HETEROTROPHIC CULTIVATION OF EUGLENA GRACILIS ON CHEMICALLY PRETREATED MEDIA

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Abstract – In this research, the impact of chemical agents on the growth of *Euglena gracilis* and contaminants (*S. cerevisiae* and *B. subtilis*) in different cultivation media was studied. *E. gracilis* was cultivated on modified Hutner and complex medium in Erlenmeyer flasks and a stirred tank bioreactor. H_2O_2 and antimycin were used as suppressors of contaminant growth activities during algae cultivation. The use of antimycin as a chemical suppressor of contaminants is not recommendable because of its significant impact on the *E. gracilis* growth. At a H_2O_2 concentration of 5 mg L^{-1} contaminant growth activities were almost completely suppressed. In these conditions, *E. gracilis* is capable to grow, but a further increase of H_2O_2 concentration is related to significant reduction of algae growth. H_2O_2 as a suppressor of contaminants has great potential for industrial application, but its optimal concentration for a particular bioprocess has to be determined in order to obtain the maximal bioprocess efficiency.

Keywords: Euglena gracilis; heterotrophic cultivation; different cultivation media;H_2O_2; antimycin

INTRODUCTION

The microalgae *Euglena gracilis* is a well studied microorganism. It has great nutritional value because it contains valuable proteins in high concentrations (Murakami *et al*., 1998), polyunsaturated fatty acids, β-carotene, and vitamins C and E (Ogbona *et al*., 1998) among others. However, this microalgae is mostly recognized for paramylon (β-1,3-glucan) production. This compound belongs to a group of biologically active polysaccharides, together with lentinan, fungal glucans, schizophyllan or pachyman, among others. Lentinan and schizophyllan are tumor suppressors and fungal glucans are well known immunostimulators (Watanabe *et al*.,

2013). Paramylon as a supplement has several human health benefits; it has anti-carcinogenic properties, it lowers blood cholesterol and its sulfonated derivatives can inhibit HIV virus activity (Koizumi *et al*., 1993). *E. gracilis* is capable of growing as a strict photoautotroph, a photo-heterotroph or a strict heterotroph utilizing organic carbon sources (Osafune *et al*., 1990). Under autotrophic conditions biomass yield is relatively low, so heterotrophic cultivation is more interesting for industrial application. *E. gracilis* is also capable of growing on several carbon sources and it has even been shown that it can grow on potato liquor and corn steep solids based media. The aim of these studies was to define simple and inexpensive complex media suitable for large scale heterotrophic cultivation of

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Euglena gracilis and paramylon production (Šantek *et al*., 2010; Šantek *et al*., 2012; Ivušić and Šantek, 2015). Medium type (composition) and medium optimization are highly important parts of any bioprocess as they can substantially reduce time and costs, as well as increase the bioprocess performance efficiency (Kennedy and Krouse, 1999). Various production modes (e.g., batch, fed-batch, continuous) or systems (e.g., shake flasks, bioreactors) can differ substantially regarding suitable medium composition due to their specific characteristics. The introduction of new mutants and strains in the bioprocess is frequently the reason for industrial optimization of medium composition (Kennedy and Krouse, 1999). Traditional or statistically designed approaches for media optimization can be used on numerous bioprocesses involving enzymes (Manivasagan *et al*., 2015), microorganisms (Li *et al*., 2014; Wang *et al*., 2014) or cell cultures (Almo and Love, 2014; Farrell *et al*., 2014). On the other hand, medium contaminations have to be avoided since their activity leads to lowering yields and other bioprocess efficiency parameters. Sterilization of equipment and medium, as well as aseptic manipulation techniques, are procedures usually used to avoid contamination during bioprocesses. In industrial bioprocesses these procedures are expensive, energy- and time-consuming and therefore the use of chemicals could be an alternative. The selection of chemicals for this purpose is a demanding process due to the fact that chemicals with desirable selective toxicity and the exact concentration to be used must be previously determined. An excessive chemical concentration may modify the performance of the working microorganism or remain in the product, altering its properties (Oliveira *et al.,* 2000). Chemical agents usually used for this purpose are: acids, basses, organic solvents, antibiotics or oxidants. An example of antibiotics application is the development of a sustainable procedure for controlling contaminant growth during the production of polyhydroxybutyric acid (PHB) by *Alcaligenes eutrophus*. In this research, erythromycin did not show a harmful effect on the bacterium growth. However, nitrofurantoin shows strong and streptomycin moderate harmful effects on *A. eutrophus* growth, respectively. Strongly harmful effect on PHB synthesis was only observed with nitrofurantoin (Oliveira *et al.,* 2000). Furthermore, the successful application of vancomycin for the growth inhibition of *Bacillus cereus* in polyhydroxyalkanoate (PHA) production with *Hydrogenophaga pseudoflava* on whey containing media was also demonstrated (Koller *et al.,* 2011).

