DOI: 10.22620/agrisci.2021.30.012

OPTIMIZATION OF EXOPOLYSACCHARIDE SYNTHESYS BY MEDICINAL FUNGUS *TRAMETES VERSICOLOR* IN SUBMERGED CULTURE

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Abstract

The species *Trametes versicolor* refers to medicinal mushroom well known in traditional Asian medicine for over 2000 years. Due to the long time required for basidiocarp formation, attention has recently been given to the submerged cultivation method for the production of mycelial biomass and various bioactive components. Exopolysaccharides (EPS) produced by *Trametes versicolor* are essential components possessing numerous functionalities and exhibiting potential medicinal applications. The subject of this investigation is higher fungus *Trametes versicolor* isolated from Bulgaria. Four well-defined culture media were studied to select the medium that maximizes production of EPS in submerged cultivation. The M3 was shown to provide the highest yields of EPS and was further investigated to optimize EPS production conditions.

The initial glucose concentration was found to be the most important factor in both EPS production and cell growth. The maximum biopolymer quantity of 1.067 g/L was obtained at 40 g/L glucose. For examination and evaluation of the correlation between the carbon source and the complex influence of the nitrogen sources over the mycelial growth and the EPS synthesis and the optimization of the media orthogonal central composition design 2^3 with star arm $\pm\alpha$=1.454671 was applied. The experimental design was based on 21 combinations. Dynamic cultivation was carried out after the optimization of the media for determination of the effect of the duration of the cultivation process over the *Trametes versicolor* growth and EPS gain. Maximum EPS yield was observed after 216 hours.
Keywords: exopolysaccharide, optimization, medicinal fungus, *Trametes versicolor*.

INTRODUCTION

The higher Basidiomycetes are in the focus of international medicinal research in recent years because they are tremendous source of different kinds of new pharmaceutical products, especially polysaccharides with antitumor, antidiabetic, and other activities (Roupas et al., 2012, Roncero-Ramos and Delgado-Andrade, 2017, Rathore et al., 2017 Habtemariam, 2019). One of the best investigated medicinal mushroom in recent years is *Trametes versicolor*. These mushroom have a remarkable history and have been used in traditional Asian medicine for thousands of years (Giavasis, 2014, Kalac, 2016, Habtemariam, 2020). *Trametes versicolor* belongs to class *Agaricomycetes*, family *Polyporales* and is able to synthesize extracellular and intracellular polysaccharides. These biopolymers have given its medicinal value in cancer therapy and are assessed by reviewing the chemistry, pharmacology and therapeutic potential at three levels: *in vitro*, *in vivo* and clinical studies (Cui and Chisti, 2003, Benson et al., 2017, Donot et al., 2016, Scarpari et al., 2020, Habtemariam, 2020). Currently fungal polysaccharides are usually isolated from the fungal fruiting bodies (basidiocarp). Fungal fruiting bodies in nature are formed during solid

state cultivation of mushrooms on specific wooden substrates, which is an extremely long and time consuming process. Submerged cultivation is an alternative and promising approach for producing mushroom biomass rich in biologically active components as well as valuable extracellular secondary metabolites (Kim et al., 2002).

The purpose of this study is to optimize the submerged culture conditions to produce exopolysaccharides (EPS) by *Trametes versicolor* using a statistically based experimental design.

MATERIALS AND METHODS

Microorganism

The fungal strain *Trametes versicolor* used in this research is a part of the microbial collection of the Biotechnology Department of the University of Food Technologies in Plovdiv. The strain is available in the National Bank of Industrial Microorganisms and Cell Cultures under No 8979. *Trametes versicolor* is maintained on MCM (Mushroom complete medium) containing (g/L): yeast extract – 2.0; KH_2PO_4 – 0.46; K_2HPO_4 – 1.0; $MgSO_4x7H_2O$ – 0.5; peptone – 2.0; glucose – 20.0; agar – 20.0 and pH 6.0. The sterilization is carried out at 121°C for 15 min.

