

Original Research Article

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Effects of Fermentation Conditions on the Production of Secondary Metabolites of *Penicillium brevicaula alba*-CC200 and *Aspergillus egypticus*-HT166 Inhibiting Pancreatic α -Amylase

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ABSTRACT

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Carbon and nitrogen sources were selected to create a new modified Chapek-Doks nutrient medium in order to increase the production of secondary metabolites, that inhibit pancreatic α -amylase, by endophytic fungi *Penicillium brevicaula alba*-CC200 and *Aspergillus egypticus*-HT166. The results showed that the highest α -amylase inhibitory activity - 96.8% and 88%, respectively, was observed during the shake flask cultivation of strains at the end of the stationary phase on a medium containing casein supplemented by yeast extract.

Introduction

Microbial fermentation is a complex process and metabolic processes in microorganisms seriously depend on cultural parameters (Scherlach *et al.*, 2009). Variations of fermentation conditions such as medium composition, aeration, temperature, or the shape of the culture vessel, can lead to changes in the metabolic profile of a strain and influence the yields of certain compounds (Yuniati *et al.*, 2018; Amna *et al.*, 2012). Recently, the term “OSMAC” (one strain

many compounds) was proposed to describe the long known effects of the fermentation parameters on secondary metabolites biosynthesis by any given microorganism, from increasing in the number of produced compounds to the accumulation of hitherto unknown natural products (Grond *et al.*, 2002; Bode *et al.*, 2000; Rateb *et al.*, 2011). It was shown that different cultural conditions lead to the discovery of new natural substances. This is especially true for endophytic microorganisms with their wide spectrum of interactions with the host plant

and other endophytes. For the last two decades endophytes have been recognized as important sources of a variety of new biologically active secondary metabolites potentially useful for human medicine, with antimicrobial, anticancer, antidiabetic and other activities (Ruzieva *et al.*, 2017; Picot *et al.*, 2014) and could serve for obtaining of novel biotechnologically valuable natural products (Bode *et al.*, 2000; Yuniati *et al.*, 2018). Even small variations under axenic cultivation can affect the composition of produced secondary metabolites (Kusari *et al.*, 2012). For example, at cultivation of endophyte *Paraphaeosphaeri aquadrisepata* replacing in medium tap water to distilled there was observed induction of the production of six new secondary metabolites (Prajjal *et al.*, 2019). Upon transition from solid to liquid medium endophyte *Chaetomium chiversii* begins to produce chaetochromin A as the major metabolite instead of radicicol (Paranagama *et al.*, 2007).

Thus, the selection of cultivation conditions is extremely important for obtaining the desired products in significant quantities and activity. Elucidating the optimal set of parameters will enable the exploitation of endophytes biosynthetic potential to achieve sustained production of a desired secondary metabolite (Kusari *et al.*, 2012).

In our previous investigations from *Celosia cristata* and *Helianthus tuberosus* there were isolated endophytic fungi producing metabolites with α -amylase inhibiting properties. So, inhibitory activity of ethylacetate extracts of *Penicillium brevicaula* *alba*-CC200 and *Aspergillus egypticus*-HT166 was about 80% and 60%, respectively (Ruzieva *et al.*, 2017; Gulyamova *et al.*, 2018) but fungal biomass and secondary metabolites yields at submerged cultivation was not high enough.

In this regard, the aim of this study was to select the cultivation conditions, in particular, the type of fermentation, carbon and nitrogen sources allowing to increase the yield of secondary metabolites and their inhibitory activity.

Materials and Methods

Penicillium brevicaula alba-CC200 and *Aspergillus egypticus*-HT166 were grown on Chapek-Dox medium as basal of the following composition (g/l): NaNO₃ - 2, MgSO₄ - 0.5, KCl - 0.5, KH₂PO₄ - 1.0, FeSO₄ - 10 mg, pH 6.0-6.6. Sucrose (20 g/l, 40 g/l, 60 g/l 80 g/l medium), glucose, lactose and maltose (20 g/l medium) were used to select a carbon source. To study the suitable nitrogen source NaNO₃, (NH₄)₂SO₄ (2g/l), casein and peptone, casein + yeast extract and peptone + yeast extract much as 10g/l in the basal medium were used.

Submerged fermentation was carried out on rotary shaker at 180 rpm and at static condition at 28°C for 7 days. The biomass of cultures was separated by centrifugation at 6000 rpm, weighed and stored until use at -40°C. Secondary metabolites were extracted from 5 g of crude homogenized biomass by 25 ml of ethyl acetate for 24 hours at room temperature on a rotary shaker. The mixture was filtered through paper (Whatman No.1) and Na₂SO₄ (40 µg/ml) was added to the filtrate. The extracts were evaporated to dryness on a vacuum evaporator and 1 ml of dimethyl sulfoxide was added. The resulting extracts were stored at 4°C until use. Fungal biomass and total metabolite production were calculated as dry weight. There were conducted 3 replications.

