Lignocellulosic Sugars as Co-substrates for Single Cell Oil and γ-Linolenic Acid Production by *Thamnidium elegans*

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ABSTRACT

Thamnidium elegans CCF-1465 was tested for its ability to produce lipid containing γ-linolenic acid (GLA), during its cultivation on mixtures of glucose and xylose, abundant sugars of lignocellulosic biomass. Experiments were conducted in both shake-flask and bioreactor trials, while in any case, nitrogen limited media were employed in order to direct the microbial metabolism towards the synthesis of intra-cellular lipid. All cultures were performed at media presenting initial total sugar concentration of 100 g/L. Analysis of intra-cellular lipid showed that the highest GLA content was observed in early stationary growth phase. Additionally, the composition of fatty acids of the neutral fraction resembled with that of total lipids, while the phospholipids, in most cases, were the most unsaturated fraction compared to the other lipid fractions of T. elegans.

Keywords: γ-linolenic acid, mixed sugars, single cell oil, *Thamnidium elegans*

1 INTRODUCTION

Lignocelluloses as agricultural, industrial and forest residuals account for the majority of the total biomass present worldwide [1]. Their abudance and renewable nature renders them as potential substrates for bioconversions, since they can serve as cheap feedstock of monosaccharides in a variety of microbial fermentations. Glucose and xylose are the principal sugars of lignocellulosic biomass, being produced *via* dilute acid hydrolysis treatment process [2]. Therefore, the capability of microorganisms to utilize both C-5 and C-6 sugars as carbon source is particularly desirable in order to increase the efficiency of high added value metabolic compounds deriving from lignocellulosic materials [3].

The production of microbial lipids, known as single cell oils (SCO) [4,5], currently attracts much interest as an alternative starting material for biodiesel production (2nd generation biodiesel), since their fatty acid composition resembles to that of vegetable oils [3, 5-7]. The principal microorganisms that are regarded as oleaginous (can accumulate more than 20% of lipids in their dried cell mass) belong to a relatively limited number of yeasts and a relatively higher number of molds [8]. Especially, various oleaginous Zygomycetes have the potential to produce high quality fats and lipids, rich in polyunsaturated fatty acids (PUFAs). Among them, γ-linolenic acid (GLA) receives much attention due to its nutritional importance and its selective pharmaceutical and anticancer properties [9-11]. However, the need of low-cost raw materials that can be utilized as carbon sources by oleaginous microorganisms still represents a challenging aspect.

Aim of the present work was to study the biochemical behaviour and ability of a Zygomycetes strain, namely *Thamnidium elegans*, to accumulate lipid rich in γ-linolenic acid (GLA) during its cultivation on glucose and xylose, either as sole carbon source or in mixture. Intracellular lipids and lipid fractions were monitored for their fatty acid composition and GLA content. Insights concerning kinetic evolutions and production of value-added metabolic compounds of the microorganism are also provided and discussed.

2 MATERIALS AND METHODS

2.1 Microorganisms and culture conditions

Thamnidium elegans CCF-1465, obtained from the collection of the Laboratory of Food Microbiology and Biotechnology of Agricultural University of Athens, was used in the present study. The strain was maintained on potato dextrose agar (PDA, Plasmatec, Dorset, UK) at 4±1 °C and sub-cultivated every month in order to maintain its viability. The salt composition of the employed growth medium was obatined by Chatzifragkou et al. (2010). Yeast extract and $(NH4)_2SO_4$ were used as nitrogen sources at concentrations of 4 and 2 g/L respectively. The pH of the medium was 6.0±0.1 after autoclaving. Glucose and xylose (analytical grade, Sigma) were used either as the sole carbon source or in mixtures, at total concentration of at 100 g/L.

Experiments were conducted in 250-mL conical flasks, containing 50±1 mL of growth medium, sterilized at 121 °C / 20 min and inoculated with 1 mL of spore suspension (around 105-107 cfu). All cultures were incubated in an orbital shaker (New Brunswick Sc, USA) at an agitation rate of 180 rpm and incubation temperature of 28±1 °C. In all experiments a pH value wa maintaned greater than 5.2, by the addition of appropriate volumes of KOH (5M) into the flasks when needed. Moreover, experimental work was carried out in a 3-L bioreactor (New Brunswick Scientific Co, USA) with a working volume of 1.5-L. Glucose was used as the sole carbon source at initial concentration of

100 g/L, while the nitrogen sources used were yeast extract and ammonium sulfate, in concentrations as reported above. The initial pH of the medium was 6.0±0.1 and maintained at the desirable value by automatic addition of 5M NaOH, through a peristaltic pump. The agitation rate of the bioreactor was 500 to 550 rpm, air supply was maintained at a constant rate of 1.0 vvm and growth temperature at 28 °C.