Inoculation and cultivation conditions

For obtaining of inoculum pre-grown culture was used. The medium contains (g/L): glucose – 30.0; malt extract – 3.0; peptone – 5.0; yeast extract – 5.0; $NaNO_3$ – 2.0; $(NH_4)_2SO_4$ – 5.0; K_2HPO_4 – 1.0; KCl – 0.5; $MgSO_4$ x $7H_2O$ – 0.5; $FeSO_4$ x $7H_2O$ – 0.01 and pH 6.0. Erlenmayer flasks with 100 mL media were sterilized at 121°C for 20 min. The cooled flasks were afterwards inoculated with 5 mL suspension of the fungal culture. The cultivation was carried out on a rotary shaker with 220 rpm at 28°C for 8 days.

Screening of media

Four media (M1-M4, table 1) were used for primary determination of the best gain of EPS and optimal mycelial growth.

One-factor-at-a-time screening of carbon sources and its concentrations

In each experiment, one factor was varied, while all other factors were holding constant. Glucose, fructose, sucrose and maltose with final concentration 30 g/L were initially studied. For the choice of the quantity of the carbon source 10, 20, 30, 40 and 50 g/L concentrations were tested, and the 30 g/L were used as control.

Table 1. Growth media compositions

	M1	M2	M3	M4
Sucrose	30 g/L	-	-	-
Glucose	-	30 g/L	30 g/L	10 g/L
YE	5 g/L	5 g/L	5 g/L	5 g/L
Malt E	-	-	3 g/L	3 g/L
peptone	-	-	5 g/L	5 g/L
$(NH_4)_2SO_4$	-	5 g/L	-	-
$NaNO_3$	-	2 g/L	-	-
K_2HPO_4	1 g/L	1 g/L	1 g/L	-
KCl	0.5 g/L	0.5 g/L	0.5 g/L	-
$MgSO_4$ x $7H_2O$	0.5 g/L	0.5 g/L	0.5 g/L	-
$FeSO_4$ x $7H_2O$	0.01 g/L	0.01 g/L	0.01 g/L	-

Orthogonal central composition design 2^3 with star arm $\pm\alpha=1.454671$

After the determination of significantly influencing factors and the compilation of a matrix plan of the experiment the initial variables were coded with the equation:

$$x_i = \frac{(Z_i - Z_i^0)}{\Delta Z_i} \quad (1)$$

Where x_i is the coded value of the variable, Z_i, Z_i^0 – the natural value of the variable in the center of the plan and ΔZ_i is the interval of the *i*-variable.

The determination of the natural values of the factors in the star points was made according to equation 2. The insurance of the orthogonality of the matrix plan of the experiment the StatGraphics XV algorithm applied in a linear transformation of the

quadratic members in the matrix of the experiment:

$$X_j = X_j^2 - \bar{X}_j^2 = X_j^2 - \frac{\sum_{i=2}^{N} X_{ij}^2}{N} \quad (2)$$

N-count of the experiments.

All other transformations and the determination of the main statistical parameters for determining the influence of individual factors and the adequacy of the mathematical model are embedded in the algorithm of StatGraphics XV.

Isolation of the exopolysaccharide

The biomass was separated from the cultural broth after filtration through Whatman filter. The broth was then evaporated on a rotary vacuum evaporator until reaching ½ of the initial volume. Ethanol in 2:1 ratio was added and mixed vigorously. The solution was then left for 24 h at 4°C and centrifuged at 6000 rpm for 20 min afterwards. The residue was washed with 75% ethanol and centrifuged again. The obtained EPS was dried at 30°C for 12 h and then the mass was determined.

Determination of the dry weight

Absolute dry weight was determined on a dry weight scale RADWAG at 105°C until constant weight.

Determination of total sugars and reducing sugars

The total sugars are determined by the phenol-sulfuric acid method (Dubois, 1956), and the reducing sugars – by the method published by Lever (Lever, 1977).

All experiments are performed in triplicate.