Inhibition of α -amylase activity of the extracts was determined according to Picot *et al.*, (Picot *et al.*, 2014). A starch solution (1 g/10 ml of water) was boiled for 2 minutes, the

volume was adjusted to 100 ml with distilled water and used as a substrate for 2-3 days. To 2 ml of the substrate there was added 100 µl of porcine pancreatic α-amylase (13 units/ml in 0.1 M Na-phosphate buffer), 2 ml of phosphate buffer pH 7.2, 100 µl of endophyte extract and incubated for 10 minutes at 37°C. No extract was added to the control samples. After incubation, the reaction was stopped by the addition of 10 ml iodine reagent and the absorbance was measured at a wavelength of 630 nm. The iodine reagent contains 0.5 g of crystalline iodine, 5 g of potassium iodide, 250 ml of water. To obtain a working solution, 2 ml of this reagent was adjusted to 100 ml with 0.1 M HCL.

Inhibitory activity was expressed by the formula: $(A_0 - A_t) / A_0 \times 100\%$, where A_0 is the absorption of the control sample, and A_t is the absorption of the experimental sample, respectively.

Acarbose (Glucobay, Bayer Pharma AG, Germany) was used as a standard inhibitor. Residual sucrose in cultivation medium was determined within 7 days according to Dubois *et al.*, (Dubois *et al.*, 1956).

Results and Discussion

In our previous investigations in order to select the growth medium for *P. brevicaula alba* CC-200 and *A. egypticus*-HT166 providing high level of inhibitory activity of metabolites, Czapek's Dox broth was selected as basal but production of biomass and total metabolites was not enough (Ruzieva *et al.*, 2017). To increase the yield of mycelium mass and metabolites containing the active inhibitor compound the influence of modifying of fermentation conditions, carbon and nitrogen sources was studied. To select carbon sources sucrose, glucose, lactose and maltose were used. As nitrogen sources sodium nitrate, ammonium sulfate, yeast

extract, peptone, and casein were added to the basal media. Fermentation was carried out under still and shake flask cultivation.

It was observed that at any carbon source used under shake flask cultivation culture lead to more high yields of mycelium mass and inhibitory activity in compare with still culture of *P. brevicaula alba*-CC200 as well as *A. egypticus*-HT166 (Table 1). At the same time, the use of sucrose for *P. brevicaula alba* CC-200 as carbon source was accompanied by the highest level of biomass accumulation, total metabolites and their inhibitory activity.

The maximum biomass production (13,7 g/l) was observed in *P. brevicaula*-CC200 in shaking cultivation using sucrose, while at static cultivation on the same media it was 4,2 g/l. As can be seen from obtained results, significant difference was observed also in the weight of total metabolites dissolved in ethyl acetate, 96 mg/g of biomass harvests in shaking condition in compare with 14 mg/g in static cultivation of *P. brevicaula*-CC200 on media with sucrose. At using of glucose, lactose and maltose inhibitory activity of total metabolites was much lower in compare with sucrose.

The level of *A. egypticus*-HT166 biomass production in shaking cultivation was significantly higher in compare with static cultivation, while the effect of carbon sources on the weight of total metabolites was not as obvious as for *P. brevicaula*-CC200. The maximum of α-amylase inhibitory activity of *P. brevicaula alba*-CC200 and *A. egypticus*-HT166 extracts on sucrose was 81% and 76% compared to 72% and 58% on glucose, 63% and 63% at lactose, and 66% and 61% when using maltose, respectively. It should be noted that at screening of carbon sources for endophyte *Athelia rolfsii* biomass production glucose was determined as most suitable for optimal growth (Yuen Yuniati *et al.*, 2018).

Since sucrose was determined the most suitable carbon source, its optimal concentration was selected via fermentation in the presence of various initial sucrose concentrations (2%, 4%, 6% and 8%) within 11 days (Figure 1 a,b).

It was observed that the growth dynamics of both studied strains within 11 days not significantly differ at various sucrose concentrations. As can be seen in Figures 1 and 2, in general the biomass growth as well as total metabolites accumulation directly

depends on the concentration of sucrose. The highest amount of biomass of *P. brevicaula alba*-CC200 (33,6 g/l) was observed at 8% of sucrose and lowest (23,1g/l) at 2% of sucrose on day 9 (Figure 1,a), while growth of *A. egypticus*-HT166 in compare with *P. brevicaula alba*-CC200 is 4-fold lower with highest weight of biomass 8,2 g/l on 7th day of growth on use of 6% of sucrose (Fig.1,b). It should be noted that biomass accumulation occurred when the sucrose in cultivation broth is almost completely depleted.