2.2 Analytical methods

Biomass (X, g/L) was determined by dry matter (90±5 ºC/ 24 h). The determination of glucose, xylose and produced xylitol was conducted by means of H.P.L.C. analysis in a Waters 600 device, as described by Chatzifragkou et al. [12]. Dissolved oxygen (D.O.) concentration in shake flasks was measured with the aid of a selective electrode as described in Chatzifragkou et al. [11], whereas in the bioreactor, determination was performed on-line by using a selective electrode (Mettler Toledo, Switzerland). Total cellular lipid (L, g/L) was extracted from the dried mycelia with a mixture of chloroform/methanol 2:1 (v/v) and weighted after evaporation of the solvent in a rotary evaporator. Lipids were converted to methyl-esters and analysed with the aid of a G.C. apparatus as in Papanikolaou et al. [13]. Furthermore, in some trials cellular lipids were fractionated into their lipid fractions, according to the method described by Fakas et al. [14].

3 RESULTS AND DISCUSSION

3.1 Growth of *T. elegans* in glucose-xylose based media

Initially, *T. elegans* was cultivated in shake-flasks, with glucose or xylose as the sole carbon source at 100 g/L, in nitrogen limited media in order to direct the microbial metabolism towards the synthesis of intra-cellular lipid [5]. Quantitative data obtained from the kinetics are presented in Table 1. On glucose-based media, the strain presented appreciable cell growth, with maximum biomass production corresponding to 31.9 g/L, after 309 h of fermentation. Intracellular oil was typically accumulated after depletion of nitrogen source from the culture medium. Specifically, remarkable lipid accumulation was carried out by the strain, namely 15.0 g/L, with the corresponding yield $Y_{L/X}$ equal to 47.1 % (w/w), while cellular lipids of *T. elegans* were found to be particularly enriched in GLA (1014.6 mg/L).

On xylose-based media, *T. elegans* was found capable of producing satisfactory amounts of biomass, namely 21.4 g/L, whereas sufficient amounts of cellular lipids were also produced accompanied by a $Y_{L/X}$ value of 41.8 % (w/w).

Only a limited number of studies have recently appeared describing xylose ulitization as carbon source by oleaginous microorganisms [3,15-16]. On the other hand, cultivation of *T. elegans* on xylose favored the production of xylitol, reaching a maximum concentration of 31.3 g/L. The secretion of xylitol can account for the relatively lower final concentrations of produced biomass and cellular lipids by the strain during growth on xylose, as compared to the performance of the microorganism during growth on glucose. According to the international literature, yeast strains and especially those belonging to the genus *Candida*, are considered to be the best xylitol producers [17]. Regarding fungal strains, there is only one report referring to *Petromyces albertensis*, which accumulated 39.8 g/L of xylitol when cultivated for 10 days on 100 g/L xylose-based media [18]. These results are in agreement with the biochemical behavior of *T. elegans* cultivated on 100 g/L xylose, which produced 31.3 g/L of xylitol about 240 h (10 days) after inoculation. Furthermore, after xylose depletion from the medium, the produced xylitol was partially consumed.

Table 1. Kinetic data of *T. elegans* cultivation on glucose, xylose or mixtures as carbon source

Substrate ratios (100 g/L in total)		Time (h)	Biomass (g/L)	Xylitol (g/L)	Oil (g/L)	GLA (mg/L)
Glu	Xyl					
1	0	304	31.9	-	15.0	1014.6
0	1	240[a]	14.9	31.3	5.1	338.9
		384[b]	21.4	22.3	8.9	487.1
1	1	429[c]	27.4	3.4	12.6	936.2
3	1	501[c]	29.5	1.4	10.8	883.5
1	3	264[a]	18.6	10.1	4.0	538.0
		504[b]	27.1	0.0	11.0	925.5

[a]:Representation when maximum xylitol was achieved
[b]:Representation when maximum lipid accumulation was achieved
[c]: Maximum concentrations of cellular lipids and xylitol were simultaneously achieved