RESULTS AND DISCUSSION

The exopolysaccharides produces by the *Basidiomycetes* are topic of serious research in the past years due to their applications in several industries like food, pharmaceutical and beauty. The controlled submerged cultivation of the basidiomycetes is preferred due to the higher yields of biomass for short amount of time, lower contamination risk and simplified isolation and purification (Kim et al, 2002, Lin et al, 2006, Fazenda et al, 2008). *Trametes versicolor* 8979 was cultivated on four nutrient media – M1, M2, M3 and M4. The concentration of biomass and EPS was monitored at the end of the cultivation. Results are given on fig.1

Trametes versicolor 8979 grows well on all used media gaining biomass concentrations between 8.48 and 10.11 g/L with optimal concentration with M1 media. There was EPS production in all media used. The highest concentrations were registered with M3. Similar results were obtained previously (Lee et al., 2007; Hsiesh et al., 2006). The EPS concentration registered with M3 media was higher than the reported by Kim et al. (2002) and Tavares et al. (2005) 0.504 g/L and 0.700 g/L respectively. In all cases *Trametes versicolor* was cultivated on media containing yeast extract and malt extract. The results shown on figure 2 state that the growth of *T. versicolor* and the EPS production takes place when the carbon source isn`t fully utilized and the pH of the cultural broth is lower in comparison with the initial value. Such pH dropping isn`t unusual for the cultivation of *Trametes versicolor* and was reported previously (Taveres et al. 2005, Wang et al. 2012). Thus M3 was chosen for further optimization.

The carbohydrates are the major component of the cytoskeleton and very important for the growth of the basidiomycetes, as well as the EPS synthesis. For the evaluation of the most suitable for the growth, biomass gain and EPS synthesis carbon source in submerged culture, a single-factor experiment was applied, were only one parameter was changed and the others were fixed at known levels. The effect of four carbon sources (glucose, fructose, sucrose and maltose) with final concentration 30 g/L was evaluated. *Trametes versicolor* 8979 could utilize all used sources with biomass gain between 9.5 and 11.43 g/L and EPS gain up to

0.960 g/L when glucose was used as carbon source.

It is again visible that the EPS synthesis isn`t related with the biomass gain and that the good mycelial growth doesn`t correlate with high EPS yields (Table 2).

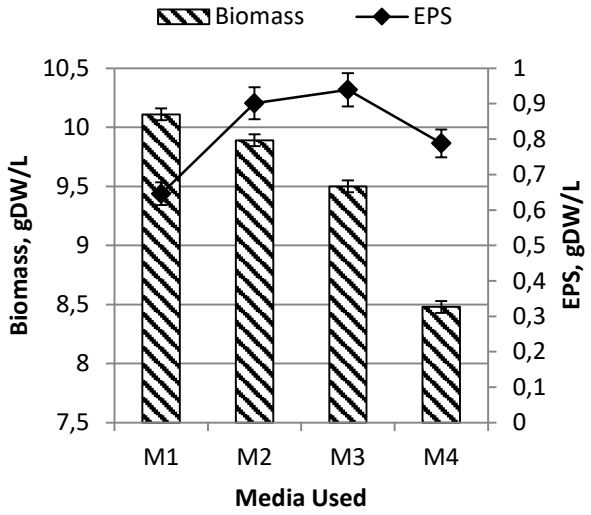

Fig.1 Biomass and EPS concentrations after 7 days at 28°C

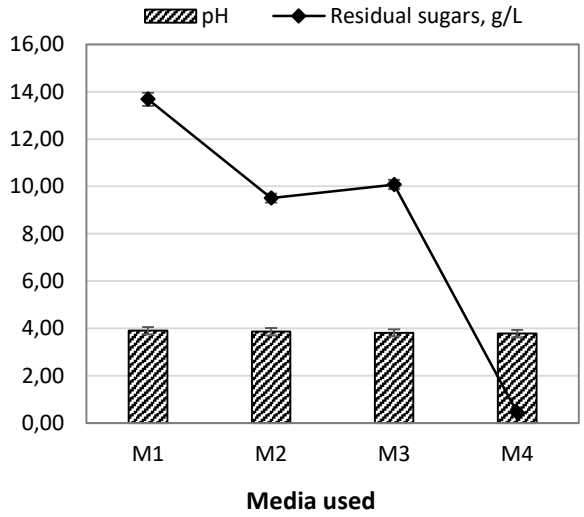

Fig.2 Final pH and residual sugars concentration

Glucose been the best carbon source for EPS production in submerged culture was reported by other science groups as well (Xu et al., 2003, Nour El-Dein et al, 2004, Lin and Sung, 2006).