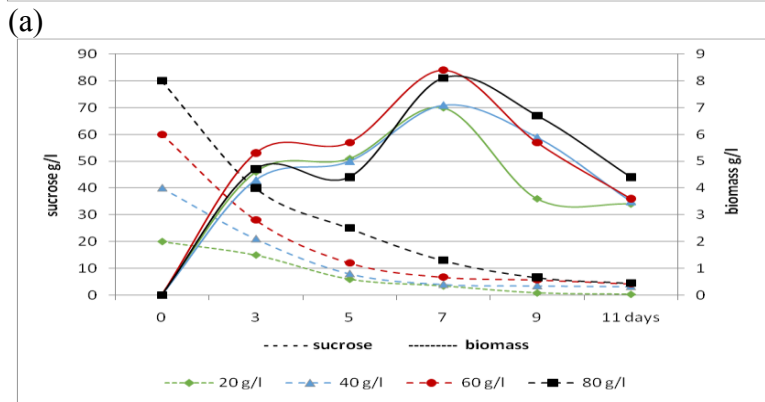
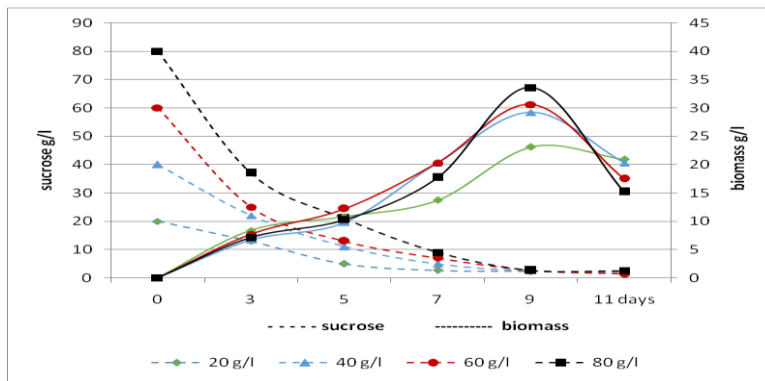
Table.1 The influence of cultivation conditions and carbon sources on *P.brevicaulealba*-CC 200 and *A.egypticus*- HT166 biomass, metabolites yield and α -amylase inhibitory activity

<i>P. brevicaula alba - CC200</i>							
Staticcultivation				Shaking cultivation			
S.No	Carbon Sources	Biomass (g)	Extract (mg/g)	Inhibition of α -amylase (%)	Biomass (g)	Extract (mg/g)	Inhibition of α -amylase (%)
1	sucrose	4,2	14	56	13,7	96	81
2	glucose	5,9	13	40	7,6	46	72
3	lactose	5,1	12,2	21	7,4	45,9	63
4	maltose	5,7	8,5	40	8,2	40	66
<i>A. egypticus -HT166</i>							
Staticcultivation				Shaking cultivation			
S.No	Carbon Sources	Biomass (g)	Extract (mg/g)	inhibition of α -amylase (%)	Biomass (g)	Extract (mg/g)	Inhibition of α -amylase (%)
1	sucrose	3,7	10,0	35	7,1	11,9	76
2	glucose	4,45	9,0	48	7,88	6,4	58
3	lactose	6,17	8,0	36	7,2	8,9	63
4	maltose	3,7	10,0	35	10,5	9,9	61

Table.2 The influence of various nitrogen sources on the accumulation of biomass, metabolites yield and production of α -amylase inhibitors of *P. brevicaula alba*– CC200 and *A. egypticus*– HT166

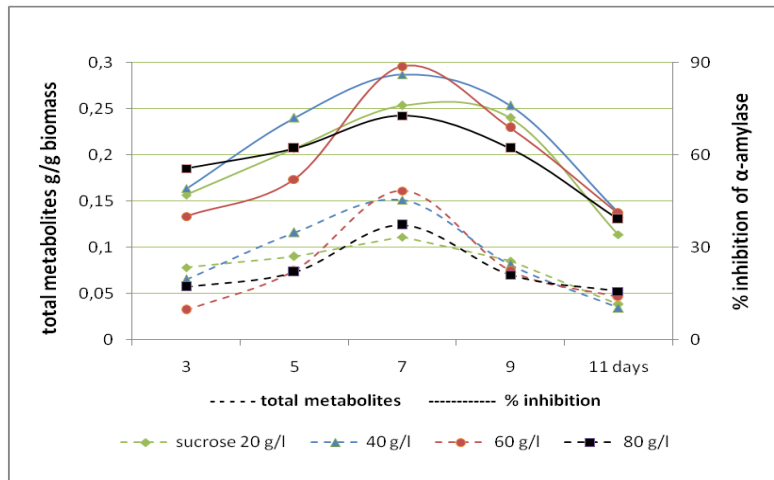
<i>P. brevicaula alba</i> – CC200					<i>A. egypticus</i> –HT166S		
S.No	Nitrogen sources	Biomass (g)	Extract (mg/g)	inhibition of α -amylase (%)	Biomass (g)	Extract (mg/g)	Inhibition of α -amylase (%)
1	NaNO ₃	30,6	161	88,7	8,4	34	85
2	(NH ₄) ₂ SO ₄	14,2	41	51,8	2,9	11	31
3	casein	16,1	190	92,3	19,8	75	87
4	peptone	12,3	226	82,6	9,6	120	84
5	NaNO ₃ + yeast extract	14,2	41	48,6	10,3	46	23,4
6	casein + yeast extract	15,8	147	96,8	10,1	68	88
7	peptone + yeast extract	13,7	156	67	11,5	55	41

