41, on medium without squalene - 0.24. Squalene promotes faster adaptation of the strain to the substrate and reduces the duration of its lag phase of growth by 2-4 hours. The number of cells increases by 2.2-2.3 times with the use of squalene in doses of 0.0005-0.001 % after 24 hours and by 1.4 times after 48 hours of incubation on the nutritional mixture based on wheat flour. The strain reached the stationary phase after 24 hours of cultivation with the use of squalene, while in the control variant - after 48 hours. This reduces the duration of cultivation by 2 times. Anolyte water contributed to the rise of generative activity of the strain due to the acid reaction by 34.3 % in comparison with the initial water. The most effective method of activation of the strain is the combined use of anolyte water and squalene in a dose of 0.0005-0.001 %.

Keywords: *anolyte water, fodder protein, growths activation, S. fibuligera Y-436, squalene*

Introduction

The way to solve the problem of deficiency of fodder protein is its production by microorganisms. The perspective group of microorganisms for obtaining the protein feed is mycelial fungus *Saccharomycopsis (Endomycopsis) fibuligera* (Smirnov et al 1997); (Tsygankova et al 1997). *S. fibuligera* is found to actively accumulate trehalose from starch. This yeast is also found to secrete a large amount of amylases, acid protease, β-glucosidase and pectinases, which have highly potential applications in fermentation industry (Chi et al 2009).

S. fibuligera is food-borne, dimorphic yeast, which has been considered, in the realm of ascomycetous yeast species, as one of the best producers of amylolytic enzymes (De Mot et al 1984). The capability of *S. fibuligera* to degrade starch relates with the production of two types of amylases: endo-acting α-amylase and exo-acting glucoamylase. Some *S. fibuligera* strains synthesize both enzymes while others produce only one type of amylase. The ability of amylases to digest raw

starch is a technologically interesting property. Such enzymes can be used for energy saving in starch-processing (Saha & Ueda 1983). Raw starch degradation is rare among yeast amylases, but one of the known yeast enzymes having this capability is the glucoamylase produced by *S. fibuligera IFO 0111* (Hostinová 2002).

It is known that additives of squalene as biochemically active substance, which has a property to capture oxygen and saturate the microorganism cells with it during the simple biochemical interaction with water, ensure the more complete use of the substrate. According to some studies, squalene stimulates the aerobic microorganisms' multiplication, which can activate the aerobic stage of wastes treatment and improve their preparation for the methanogenesis process. Additionally, the adding of amaranth seeds to the methanogenic bioreactor stimulates the process of methanogenesis also (Covaliov et al 2015).

Thus, it was assumed that biologically active additives obtained from the amaranth and added in the nutrient medium for cultivation of *S. fibuligera* can be used as one of the essential elements of the technology for producing fodder protein. In order to simplify and reduce the cost of technology the further research has been planned to conduct with natural sources of squalene - with amaranth oil.

The method of water activation by electric field is also interesting in terms of its application on the technology of obtaining protein feed products. Electrochemically activated water (catolyte, anolyte and mixed water) acquires new properties that affect its biological characteristics, kinetics of the reactions and the solubility of substances. It has been established that electrochemically activated water can increase the yeast fermentation and their generative activity in comparison with the initial water (Pankiv et al 2013). The authors explain this fact by increasing the permeability of the membrane of the yeast cell by providing a potential difference on its surface, which contributes to a better transport of nutrients into the cell. The particular interest is the anolyte water, because it has an acidic reaction of medium and a high redox potential.

The purpose of the research was to investigate the effect of squalene and electrochemically activated water on the strain of *S. fibuligera Y-436* in connection with its application as a producer of fodder protein for animals.

Materials and Methods

Inoculum preparation of *S. fibuligera Y-436.* Industrial strain of mycelial fungus *Saccharomycopsis (Endomycopsis) fibuligera Y-436* was purchased from the Russian National Collection of Industrial Microorganisms of the Federal Institution "State Research Institute of Genetics and Selection of Industrial Microorganisms of the National Research Center "Kurchatov Institute" (Moscow, Russian Federation). The strain *S. fibuligera Y-436* is not genetically modified.