Table 2. Effect of the carbon source over the biomass and EPS yields

Carbon source	Final pH	DW BM, g/L	EPS, g/L	Residual sugars, g/L
Glucose	3.84	11.03	**0.960**	10.51
Fructose	4.08	10.45	0.930	14.51
Sucrose	3.89	9.5	0.856	12.34
Maltose	4.04	11.43	0.586	9.86

Carbon source concentration in the medium is important regarding the growth and the development of the producer as well as the synthesis of significant metabolites. Some studies state that enzyme production from *Trametes versicolor* is extremely dependent on the glucose concentration (Tavares et al., 2005).

The obtained results showed highest EPS yield of 1.067 g/L when the glucose concentration was 40 g/L. The biomass DW grows with the escalation of the glucose concentration and the maximum biomass yield was detected at the highest concentration used (50 g/L). Similar results were reported from Lee et al. (2007) and Hiesh et al. (2006) for the fungal specie *Ganoderma lucidium*. It must be noted that the different *Trametes versicolor* species possess different metabolic characteristics which could result in different results between the studies.

Based on the data obtained from the single-factor experiment the following components were further varied – glucose, yeast extract, peptone and ammonium sulphate with determined limits (of variation) (Table 3).

The area, where the optimum of a regression mathematical model was located, was defined as nearly stationary, substantially nonlinear and defined by the equation:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{1<j<i<k}^{k} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (3)$$

Table 3. Limits of variation of the basic components, g/L

Component, g/L	-α	Lower level	Basic level	Higher level	+α
	-1.54671	-1	**0**	+1	+1.54671
Glucose	9.1	20	**40**	60	70.9
Yeast extract	0.41	1.5	**3.5**	5.5	6.59
Peptone	0.41	1.5	**3.5**	5.5	6.59
Ammonium sulphate	0.91	2	**4**	6	7.09

Table 4. Design of experiment for the optimization of the medium experiment

№	Block	Glucose	Yeast extract	Peptone	Ammonium sulphate	Biomass, g/L	EPS, g/L
1	1	0	0	0	0	9,39	1,23
2	1	-1	-1	-1	1	5,41	0,76
3	1	1	1	1	1	16,24	1,39
4	1	0	0	1,54671	0	10,43	0,803
5	1	0	1,54671	0	0	10,29	0,85
6	1	-1	1	1	1	8,31	0,727
7	1	0	0	0	-1,54671	9,55	1,979
8	1	1	1	1	-1	12,1	0,731
9	1	-1,54671	0	0	0	5,37	0,63
10	1	1,54671	0	0	0	13,61	0,758
11	1	-1	1	-1	1	8,4	0,738
12	1	0	-1,54671	0	0	8,29	0,827
13	1	-1	-1	-1	-1	7,99	0,81
14	1	-1	-1	1	-1	8,826	0,868
15	1	1	-1	1	1	10,06	1,424
16	1	0	0	0	0	9,04	1,35
17	1	1	-1	1	-1	11,964	1,175
18	1	1	-1	-1	1	3,37	0,998
19	1	-1	-1	1	1	8,63	0,886
20	1	0	0	1,54671	0	4,369	0,653
21	1	-1	1	1	-1	8,98	0,778
22	1	0	0	0	1,54671	9,05	0,86
23	1	0	0	0	0	7,35	1,36
24	1	-1	1	-1	-1	8,72	0,982
25	1	1	1	-1	-1	8,939	1,405
26	1	1	1	-1	1	11	1,123
27	1	1	-1	-1	-1	8,648	1,194