The aim of this study was to investigate the effect of chemical agents (H_2O_2 and antimycin) on the heterotrophic cultivation of *E. gracilis* in different experimental conditions. This study also shows how to suppress further growth of contaminants during the bioprocess if contamination happens after initial medium and bioreactor sterilization. In this research, heterotrophic cultivation of *E. gracilis* was performed in Erlenmeyer flasks and additionally verified in the stirred tank bioreactor.

MATERIALS AND METHODS

Microorganisms and cultivation media

Euglena gracilis strain Z (Klebs SAG 1224-5/25) was obtained from the Algensammlung Göttingen, Germany. The yeast *S. cerevisiae* and the bacterium *B. subtilis* were from the Culture collection of the Faculty of Food Technology and Biotechnology, University of Zagreb. Working microorganisms were maintained as follows: *E. gracilis* on modified Hutner medium (Hutner *et al*., 1966), *S. cerevisiae* on malt extract agar and *B. subtilis* on standard (containing beef, yeast, peptone and casein extract) agar, respectively. In this research, modified Hutner and complex medium [consisting of 20 g L^{-1} of glucose and 60 g L^{-1} of corn steep liquor (CSL)] were used for heterotrophic cultivation of *E. gracilis*. All media were sterilized at 121°C for 20 min, then cooled down prior to inoculation and addition of chemical agents (with or without contaminants addition). Inocula of *E. gracilis* and contaminants (*S. cerevisiae* and *B. subtilis*) were prepared separately on the medium (modified Hutner or complex medium) that was used in further research. Inocula were propagated on the rotary shaker in 500 mL Erlenmeyer flasks with 200 mL medium at 28°C for 72 hours and rotation speed of 150 min^{-1}. The cell number concentration in the *E. gracilis* inoculum was approx. 10^7 CFU/mL and in *S. cerevisiae* and *B. subtilis* inocula in the range of 5 · 10^7- 10^8 CFU/mL, respectively.

Heterotrophic cultivation of *E. gracilis* in Erlenmeyer flasks under different conditions

Cultivations of *E. gracilis* were performed on modified Hutner medium in Erlenmeyer flasks (200 mL of working volume in 500 mL flask; inoculum 10 % v/v) on a rotary shaker during five days at 28°C and rotation speed of 150 min^{-1}. H_2O_2 (range of 1.5-15.0mg L^{-1}) and antimycin (range of 0.2-1.2g L^{-1}) were selected as suppressors of contaminants. Prior to inoculation, contaminants and chemicals addition, cultivation media were prepared and sterilized (121°C/20 min). The whole study consisted of different experimental set-ups that were characterized by different *E. gracilis* cultivation conditions. The same algae cultivation conditions were present inside a particular experimental setup (5 flasks).The control setup was the setup inoculated only with *E. gracilis* culture. Inoculum for separate *E. gracilis* co-cultivation with contaminants contains cell suspension of algae (15 mL) and contaminant (5 mL). Every 24 hours, one flask (from each experimental setup) was removed from the rotary shaker and used for analytical purposes.

During the second part of this research *E. gracilis* was cultivated on complex (20 g L^{-1}of glucose and 60 g L^{-1}of CSL) medium in the selected experimental conditions according to the results obtained from research performed on the modified Hutner medium. In this part of research, *E. gracilis* was co-cultivated with contaminants and H$_2$O$_2$ addition (5 mg L^{-1}). Inoculum for *E. gracilis* co-cultivation with both contaminants consisted of the cell suspension of algae (10 mL), *S. cerevisiae* (5 mL) and *B. subtilis* (5 mL). Every 24 hours, one flask (from each experimental setup) was taken off from the rotary shaker for analytical purposes. During flask cultivations *E. gracilis* was not exposed to light. All flask cultivations of *E. gracilis* were done in triplicate and the standard deviation of experimental data was calculated.