The strain was maintained on Yeast Extract Peptone Dextrose (YEPD) Agar slant consisted of 5 g l^{-1} yeast extract, 10 g l^{-1} peptone, and 20 g l^{-1} dextrose. Then incubated at 28°C for 48 hours and stored at 5°C. The slant culture was transferred to YEPD Broth. Cultivation conditions were 28°C with 100 rpm shaking for 48 hours. Next, 10% v/v submerged culture was used as the fodder protein producer**.**

Cultural-morphological characteristics of the strain. On a complete yeast medium it forms white, smooth, matte colonies with pasty consistency, which with aging begins to flutter and starts to turn into fungal colonies with a true mycelium. Cells

have an oval or elongated shape with sizes of 4.0-8.0 * 6.0-18.0 μm. *S. fibuligera Y-436* strain assimilates soluble starch, glucose, arabinose, galactose, sucrose, maltose, lactose, raffinose, glycerol and mannitol. The strain uses organic and inorganic nitrogen and forms sediment and films on liquid media. The rate of reproduction in the YEPD Broth is 0.40 cells $hour^{-1}$. The optimal growth temperature of the strain is t=28°C, pH = 5.0-5.5.

Substrate preparation. The wheat flour was selected as substrate in the present study to produce fodder protein. The hydro-module contained 12.5 % w/v wheat flour and 5.0 % $(NH_4)_2SO_4$. The stages of preparation of the hydro-module consisted of heating water to 50° C, adding wheat flour, heating the mixture to 60° C, steaming 40 minutes, heating to 95° C, steaming 60 minutes and cooling.

Water preparation. Electrolyzed water (catolyte, anolyte and anolyte+catolyte) for experiments was obtained from the tap water with the aid of a device of "Ecovod-6" (Ecovod LTD, Ucraine).

Experiments preparation. All experiments were conducted in shaken flasks using 1000 ml Erlenmeyer flasks with a working volume of 500 ml. Benchtop thermostatic shaker-incubator ES-20/60 (Biosan, Latvia) has been used in studies for controlled incubation.

Experiment 1. In this experiment, various doses of squalene contained in the amaranth oil were tested with in relation to the strain of *S. fibuligera Y-436* cultivated on the YEPD Broth. As the source of squalene was used oil of amaranth. Amaranth oil contained 8 % of squalene. The following concentrations of squalene have been selected for the research: 0.0005%, 0.001% and 0.002% which were equivalent to the introduction in each shaking flask of 5.0, 10.0 and 20.0 mg of active substance (squalene) per liter of the nutrient mixture. The introduction of amaranth oil and mycelial fungus *S. fibuligera Y-436* was carried out after cooling the nutrient mixture to 28° C. The culture suspension was added to the medium once at the start of incubation in a volume of 10 % v/v. Cultivations condition were 28°C with 100 rpm for 78 hours.

Experiment 2. In this experiment, various doses of squalene contained in the amaranth oil were tested with in relation to the strain *Y-436* cultivated on a nutrient medium with wheat flour.

The experimental procedure is the same as in experiment 1. Cultivations condition were 28° C with 120 rpm for 48 hours.

Experiment 3. The experiment examined the effect of the electrolyzed water (anolyte, catolyte and mixture of catolyte and anolyte) on the strain of mycelial fungus *S. fibuligera Y-436* cultivated on a nutrient medium with wheat flour. Cultivations condition were 28° C with 120 rpm for 48 hours.

Experiment 4. The objective of this experiment was to investigate the combined effect of anolyte water and various doses of squalene on the growth of the strain *S. fibuligera Y-436* cultivated on a nutrient medium with wheat flour. Cultivations condition were 28° C with 120 rpm for 48 hours.