Biomass = 9,04911 + 1,43375*Glucose + 1,0048*Yeats extarct + 1,53997*Peptone + 0,886312*Glucose*Peptone + 0,948062*Yeats extarct*Amonium sulphate (4)

Polysacharide = 1,10881 + 0,148618*Glucose - 0,148307*Glucose^2 - 0,134095*Peptone^2 + 0,154956*Amonium sulphate^2 (5)

Table 5. Analysis of Variance (ANOVA) for Biomass

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*	*Significance*
A:Glucose	42,7255	1	42,7255	21,02	**0,0006**	**Significant**
B:Yeast extract	20,9847	1	20,9847	10,32	**0,0075**	**Significant**
C:Peptone	49,2906	1	49,2906	24,24	**0,0004**	**Significant**
D:Ammonium sulphate	1,4662	1	1,4662	0,72	0,4124	Non-significant
AA	2,10681	1	2,10681	1,04	0,3288	Non-significant
AB	7,13291	1	7,13291	3,51	0,0856	Non-significant
AC	12,5688	1	12,5688	6,18	**0,0286**	**Significant**
AD	0,484764	1	0,484764	0,24	0,6341	Non-significant
BB	1,36572	1	1,36572	0,67	0,4284	Non-significant
BC	1,88444	1	1,88444	0,93	0,3547	Non-significant
BD	14,3812	1	14,3812	7,07	**0,0208**	**Significant**
CC	2,2648	1	2,2648	1,11	0,3120	Non-significant
CD	3,50345	1	3,50345	1,72	0,2138	Non-significant
DD	1,39898	1	1,39898	0,69	0,4230	Non-significant
Total error	24,3968	12	2,03307			
Total	185,956	26				

R-squared = 86,88%; R-squared (adjusted for d.f.) = 71,57%; Standard Error of Est. = 1,426; Mean absolute error = 0,833; Durbin-Watson statistic = 2,19469 (P=0,7360); Lag 1 residual autocorrelation = -0,13087

Table 6. Analysis of Variance (ANOVA) for Polysaccharide

Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*	*Significance*
A:Glucose	0,459079	1	0,459079	5,82	**0,0328**	**Significant**
B:Yeast extract	0,00203033	1	0,00203033	0,03	0,8752	Non-significant
C:Peptone	0,00194392	1	0,00194392	0,02	0,8779	Non-significant
D:Ammonium sulphate	0,12748	1	0,12748	1,62	0,2277	Non-significant
AA	0,251762	1	0,251762	3,19	**0,0993**	**Significant**
AB	0,000115562	1	0,000115562	0,00	0,9701	Non-significant
AC	0,0000600625	1	0,0000600625	0,00	0,9784	Non-significant
AD	0,0358156	1	0,0358156	0,45	0,5132	Non-significant
BB	0,0884498	1	0,0884498	1,12	0,3105	Non-significant
BC	0,0919606	1	0,0919606	1,17	0,3015	Non-significant
BD	0,000232563	1	0,000232563	0,00	0,9576	Non-significant
CC	0,205822	1	0,205822	2,61	**0,1322**	**Significant**
CD	0,169538	1	0,169538	2,15	0,1683	Non-significant
DD	0,27484	1	0,27484	3,48	**0,0866**	**Significant**
Total error	0,946485	12	0,0788738			
Total	2,65562	26				

R-squared = 64,36%; R-squared (adjusted for d.f.) = 62,78 %; Standard Error of Est. = 0,280; Mean absolute error = 0,143; Durbin-Watson statistic = 2,28875 (P=0,8149); Lag 1 residual autocorrelation = -0,14613

A second degree model was obtained by the usage of central composition design 2^3 with star arm ±α=1.454671. The design allows the evaluation of the correlation between the carbon source and the combinative effect of nitrogen sources over the mycelial growth and the EPS synthesis. The plan-matrix of the experiment was based on 21 combinations and together with the data observed are given in Table 4.