Heterotrophic cultivation of *E. gracilis* in the stirred tank bioreactor under different conditions

Batch cultivation of *E. gracilis* in the stirred tank bioreactor on both media (modified Hutner and complex medium) was done after sterilization (together medium and bioreactor) at 121°C for 20 min. All cultivations were performed at 28 °C and a bioreactor working volume of 5 L, respectively. For *E. gracilis* co-cultivation on modified Hutner medium the inoculum contained the cell suspension of *E. gracilis* (300 mL), *S. cerevisiae* (150 mL) and *B. subtilis* (150 mL). Inoculum for separate *E. gracilis* co-cultivation with contaminants on complex medium contained cell suspension of algae (400 mL) and contaminant (200 mL). In these cultivations, dissolved oxygen tension was maintained at 30 % of air saturation by changing the air flow and stirrer speed rate. To ensure heterotrophic growth, the bioreactor was wrapped in aluminum foil in order to avoid light penetration. The monitoring of bioprocess performance was done by taking broth samples during *E. gracilis* cultivations. All algae cultivations in the bioreactor were repeated and the standard deviation of experimental data was determined.

Analytical procedures and bioprocess efficiency

Cell number density of *E. gracilis* was determined by microscopic cell counting in a Thoma chamber. Due to high mobility of algae cells, deactivation was done by addition of 1 % H$_2$SO$_4$ solution. Cell number density of *S. cerevisiae* and *B. subtilis* was determined by standard microbiological methods (medium for maintenance; Petri dishes at 28°C; 72 h for *S. cerevisiae* and 96 h for *B. subtilis*). Determination of biomass concentration was done gravimetrically. For this purpose, homogenized samples were centrifuged at 3629×g (Sanyo, Harrier 18/80) for 15 minutes and pellets were dried at 75 °C to the constant mass. Supernatants were used for determination of glucose concentration with a Shimadzu CLASS-VP LC-10AVP chromatograph with RI detector and Supelcogel

C-610H column. Phosphoric acid solution (0.02 mol L^{-1}) in demineralized water (conductivity <1 μS cm^{-1}) was used as the mobile phase. Before chromatographic analysis the prepared supernatant solution was filtered through a nylon filter (0.22 μm) and subsequently degassed in an ultrasonic bath for 20 minutes. Mobile phase flow rate was 0.5 mL min^{-1}. Hydrogen peroxide concentration was determined by the CEFIC method (Anonymous 1, 2003).

Bioprocess efficiency parameters [biomass production (X_T), conversion yield ($Y_{X/S}$) and productivity (Pr)] were calculated by standard procedures (Ivušić and Šantek, 2015).

The production of biomass (X_T) was calculated by following equation:

$$X_T = X\text{-}X_0 \qquad (1)$$

where X and X_0 are biomass concentrations at the end and at the beginning of bioprocess, respectively.

Conversion yield of substrate into biomass ($Y_{X/S}$) was estimated by following equation:

$$Y_{X/S} = X_T / (S_0 - S) \qquad (2)$$

where S_0 and S are substrate concentrations at the beginning and the end of bioprocess, respectively.

Bioprocess productivity (Pr) was determined by the following equation:

$$Pr = X_T / (t\text{-}t_0) \qquad (3)$$

where t is the end and t_0 the start time of cultivation, respectively.

For calculation of $Y_{X/S}$ and Pr average values of X_T (without standard deviation) were used. In this investigation, the standard deviation of substrate concentration was in the range of ± 0.1 - 0.6 g L^{-1} and the average values were used for $Y_{X/S}$ calculation (data presented in Tables 1 - 3).