Methods. Cells of *S. fibuligera Y-436* were counted using the Gorjaev's chamber at least in five fields of vision. Dissolved oxygen, total dissolved solids (TDS) and pH measurements were determined by use of multi-channel analysers "Consort C3010'' (Belgium). The product mass was measured by a weight method after separation of the solid and liquid phases by centrifugation. The biomass was determined by the calculation method.

Results and Discussion

Effect of squalene on S. fibuligera Y-436 strain. Squalene had a stimulating effect in all doses on the growth of the strain *Y-436* on YEPD Broth (Figure 1).

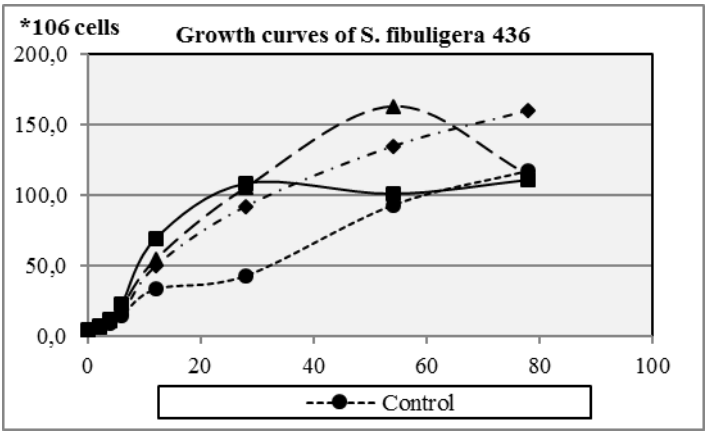

Figure 1. Growth curves of the *S. fibuligera Y-436* strain on YEPD Broth as a function of squalene doses (experiment 1)

The stimulating effect of squalene was maintained for 54 hours, in a dose of 0.0005% - up to 78 hours of the strain cultivation under aerobic conditions. Differences in the growth of the strain on a medium without squalene and with it were manifested already after 4 hours of cultivation of the strain. The strain reached the stationary phase after 54 hours of cultivation under use of squalene in a dose of 0.001% and after 28 hours of culture - in a dose of 0.002%. Activation of the growth of the strain lasted up to 78 hours under conditions of application of 0.0005% squalene. The dynamics of growth at this dose of squalene was similar to control. However, the number of cells exceeded the control by 1.3-2.1 times throughout the experiment. Apparently, the substrate consumption by culture was more gradual in comparison with doses of squalene of 0.001% and 0.002%. The growth rate of the strain in the exponential phase of growth between 4 and 6 hours on medium with a squalene of 0.0005% is 0.30, 0.001% - 0.44, 0.002% - 0.41, while on medium without squalene - 0.24. A similar regularity was observed up to 28 hours in the growth of the culture. Squalene promotes faster adaptation of the strain to the substrate and reduces the duration of its lag-phase of growth by 2-4 hours.

Squalene had a rather strong influence on the physicochemical parameters of the nutritional mixture with wheat flour and the growth of the strain *Y-436* on it. The pH in all variants of the experiment decreased during the growth of the strain *S. fibuligera Y-436* on nutritional mixture as a result of carbohydrates use (Table 1). However, in the nutritional mixture with squalene, the pH values decreased more significantly than in the control. The strongest effect was observed with the growth of the strain on the medium from 0.002% squalene, the pH value was 3.78, while on the medium without squalene - 4.42. This indirectly indicates the faster use of carbohydrates by the strain when 0.002% squalene was added to the medium.

A faster growth of the strain was also recorded in terms of the oxygen content in the medium. Its amount in the medium with squalene supplements was 12.9-31.0 times less than in the medium without squalene after 24 hours of strain cultivation. The total dissolved solids (TDS) also decreased during the experiment that indicates their use by the strain for cells growth and reproduction.