An interesting observation was that the increase of the ammonium sulphate concentration led to increased EPS synthesis (equation 5). The obtained data showed that the yeast extract does not affect the EPS yields, but was a key component providing the culture with valuable amino acids and important elements for the growth (equation 4). Other studies showed that organic nitrogen sources are preferred in media for submerged cultivation of basidiomycetes for EPS synthesis (Yadav et al., 2014).

Considering the data received from the experiment, Multiple Response Optimization (fig.3, 4, 5) – minimum biomass and maximum EPS yield, minimum biomass and EPS yield and maximum biomass and EPS yield was made (table 7).

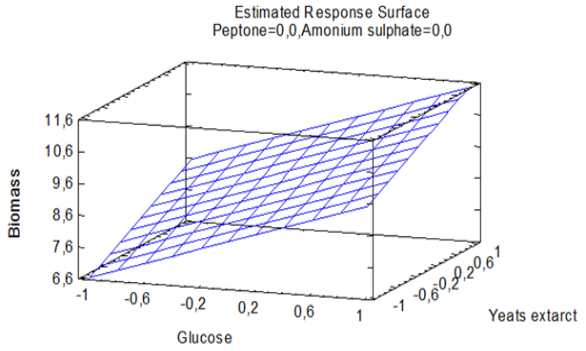

Fig.3 Estimate response surface of the glucose and ammonium sulphate effect over the biomass yield

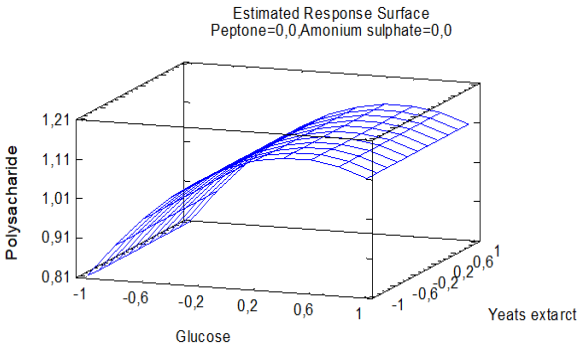

Fig. 4 Estimated response surface of the glucose and yeast extract effect on the EPS gain

It was proven that the model covers accurately the experimental data (equation 4 and equation 5). The results from the cultivations justified that the increasing the glucose concentration above certain levels led to negative effect over the EPS concentration in the medium.

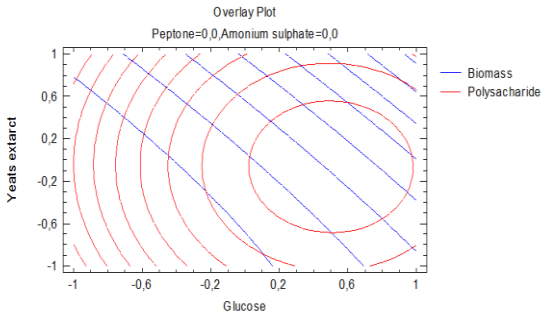

Fig. 5 Multiple Response Optimization

Based on the data given in table 7 a conclusion could be made that the optimal glucose concentration should be 40.32 g/L .With that and maximal ammonium sulphate concertation (7.09 g/L) and minimal values for the yeast extract and the peptone (3.5 g/L) the biomass concertation was at its lowest level and the EPS concentration at its highest – 1.49 g/L.

The effect of the cultivation duration over the EPS synthesis was also evaluated and was performed during 14 days (figure 6).

The EPS synthesis starts with the beginning of the cultivation process and the EPS

concentration at the 24th hour was 0.069 g/L and 0.432 g/L at the 48th hour. The EPS synthesis was the most intensive during the exponential phase of the culture growth and the maximum concentration (1.69 g/L) was detected at the 216th hour or at the beginning of the stationary phase of growth. The concentration of EPS on the 9th day of the cultivation was 20% higher than the one registered on the 7th day. It is interesting to be noticed that the glucose was utilized until the 8th day (reducing sugars concentration 0.44 g/L) while the EPS concentration continue to grow for a day further.