RESULTS AND DISCUSSION

The main purpose of this study was to examine the possibility to suppress the growth of different microorganisms during algae cultivation (or medium preparation) by using different chemical agents. H$_2$O$_2$ and antimycin were chosen as possible microorganism growth suppressors because of their properties. H$_2$O$_2$ was selected due to the fact that it can be very effective in relatively low concentrations because of its high oxidative properties. These properties are a consequence of H$_2$O$_2$ decomposition on water and nascent oxygen (Finnegan *et al*., 2010). Antimycin on the other hand was chosen based on its ability to inhibit microorganism (bacteria, molds and yeasts) growth even at low concentrations for a longer period of time (Franklin and Snow, 1975). In this research,

E. gracilis was cultivated in different experimental conditions in order to define the effect of chemical agents on the growth of algae and representative contaminants (*S. cerevisiae* and *B. subtilis*). Yeast *S. cerevisiae* was selected as a representative of eukaryotic microorganisms (prefer acidic pH value = 4.5 - 5.0) and *B. subtilis* as a representative of prokaryotic microorganisms (prefer a neutral pH value). During *E. gracilis* cultivation on modified Hutner medium, H_2O_2 concentration was also monitored and it was observed that its initial concentration was reduced by 25.48 % in Erlenmeyer flasks and 32.56 % in the stirred tank bioreactor after 120 hours of bioprocess, respectively. Furthermore, similar tends of H_2O_2 concentrations were also observed during *E. gracilis* cultivation on complex medium where the initial H_2O_2 concentration was reduced by 29.23 % in Erlenmeyer flasks and 35.64 % in the stirred tank bioreactor after 120 hours of bioprocess, respectively.

Heterotrophic cultivation of *E. gracilis* on modified Hutner medium in different conditions

At the beginning of this research, *E. gracilis* was cultivated in Erlenmeyer flasks on modified Hutner medium with different concentrations of H_2O_2 (1.5 - 15 mg L^{-1}) in order to define the impact of H_2O_2 concentration on the algae growth. The control cultivation of *E. gracilis* was done without H_2O_2 addition and, consequently, the highest bioprocess efficiency parameters were observed. These parameter values are in agreement with literature data (Šantek*et al*., 2009). As can be seen in Table 1, the increase of H_2O_2 concentration was related to the decrease of bioprocess efficiency parameters compared to the control cultivation of *E. gracilis*.

Considerable suppression of *E. gracilis* growth was detected during cultivation on modified Hutner medium with H_2O_2 concentration in the range of 1.5 - 7.5 mg L^{-1}. This phenomenon is most obvious in the biomass production (X_T) that was reduced by 42 - 48 % compared to the control algae cultivation. Furthermore, parameters $Y_{X/S}$ and *Pr* were also reduced, but to a lower extent compared to the control cultivation. Further increase of H_2O_2 concentration was related to the almost complete suppression of algae growth, which was the most obvious for the cultivation on the modified Hutner medium with 15 mg L^{-1} of H_2O_2. In these experiments, bioprocess efficiency parameters were considerably reduced compared to the control cultivation. On the basis of these results it is clear that H_2O_2 has considerable impact on the *E. gracilis* growth. However, the growth of contaminants during *E. gracilis* cultivation was also suppressed and therefore for further research the H_2O_2 concentration of 5 mg L^{-1} was selected due to the fact that the highest bioprocess efficiency parameters were observed in this cultivation.

In our further research *E. gracilis* was cultivated on the modified Hutner medium with different antimycin

Table 1. Bioprocess efficiency parameters obtained during heterotrophic cultivation of *E. gracilis* in Erlenmeyer flasks on modified Hutner medium with H_2O_2 addition.

H_2O_2 concentration (mg L^{-1})	X_T (g L^{-1})	$Y_{X/S}$ (g g^{-1})	*Pr* (g L^{-1} h^{-1})
0	12.87 ± 0.62	0.660	0.107
1.5	6.75 ± 0.42	0.373	0.056
3	6.63 ± 0.32	0.368	0.055
4.5	7.30 ± 0.48	0.405	0.061
5	7.53 ± 0.28	0.560	0.063
6	7.07 ± 0.36	0.413	0.059
7.5	6.86 ± 0.34	0.387	0.057
9	5.97 ± 0.27	0.364	0.050
12	2.86 ± 0.22	0.062	0.024
15	1.26 ± 0.18	0.031	0.011