Fig.1 The effect of initial sucrose concentrations on *P.brevicaulealba*-CC 200 (a) and *A.egypticus*-HT166 (b) growth dynamics

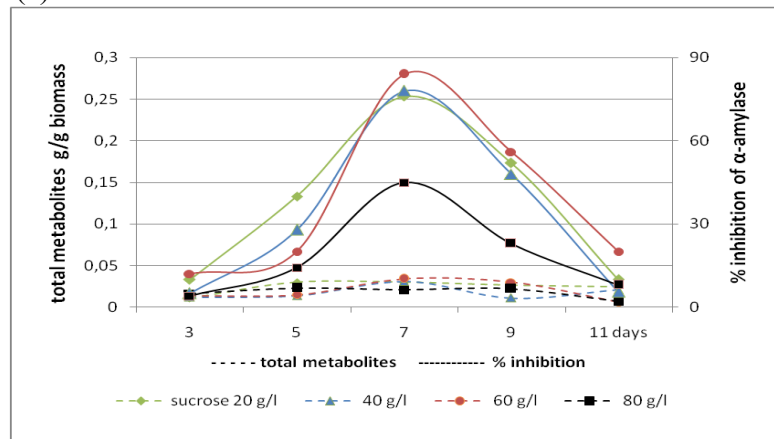


(b)

Fig.2. The effect of initial sucrose concentrations on inhibitory activity of total metabolites of *P.brevicaule alba* CC-200 (a) and *A.egypticus*-HT166 (b) during growth dynamics



(a)



(b)

It was found that at variation of sucrose within the range of 2%-8% highest level of metabolites accumulation and inhibitory activity of extracts was observed at 6% sucrose on day 7 for both strains (Figure 2 a, b), and the lowest activity and metabolites level was observed at 8% sucrose for *A.egypticus*- HT166 (Figure 2 b). It should be noted that within the range of 2 - 6% sucrose, there is a correlative dependence of inhibitory activity of metabolites increase with increasing its accumulation, while at 8% concentration the weight of extracts and their activity decreases. Thus, the concentration of carbohydrate substrate plays an important role in optimizing the fermentation process.

Given the obtained data, selection of nitrogen sources, fermentation of *P. brevicaula alba*-CC200 and *A. egypticus*-HT166 was carried out in presence of 1% NaNO₃, (NH₄)₂SO₄, casein, peptone, and casein and peptone supplemented by yeast extract.

Fermentation results with indicated nitrogen sources showed that NaNO₃ was the best nitrogen source for the production of *P. brevicaula alba*-CC200 biomass (30,6 g/l). Maximum of total metabolites dissolved in ethyl acetate, was from media supplemented with peptone (226 mg/g biomass) followed by casein (190 mg/g) (Table 2). For *A. egypticus*-HT166 casein provided maximum biomass

(19,8 g/l), while peptone was the best for accumulation of total metabolites (120 mg/g biomass).

The highest inhibitory activity is observed in *P.brevicaule*-CC200 and *A. egypticus*-HT166 extracts on a medium containing casein (92,3% and 87%, respectively) or casein supplemented by yeast extract (96,8% and 88%, respectively).

Thus, obtained data show that fermentation *P.brevicaule*-CC200 and *A. egypticus*-HT166 in media containing sucrose as a source of carbon and organic sources of nitrogen, in particular casein and peptone, are optimal, allowing significantly increase the level of production of metabolites with high inhibitory activity.

It should be noted that despite the difficulties of growing endophytes known from the literature (Kusari *et al.*, 2011), *P. brevicaule*-CC200 isolated from *Celosia cristata* and *A. egypticus*-HT166 from *Helianthus tuberosus* in general utilize various nutrient sources and preserving the inhibitory properties of their metabolites.

Thus, as a result of the studies, it was found that during the cultivation of endophytic micromycetes by optimizing nutrient media using carbon and nitrogen sources, a significant increase in the synthesis of secondary metabolites, α -amylase inhibitors is possible, which opens up new prospects for obtaining natural and effective hypoglycemic drugs.

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