As a next step, *T. elegans* was tested for its ability to accumulate oil during its cultivation on glucose-xylose mixtures, under nitrogen limited conditions. Overall, the microorganism grew particularly well in all glucose-xylose mixtures, with maximum biomass production ranging from 27.4 to 29.5 g/L, while in all cases total substrate assimilation occurred. Literature indicates that in some cases, the existence of glucose in the fermentation medium as co-substrate has been reported to partially inhibit xylose consumption, while the catabolism of the latter starts after the preferred sugar (i.e. glucose) is depleted [19-20]. In our case, glucose and xylose were simultaneously consumed, regardless of their initial concentrations. In terms of lipid accumulation, *T. elegans* proved capable of producing significant amounts of oil, accompanied by sufficient $Y_{L/X}$ bioconversion yields of 36.7-45.9% (w/w). As far as GLA production is concerned, the mixture of glucose-xylose 1:1 proved to be the most promising substrate among others tested for the accumulation of the aforementioned fatty acid, reaching the maximum value of 974.9 mg/L about 381 h after inoculation. Furthermore, it should be mentioned that xylitol production took place in all mixtures. However,

the amount of accumulated xylitol remained lower than that achieved on the substrate with xylose as sole carbon source, indicating that xylitol production could be potentially associated with the presence of glucose in the fermentation medium. Worth also mentioning was the fact that during cultivation on glucose-xylose mixtures, all or part of the secreted xylitol was re-consumed by the strain during the fermentation process, in favor of lipid production. As far as the specificity of sugar uptake rates by the fungus is concerned, in the case of glucose or xylose utilization as sole carbon sources, glucose was noticeably faster consumed as compared to xylose. On the contrary, during cultivation of the fungus in equal amounts of glucose-xylose mixtures, the specific uptake rates (r_s''', g/L•h) for both sugars were similar (0.13 and 0.11 g/L•h, for glucose and xylose respectively).

Based on the obtained results *T. elegans* cultivation was performed in a 3-L bioreactor, utilizing glucose as the sole carbon source. the performance of the microorganism was satisfactory, by producing fair amounts of biomass up to 30.1 g/L, with a noticeable lipid content of 10.6 g/L (Figure 1).

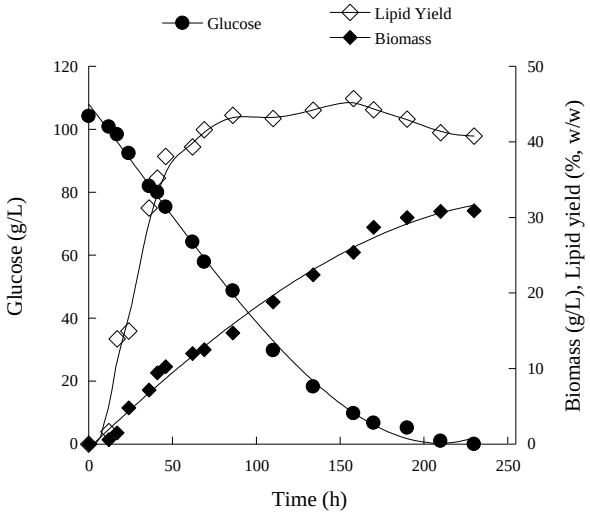

Figure 1. Kinetics of growth, substrate consumption and lipid production yield, during cultivation of *T. elegans* on glucose (S_0=100 g/L) in a bioreactor.

However, the duration of the bioprocess was noticeably shorter in the bioreactor, while the accumulated lipid in the bioreactor was slightly lower than that produced in the shake-flask trial with the same carbon source. Therefore, it can be deduced that the preservation of optimum conditions in the bioreactor resulted in the direction of microbial metabolism firstly towards biomass production and secondly towards intra-cellular lipid accumulation. Furthermore, it should be mentioned that the GLA content in the bioreactor trial was slightly reduced compared with the corresponding shake-flask experiment, due to the relatively lower lipid production of the microorganism obtained in the bioreactor experiment.

3.2 *T. elegans* lipid analysis

Analysis of *T. elegans* lipids was conducted during evolution of fermentation time in all trials, in order to monitor possible alterations of the lipid profile of the microorganism, in terms of fatty acid composition. The predominant fatty acid produced was oleic acid ($^{\Delta 9}$C18:1), followed by palmitic acid (C16:0), linoleic acid ($^{\Delta 9,12}$C18:2) and γ-linolenic acid (GLA,$^{\Delta 6,9,12}$C18:3) in significant quantities. On the contrary, palmitoleic (C16:1) and stearic acid (C18:0) were detected in lower amounts. GLA ($^{\Delta 6,9,12}$C18:3) was found in large contents (up to 18%) in the produced oil, regardless of the substrate employed (Fig. 2). During cultivation of the strain in glucose-xylose mixtures, the percentage of oleic acid was found to be lower, as compared to those obtained during cultivation on either glucose or xylose as the sole carbon source. The opposite trend was observed for linoleic acid.