concentrations (0.2 - 1.2 g L^{-1}) in order to define the impact of antimycin on the algae growth. The control cultivation of *E. gracilis* was done on the modified Hutner medium without antimycin addition. As can be seen in Table 2, a significantly higher impact on the *E. gracilis* growth was already observed at the lowest antimycin concentration (0.2 gL^{-1}) compared to the algae cultivation with H_2O_2 addition. At the lowest antimycin concentration biomass production was reduced by 73.4 % compared to the control cultivation and a similar phenomenon was also detected for two other bioprocess efficiency parameters. The complete suppression of *E. gracilis* growth was detected at antimycin concentration higher than 0.3 g L^{-1} where negligible algae growth was observed. On the basis of these results it was decided that antimycin would not be used in our further research due to its significant inhibition of the *E. gracilis* respiratory chain. Furthermore, the application of antimycin on an industrial scale could enlarge antibiotic resistance in the environment (Franklin and Snow, 1975).

Table 2. Bioprocess efficiency parameters obtained during heterotrophic cultivation of *E. gracilis* in Erlenmeyer flask on modified Hutner medium with antimycin addition.

Antimycin concentration (g L^{-1})	X_T (g L^{-1})	$Y_{X/S}$ (g g^{-1})	*Pr* (g L^{-1} h^{-1})
0	12.32 ± 0.58	0.645	0.103
0.2	3.28 ± 0.27	0.196	0.027
0.3	2.71 ± 0.21	0.166	0.023
0.35	0.80 ± 0.12	0.050	0.007
0.5	0.14 ± 0.04	0.009	0.001
0.6	0.06 ± 0.01	0.003	0.0005
0.9	0	0	0
1.2	0	0	0

During *E. gracilis* co-cultivation with representative contaminants (*S. cerevisiae* and *B. subtilis*) on the modified Hutner medium their growth behavior was studied by H_2O_2 addition (5 mg L^{-1}). In order to have a clear impact of H_2O_2 on *S. cerevisiae* and *B. subtilis* their separate co-cultivations with *E. gracilis* were performed. As can be seen in Figure 1, the cell number of *E. gracilis* (log N1) and dry biomass concentration were increased during cultivation as a consequence of glucose consumption. In this experiment, the cell number concentration of *S. cerevisiae* (log N2) was very slightly reduced as a consequence of H_2O_2 concentration decrease during the bioprocess because of its degradation. However, it has to be pointed out that dry biomass concentration represents the biomass of *E. gracilis* and *S. cerevisiae* due to the fact that it was not possible to separate these two microorganisms.

Therefore, it was not possible to determine the correct bioprocess efficiency parameters for *E. gracilis* co-cultivations with contaminants. On the basis of these results it is clear that the H_2O_2 concentration of 5 mg L^{-1} is sufficient to suppress the yeast growth during *E. gracilis* cultivation.
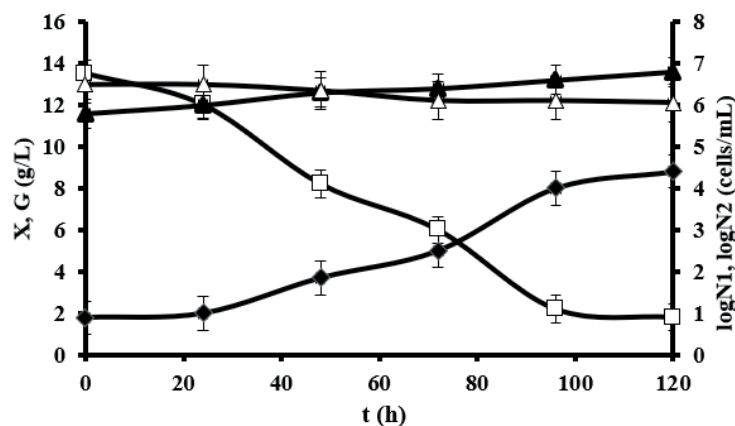
During cultivation of *E. gracilis* and *B. subtilis* on the modified Hutner medium (Figure 2) with 5 mg L^{-1} of H_2O_2 it was observed that the growth of *B. subtilis* cells was almost completely suppressed. Furthermore, the cell number concentration of *B. subtilis* was very slightly reduced due to the H_2O_2 activity although its concentration was also reduced during the bioprocess. The growth of *E. gracilis* was a consequence of glucose consumption and corresponds with the increase of dry biomass concentration. As mentioned above, the dry biomass concentration contains the dry biomass of *E. gracilis* and *B. subtilis*. Due to the fact that the bacterial cell number concentration was relatively low its portion in the total dry

biomass concentration was also low and can be neglected.