Table 1. The physicochemical characteristics of the nutritional mixture with squalene during the growth of the strain *S. fibuligera Y-436* (experiment 2)

Variant	pH		O^2, ppm		TDS, g l^{-1}		Carbohydrates, %	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Control	5.00	4.42	1.55	0	3.95	3.79	8.0	7.2
Squalene 0,0005%	4.57	3.85	0.12	0	3.78	3.59	8.0	7.2
Squalene 0,001%	4.53	3.83	0.05	0.03	3.76	3.78	8.0	7.2
Squalene 0,002%	4.43	3.78	0.11	0.04	3.73	3.75	8.0	6.8

* Initial values: pH=6.09; O^2 = 2.40 ppm; TDS = 0.408 g l^{-1}; carbohydrates = 7.0 %.

The additive of squalene in the composition of amaranth oil in all doses has increased the number of cells of the strain *Y-436* after 24 and 48 hours of the incubation (Table 2). The number of cells increases by 2.2-2.3 times with the use of squalene in doses of 0.0005-0.001% after 24 hours of incubation and by 1.4 times after 48 hours of incubation. It should be noted that with the use of squalene, the strain reached the stationary phase after 24 hours of cultivation, while in the control variant - after 48 hours. In other words, squalene contributes not only to a faster growth of the strain, but also reduces the time of passage through the growth phase.

Table 2. The number of cells of *S. fibuligera Y-436* and the sediment weight (experiment 2)

Variant	Number of cells·10^6 cc^{-1}		Sediment weight, g l^{-1}	
			total	biomass
	24 h	48 h	48 h	
Control	63.0	90.0	181.1	51.1
Squalene 0,0005%	138.0	123.8	210.5	80.5
Squalene 0,001%	141.5	124.3	225.5	95.5
Squalene 0,002%	113.3	129.3	244.3	114.3

***** initial number of cells in the hydro-module is 34.5 ·10^6 cc^{-1} .

This reduces the duration of cultivation by 2 times. As a result, the biomass of the strain increased by 1.6-2.2 times, and the total weigh of the product - by 1.2-1.4 times, respectively.

Effect of electrolyzed water on S. fibuligera Y-436 strain. Parameters of electrolyzed water are presented in the Table 3. The initial pH of the catolyte was 6.85; anolyte - 4.38; anolyte+catolyte water - 6.16, while the tap water taken for the experiment (control) - 6.59. As a result of the growth of the strain and its consumption of substrate, the pH of the nutrient medium was shifted to the acidic side, and with the use of anolyte - up to 3.71. The strain sustained an acidic reaction of the medium. This is mainly due to the contribution of plasma membrane ATPase activity which seems to be implicated in internal pH regulation (Serrano 1984, Eraso & Gancedo 1987). For example, yeast cells are able to maintain their internal pH between 6 and

7.5 when the extracellular pH varies from 3.5 to 9 (Borst-Pauwels & Peters 1977). During the growth of yeast on acid media was observed the activation of the plasma membrane ATPase and that is a mechanism for regulating internal pH.
The oxygen content during the cultivation also decreased significantly due to its use by the strain. TDS changed slightly.

Table 3. The physicochemical characteristics of the nutritional mixture on electrolyzed water during the growth of the strain *S. fibuligera Y-436* (experiment 3)

Variant	pH			O^2, ppm			TDS, g l^{-1}			Carbohydrates, %		
	0 h	24 h	48 h	0 h	24 h	48 h	0 h*	24 h	48 h	0 h	24 h	48 h
Control	6.59	4.71	4.00	2.92	0.12	0.12	0.404	6.91	6.67	7.5	8.0	7.8
Catolyte water	6.85	4.97	3.91	3.93	0.13	0.11	0.363	7.12	7.17	7.5	7.5	6.5
Anolyte water	4.38	3.98	3.71	2.35	0.15	0.43	0.553	8.63	8.60	7.5	7.8	7.0
Anolyte + catolyte water	6.16	4.51	3.99	5.08	0.20	0.16	0.363	7.06	7.11	7.5	7.7	7.1

* $(NH_4)_2SO_4$ was added after the measurement and before culturing.