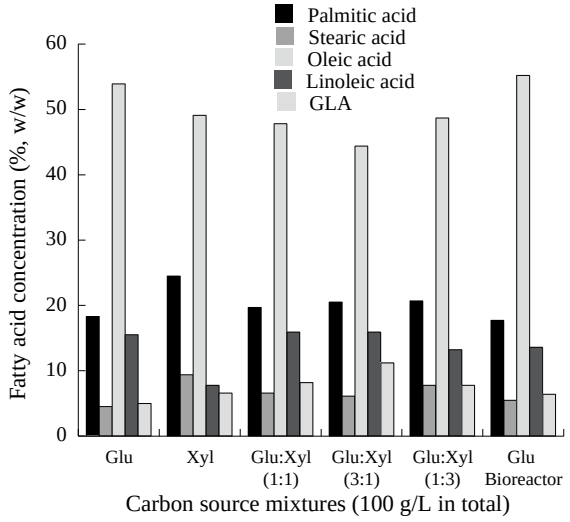

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Figure 2. Fatty acid concentration of accumulated oil in stationary growth phase during cultivation of *T. elegans* on various glucose and xylose mixtures.

Fractionation of *T. elegans* into neutral (N), sphingolipids plus glycolipids (S+G) and phospholipids (P), during stationary growth phase at all experiments with glucose and xylose, either as sole carbon source or as a mixture, revealed that the fraction of neutral lipids (N) was the major constituent of total lipids. As a result, neutral fraction composition was found alike with fatty acid profile of total lipids. The proportion of neutral lipids (N) increased during the late stationary phase (LS), whereas a further increase occurred at stationary phase (S). However, the percentage of sphingolipids plus glycolipids (S+G) and phospholipids (P) were high at the beginning of growth and declined thereafter. These results are in agreement with the general accepted energy storage role of neutral lipids, while phospholipids are considered as structural lipids of cell membranes and for this reason they are synthesized during active growth [10,14]. Also, as indicated by Fakas et al. [14], the decrease of the GLA amount in polar lipids of *Cunninghamella echinulata* ATHUM 4411 was

accompanied by an increase in the N fraction. This phenomenon occurs due to the fact that lipid accumulation constitutes a secondary metabolic process [22] and as a result the produced lipid is stored in neutral fraction as triacylglycerol (TAG) [10,14]. Analysis of intra-cellular lipids into their lipid fractions (N, S+G and P) of *T. elegans* growing in the bioreactor showed that the proportion of neutral lipids (N) was lower compared with the corresponding percentages in shake-flasks experiments. Fatty acid analysis of the lipid fractions of *T. elegans* showed that neutral lipids (N) contained high amounts of oleic acid, while sphingolipids and glycolipids (S+G) had the highest palmitic acid content. Furthermore, neutral fraction fatty acid composition remained almost invariable with age. However, the phospholipids fraction (P) was of great interest as it was particularly enriched in PUFAs and especially in GLA (almost 20% w/w) (Figure 3).

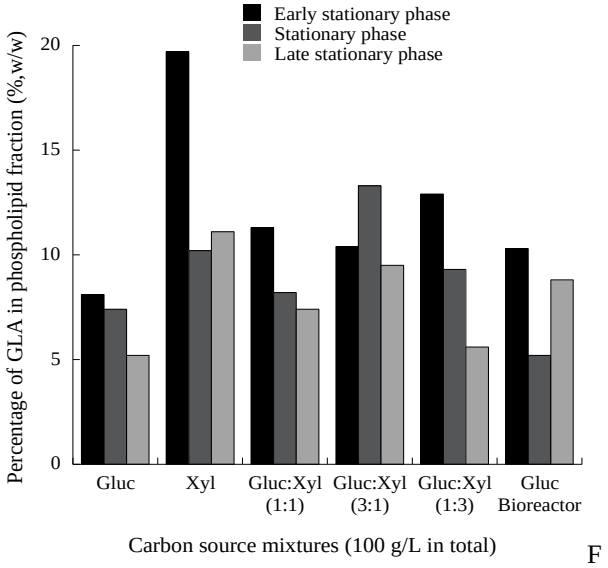

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Figure 3. GLA content of P fractions of *T. elegans* oil during different growth phases on sugar mixtures

As a result, the P fraction was less saturated than S+G and N fractions due to the high proportion of GLA and linoleic acid.

4 CONCLUSIONS

The Zygomycetes strain *T. elegans* proved to be a promising microorganism during its cultivation on glucose and xylose substrates, principal sugars of hydrolyzed lignocellulosic biomass, yielding significant amounts of intracellular oil, rich in GLA. By taking into account that microbial lipid production still represents an expensive process, the successful bio-transformation of sugar-based lignocellulosic residues as substrates represents an attractive alternative.

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