In order to verify the results obtained from Erlenmeyer flasks the cultivation of *E. gracilis* in co-culture with *S. cerevisiae* and *B. subtilis* was performed in the stirred tank bioreactor on the modified Hutner medium with 5 mg L^{-1} of H_2O_2. As can be seen in Figure 3, the cell numbers concentration of *S. cerevisiae* and *B. subtilis* were very slightly reduced during the whole heterotrophic cultivation of *E. gracilis* because of H_2O_2 activity. In the stirred tank bioreactor similar trends of cell number, dry biomass and glucose concentration were observed as during *E. gracilis* flask cultivation. On the basis of these results it is clear that the cultivation of *E. gracilis* in the stirred tank bioreactor confirms the results of algae cultivation in Erlenmeyer flasks.

**Heterotrophic cultivation of *E. gracilis* on complex medium in different conditions**

In this study, simple complex medium (20 g L^{-1} glucose and 60 g L^{-1} CSL) was used for *E. gracilis* cultivation in heterotrophic conditions in order to define its potential for industrial application as well as to define the capacity of H_2O_2 to prevent contaminant activities in these conditions. For *E. gracilis* cultivation on the complex medium the H_2O_2 concentration of 5 mg L^{-1} was selected due to the fact that this concentration was sufficient to prevent the growth of contaminants during algae cultivation on modified Hutner medium. The flask cultivation of *E. gracilis* on the complex medium was performed in different experimental conditions (Table 3; A). The control cultivation of *E. gracilis* was done without H_2O_2 and contaminants addition and the obtained bioprocess efficiency parameters were in the range of literature data (Šantek *et al.*, 2009; Ivušić and Šantek, 2015).

Figure 1. Changes of dry biomass (X; ♦) and glucose (G; □) concentration and cell number concentration of *E. gracilis* (log N1; ▲) and *S. cerevisiae* (log N2; Δ) during cultivation on modified Hutner medium with 5 mg L^{-1} H_2O_2 in Erlenmeyer flasks.
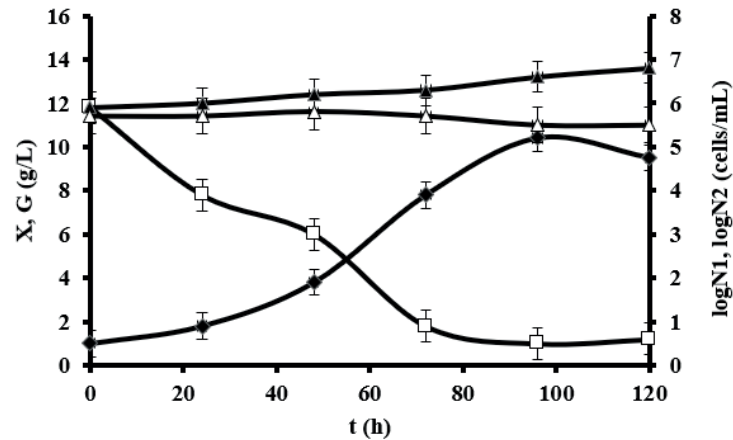
Figure 2. Changes of dry biomass (X; ♦) and glucose (G; □) concentration and cell number concentration of *E. gracilis* (log N1; ▲) and *B. subtilis* (log N2; △) during cultivation on modified Hutner medium with 5 mg L^{-1} H$_2$O$_2$ in Erlenmeyer flasks.
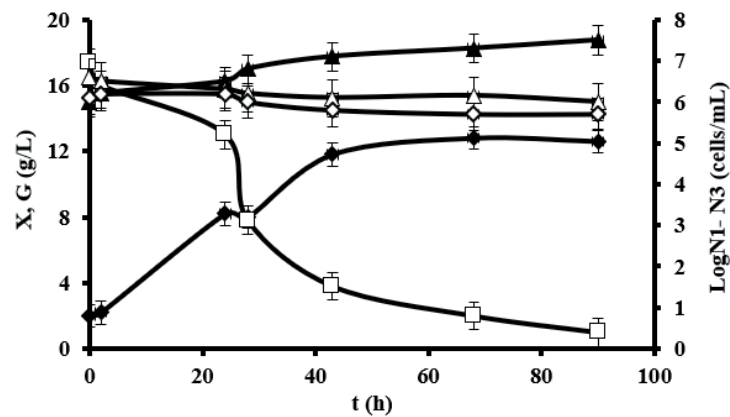
Figure 3: Changes of dry biomass (X; ♦) and glucose (G; □) concentration and cell number concentration of *E. gracilis* (log N1; ▲), *S. cerevisiae* (log N2; △) and *B. subtilis* (log N3; ◊) during cultivation on modified Hutner medium with 5 mg L^{-1}H$_2$O$_2$ in the stirred tank bioreactor.