The most effective for growth and reproduction of the strain was the use of anolyte. The anolyte due to the acid reaction of the medium, contributed to the increase of the generative activity of the strain *S. fibuligera Y-436* by 28.5 % compared to the initial water after 24 hours and by 34.3 % after 48 hours of growth (Table 4). The number of cells, when catolyte was used remained at the control level. The use of a mixture of catolyte and anolyte stimulated growth by 17.9% only after 24 hours.

Table 4. The dynamics of growth of the *S. fibuligera Y-436* strain on the nutritional mixture in the hydromodule prepared with electrolyzed water (experiment 3)

Variant	Number of cells·10^6 cc^{-1}		
	0 h	24 h	48 h
Control	8.9	61.5	68.0
Catolyte water	8.9	52.0	70.0
Anolyte water	8.9	79.0	91.3
Anolyte + catolyte water	8.9	74.5	48.0

Growth activation of the *S. fibuligera 436* strain in conditions of combined use of squalene and anolyte. The dynamics of changes in the physicochemical parameters of the nutrient mixture, prepared on the basis of anolyte was almost identical in the control and in the variants with squalene (Table 5). The reaction of the medium shifted to the acidic side, the content of oxygen, TDS and carbohydrates decreased.

Table 5. The physicochemical characteristics of the nutritional mixture on anolyte water with squalene during the growth of the strain *S. fibuligera 436* (experiment 4)

Variant	pH			O^2, ppm			TDS, g l^{-1}			Carbohydrates, %		
	0 h	24 h	48 h	0 h	24 h	48 h	0 h*	24 h	48 h	0 h	24 h	48 h
Control	4.90	3.62	3.31	2.20	0.13	0.11	0.398	4.42	4.39	7.0	7.5	6.5
Squalene 0,0005%	4.90	3.60	3.31	2.20	0.13	0.11	0.398	4.28	4.24	7.0	7.4	6.4
Squalene 0,001%	4.90	3.61	3.31	2.20	0.17	0.03	0.398	4.17	4.08	7.0	7.6	6.4

* $(NH_4)_2SO_4$ was added after the measurement and before culturing.

The use of squalene in doses of 0.0005-0.001% as an additive in a nutritional mixture prepared on the basis of anolyte, promoted activation of the growth and reproduction of the strain *S. fibuligera Y-436* (Table 6). The number of cells increases by 1.6-1.7 times with the use of squalene in doses of 0.0005-0.001% after 48 hours of incubation. The biomass of the strain increased by 23.3-34.9 %, and the total weight of the product (sediment weight) – by 5.8-8.7 %.

Table 6. The number of cells of *S. fibuligera Y-436* and sediment weight (experiment 4)

Variant	Number of cells·10^6 cc^{-1}		Sediment weight, g l^{-1}	
			total	biomass
	24 h	48 h	48 h	
Control	80.8	122.5	173.0	43.0
Squalene 0,0005%	76.0	195.0	188.0	58.0
Squalene 0,001%	77.8	212.5	183.0	53.0