Table 7. Medium variations

Variant	Glucose	Yeast extract	Peptone	Ammonium sulphate	Calculated biomass yield	Calculated EPS yield	Experimental biomass yield	Experimental EPS yield
	g/dm^3							
1	51.54	0.948	1.62	7,09	3,58	1.00	3.64	1.20
2	**40.32**	**3.51**	**3.51**	**7.09**	**9.0**	**1.43**	**9.56**	**1.49**
3	62.2	5.66	5.50	5.95	16.38	16.38	1.06	1.10

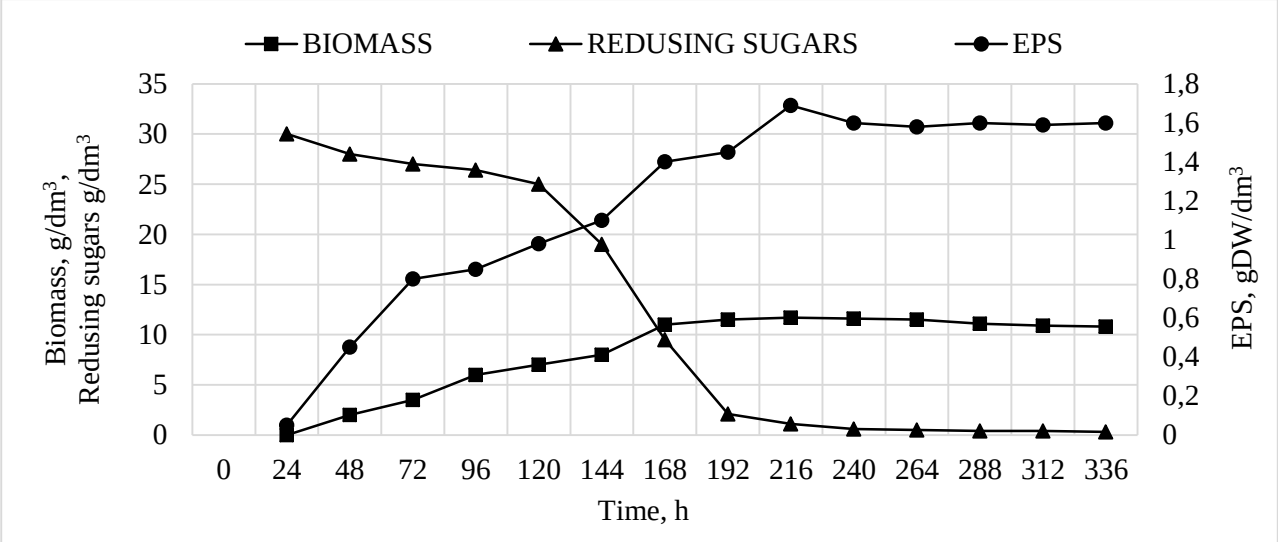

Fig. 6. Effect of cultivation duration over the growth of *Trametes versicolor* 8979 and the EPS synthesis

More research regarding the cultivation conditions effect over the EPS synthesis needs to be performed – pH, temperature, DO_2, mixing rate. These factors should be taken into account for the final optimization of the process and will be topic of following researches.

CONCLUSIONS

Trametes versicolor 8979 utilizes glucose, fructose, maltose and sucrose. Maltose leads to better growth of the culture and the highest biomass yield – 11.43 g/L. Glucose was proven to be the optimal carbon source for EPS

production where 1.067 g/L EPS were obtained with 40g/L glucose in the medium. The gain of EPS was optimized with orthogonal central composition plan 2^3 and 1.4-fold increase of the concentration of EPS was reached (up to 1.49 g/L) at the 216th hour of the cultivation process.

ACKNOWLEGMENTS

This work was supported by the Bulgarian Ministry of Education and Science under the National Research Programme "Healthy Foods for a Strong Bio-Economy and Quality of Life" approved by DCM #577/17.08.2018".

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