Table 3. Bioprocess efficiency parameters obtained during heterotrophic cultivation of *E. gracilis* in Erlenmeyer flask (A) and stirred tank bioreactor (B) on complex medium (CM) in different conditions.

Cultivation system: A) *Erlenmeyer flasks*	X_T (g L^{-1})	$Y_{X/S}$ (g g^{-1})	Pr (g L^{-1} h^{-1})
CM	10.80 ± 0.54	0.600	0.090
CM + 5 mg L^{-1} H$_2$O$_2$	7.94 ± 0.33	0.490	0.066
CM + 5 mg L^{-1} H$_2$O$_2$ + *S. cerevisiae* + *B. subtilis*	8.01 ± 0.39	0.410	0.066
B) *Stirred tank bioreactor*			
CM	10.74 ± 0.58	0.594	0.086
CM + *S. cerevisiae* + 5 mg L^{-1} H$_2$O$_2$	7.40 ± 0.39	0.416	0.062
CM + *B. subtilis* + 5 mg L^{-1} H$_2$O$_2$	6.95 ± 0.31	0.405	0.059

In our further research, *E. gracilis* was cultivated on complex medium in the presence of 5 mgL^{-1} H$_2$O$_2$ and consequently a reduction of bioprocess efficiency parameters was observed. Bioprocess efficiency parameters were diminished compared to the control cultivation as follows: X_T for 26.5 %, $Y_{X/S}$ for 18.3% and Pr for 26.7 %.

However, this reduction of bioprocess efficiency parameters was lower compared to the algae cultivation on the modified Hutner medium. This phenomenon can be explained as a consequence of higher capacity of complex medium (considerably higher content of compounds that have higher potential to prevent oxidation than in the modified Hunter

medium) to neutralize the nascent oxygen activity. During *E. gracilis* flask cultivation on complex medium in co-culture with both contaminants (*S. cerevisiae* and *B. subtilis*) and H_2O_2 addition, similar values of bioprocess efficiency parameters were obtained as in previous experiments. It has to be pointed out that these values are only estimates due to the fact that it was not possible to separate algae and contaminants during dry biomass determination. As can be seen in Figure 4, the cell numbers of *S. cerevisiae* and *B. subtilis* were very slightly reduced, which clearly indicates that H_2O_2 can successfully supress the growth of both microorganisms. Similar growth suppression of both contaminants was also observed during *E. gracilis* cultivation on the modified Hutner medium.

In our further research, *E. gracilis* was cultivated in the stirred tank bioreactor in order to verify results obtained during algae flask cultivation on complex medium. As can be seen in Table 3 (B), control cultivation of *E. gracilis* was done in the stirred tank bioreactor without H_2O_2 addition. In these conditions, the range of bioprocess efficiency parameters was similar to the algae flasks cultivation on

complex medium. On the basis of this result it is obvious that complex medium (consisting of 20 g L^{-1} glucose and 60 g L^{-1} CSL) can be successfully used for *E. gracilis* cultivation instead of chemically defined Hutner medium. In order to define the impact of H_2O_2 on the growth of contaminants, separate co-culture with *E. gracilis* were done in the stirred tank bioreactor on complex medium. As can be seen in Table 3 (B), the similar range of bioprocess efficiency parameters was observed in these cultivations as during algae flasks cultivations on complex medium. The *E. gracilis* co-culture with *B. subtilis* on complex medium was chosen (Figure 5) as an example of algae cultivation in the stirred tank bioreactor.