***** initial number of cells in the hydro-module is 8.0 ·10^6 cc^{-1} .

Conclusions

The phenomenon of growth activation of the industrial strain of *S. fibuligera Y-436* under the influence of squalene and anolyte water results from the faster adaptation and use of substrate by the strain and acid reaction of the medium, optimal for growth and reproduction mycelial fungus. Squalene had a stimulating effect on the growth of the strain *S. fibuligera Y-436* under aerobic conditions as on the YEPD Broth and on the nutritional mixture based on wheat flour in all doses. The stimulating effect of squalene was maintained on YEPD Broth for 54 hours, in a dose of 0.0005 % - up to 78 hours of the strain cultivation. The growth rate of the strain in the exponential phase of growth between 4 and 6 hours on medium with a squalene of 0.0005 % is 0.30, 0.001 % - 0.44, 0.002 % - 0.41, while on medium without squalene - 0.24. Squalene reduces the duration of its lag-phase of growth by 2-4 hours. The biomass of the strain and the total weigh of the product increased by 1.6-2.2 times and 1.2-1.4 times. Squalene had a rather strong influence on the physicochemical parameters of the nutritional mixture based on wheat flour and the growth of the strain *S. fibuligera Y-436*. Due to the shift in pH to the acid side, the strain used substrates and absorbed oxygen more rapidly. The additive of squalene increased the number of cells by 2.2-2.3 times with the use of squalene in doses of 0.0005-0.001 % after 24 hours and by 1.4 times after 48 hours. With the use of squalene, the strain reached the stationary phase after 24 hours, while in the control variant - after 48 hours, thereby reducing the time of passage through the growth phase. This reduces the duration of cultivation by 2 times. This fact is important for the decrease of energy consumption and the reduction of fodder protein production cost. The most optimal electrolyzed water for the preparation of a nutritional mixture based on the wheat flour for the strain of *S. fibuligera Y-436* was anolyte. Anolyte water contributed to the rise of generative activity of the strain due to the acid reaction by 34.3 % in comparison with the initial water. The most effective method of activation of the strain is the combined use of anolyte water and squalene in a dose of 0.0005-0.001 %.

References

Borst-Pauwels, GWFH & Peters, PHJ 1977, 'Effect of the medium pH and the cell pH upon the kinetical parameters of phosphate uptake by yeast', *Biochimica et Biophysica Acta – Biomembranes, Elsevier*, vol. 466, no. 3, pp. 488-495.

Chi, Z, Liu, G, Wang, F, Ju, L & Zhang, T 2009, '*Saccharomycopsis fibuligera* and its applications in biotechnology', *Biotechnology Advances*, vol. 27, no. 4, pp. 423-431.

Covaliov, V, Senicovscaia, I, Nenno, V, Bobeica, V & Covaliova, O 2015, 'Effects of amaranths' seeds on dehydrogenase activity and gases emission in methanogenic bioreactors', *Studia Universitatis Moldaviae, Seria Stiinte reale si ale naturii*, Kishinev, vol. 81, no. 1, pp. 230-235.

De Mot, R, Andries, K & Verachtert, H 1984, 'Comparative study of starch degradation and amylase production by ascomycetous yeast', *Systematic and Applied Microbiology*, vol. 5, no. 1, pp. 106-118.

Eraso, P & Gancedo, C 1987, 'Activation of yeast plasma membrane ATPase by acid pH during growth', *Elsevier Science Publishers B. V. (Biomedical Division)*, vol. 224, no. 1, pp. 187-192.

Hostinová, E 2002, 'Amylolytic enzymes produced by the yeast *Saccharomycopsis fibuligera* (review)', *Biologia*, Bratislava, vol. 57, no. 11, pp. 247-251.

Pankiv, NO, Palyanitsa, LYa, Kosiv, RB & Berezovska, NI 2013, 'Effect of electrochemically activated water on the enzymatic activity of alcoholic yeasts', *East European Journal of Advanced Technology*, vol. 63, no. 3-6, pp. 29-33 (UA).

Saha, BC & Ueda, S 1983, 'Alcoholic fermentation of raw sweet potato by a nonconventional method using *Endomycopsis fibuligera* glucoamylase preparation', *Biotechnology Bioengineering*, vol. 25, no. 4, pp. 1181-1186.

Serrano, R 1984, 'Plasma membrane ATPase of fungi and plants as a novel type of proton pump', *Current Topics in Cellular Regulation*, no. 23, pp. 87-126.

Smirnov, VN, Vinarov, AYu, Borisenko, EG, Tsygankova, NI, Zakharychev, AP & Smetanina, SE 1997, 'Yeast strain *Endomycopsis fibuligera* is a producer of protein biomass', *Russia Patent*, no. 2092549 (RU).

Tsygankova, NI, Vinarov, AYu, Gordeeva, EI, Smirnov, VN & Ipatova, TV 1997, 'Method for producing the protein product from starch and cellulose-containing plant raw materials', *Russia Patent*, no. 2081166 (RU).