As can be seen in Figure 5, similar bioprocess behavior was observed as during *E. gracilis* flask cultivations on complex medium (Figure 4) and modified Hutner medium in the stirred tank bioreactor (Figure 3), respectively. On the basis of these results it is clear that *E. gracilis* flask cultivations on the complex medium are verified during algae cultivations in the stirred tank bioreactor.
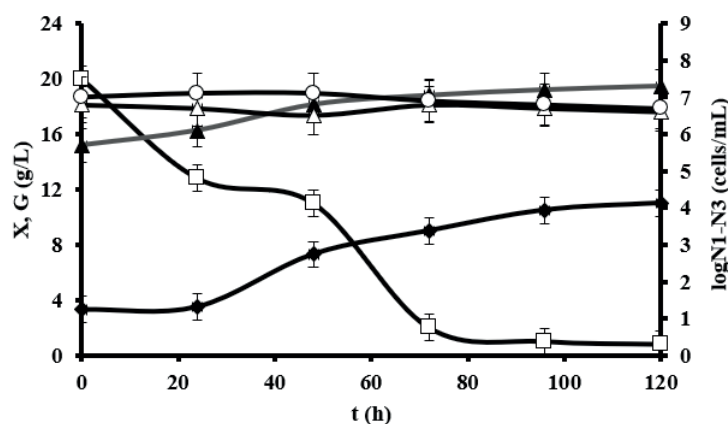
Figure 4. Changes of dry biomass (X; ♦) and glucose (G; □) concentration and cell number concentration of *E. gracilis* (log N1; ▲), *S. cerevisiae* (log N2; Δ) and *B. subtilis* (log N3; ○) during cultivation on complex medium with 5 mg L^{-1} H_2O_2 in Erlenmeyer flasks.
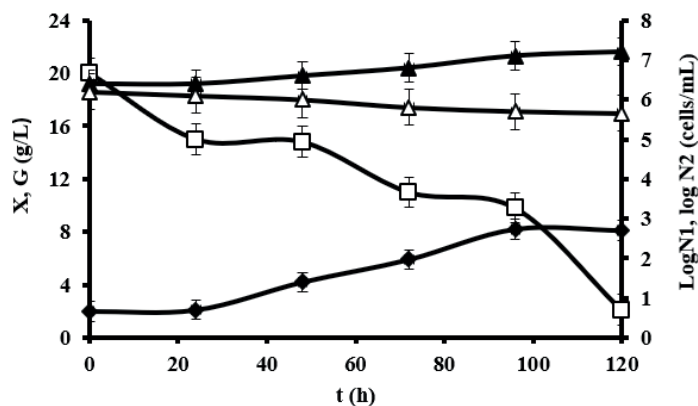
Figure 5. Changes of dry biomass (X; ♦) and glucose (G; □) concentration and cell number concentration of *E. gracilis* (log N1; ▲) and *B. subtilis* (log N2; Δ) during cultivation on complex medium with 5 mg L^{-1} H_2O_2 in the stirred tank bioreactor.

CONCLUSIONS

On the basis of obtained results it can be concluded that complex medium (consisting of 20 g L^{-1} glucose and 60 g L^{-1} CSL) can be successfully used as a substitute for chemically defined Hutner medium. This observation is confirmed by the similar values of bioprocess efficiency parameters for *E. gracilis* cultivation on both media. Antimycin as a chemical agent significantly suppresses the growth of *E. gracilis* and contaminants already at relatively low concentrations (below 0.2 gL^{-1}). Therefore, it is not recommendable to use antimycin as a chemical suppressor of contaminants during *E. gracilis* cultivation. H_2O_2 as a chemical suppressor has lower impact on *E. gracilis* and contaminant growth than antimycin. At H_2O_2 concentrations up to 5 mg L^{-1}, *E. gracilis* has the capability to grow, but the growth of representative contaminants was almost completely stopped. However, further increase of H_2O_2 concentration was related to the complete suppression of *E. gracilis* growth, especially for H_2O_2 concentration higher than 9 mg L^{-1}. On the basis of these results it is obvious that H_2O_2 has great potential for application as a chemical suppressor of contaminants during *E. gracilis* cultivation (or medium preparation) on an industrial scale, but it is necessary to determine the optimal H_2O_2 concentration for particular bioprocess conditions.

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