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INFLUENCE OF THE FERMENTATION MEDIUM COMPOSITION ON LIPASE PRODUCTION BY *RHIZOPUS ARRHIZUS*

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Abstract

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The influence of fermentation medium composition on lipase production by *Rhizopus arrhizus* was investigated. It was found that the strain did not require a lipid nature component as an inducer. The most appropriate carbon source was corn starch in 10.0 g.dm^{-3} concentration. Nitrogen sources had also crucial role in biosynthesis of lipase. The highest enzyme activity was measured when 5.0 g.dm^{-3} casein trypsic peptone (tryptone) as organic nitrogen and 0.5 g.dm^{-3} ammonium oxalate as inorganic nitrogen sources were added in the fermentation medium. Using of 3.0 g.dm^{-3} $NH_4H_2PO_4$ as a combined source of nitrogen and phosphorus in the medium for submerged fermentation and addition of 0.5 g.dm^{-3} $MgSO_4$ and 0.5 g.dm^{-3} KCl significantly increased the lipolytical activity.

Key words: lipase, *Rhizopus arrhizus*, medium, submerged fermentation

Introduction

Lipases (triacylglycerolhydrolase, E.C. 3.1.1.3) are enzymes which catalyze the hydrolysis of long chain triglycerides into free fatty acids and glycerol at the water-lipid interface and can catalyze the reverse reaction in non-aqueous medium. Those enzymes attract a great attention because of their biotechnological potential. They have a large market share because of their wide application in different industries. That leads to the necessity of developing new production schemes (Hasan et al., 2006).

Microbial lipases are most widely used because of their easily production, higher temperature and pH stability, wide substrate specificity and organic solvents resistance. There is a great interest to the fungal *Rhizopus* lipases because of their high 1,3-specificity. (Haq et al., 2002).

Lipase production is highly affected by the media composition and cultivation factors. Defining of the optimal medium components, such as lipid inducers, carbon source, organic and inorganic nitrogen sources, phosphorus source, also the presence of additional salts, has a crucial role in increasing lipase biosynthesis (Ghosh et al., 1996).

Carbon source is one of the most important components for lipase production. There are two strategies in lipase production in submerged fermentation. According to the first strategy, two carbon sources in the medium were used – carbohydrates and additional lipid inducers (Bisht et al., 2012). The second one was based on carbohydrates only (Chander et al., 1980).

Most of the microbial producers are saprophytes and the organic nitrogen source has crucial role in lipase production. Most wide used products are yeast extract (Bisht et al., 2012) and peptone (Chander, 1980).

The aim of this study is improving lipase production from *Rhizopus arrhizus* in submerged fermentation by modification of medium components.

Materials and Methods

Microorganism. Maintenance and storage

The studied *Rhizopus arrhizus* strain used in this study was provided by Biovet® Peshtera. It was grown in the following medium, g.dm^{-3}: malt extract, 10.0; yeast extract,

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4.0; glucose, 4.0; agar-agar 20.0. pH was adjusted to 7.0. The strain was cultivated at 28°C for 14 days and stored at 4°C.

Rhodamine B-olive oil plate assay for lipase activity detection

Rhodamine B-olive oil plate assay proposed by Kouker et al. (1987) was used. Lipase activity was identified as an orange fluorescent halo under UV light at 350 nm around active colonies after 48 h of incubation at 28°C.

Vegetative inoculum preparation

0.5 cm^3 spore suspension was added to 100 cm^3 sterilized (at 121°C for 30 min) medium with pH 7.0 with the following composition ($g.dm^{-3}$): glucose 30.0, peptone 20.0, $MgSO_4.7H_2O$ 0.5, KH_2PO_4 1.0, $NaNO_3$ 1.0, $CaCO_3$, 1.0. The strain was cultivated on a rotary shaker (180 min^{-1}) at 28°C for 24 h.

Submerged cultivation

Submerged cultivation was carried out in 500 cm^3 flasks containing 100 cm^3 medium with defined composition depending on the aim of the study. pH of the medium was adjusted to 7.0, then the medium was sterilized at 121°C for 30 min. 5.0 cm^3 vegetative inoculum was used for inoculating of each flask and cultivation was carried out at 28°C for 64 h at a rotary shaker (180 min^{-1}). The initial medium composition was ($g.dm^{-3}$): lipid inducer 20.0, glucose 10.0, peptone 5.0, KH_2PO_4 3.0, $NaNO_3$ 0.5, $MgSO_4$ 0.5. The influence of lipid inducers, carbon sources, both organic and inorganic nitrogen sources, phosphorus sources and the effect of additional salts and their concentrations was investigated by one-factor-at-a-time experiments. Different inorganic nitrogen and phosphorus sources were added in the fermentation medium in a concentration that insured such quantity of nitrogen/phosphorus which is added with 0.5 $g.dm^{-3}$ $NaNO_3$/3.0 $g.dm^{-3}$ KH_2PO_4. Lipid inducers, carbon and organic nitrogen sources were added in the same concentration as in the initial medium.

Lipase assay

Lipase activity was measured by spectrophotometric method using p-nitrophenyl palmitate as substrate buffered with Tris-HCl pH 9.0, (Kaushik et al., 2006). Reaction mixture was incubated for 15 min at 35°C and the reaction was stopped by adding 1 cm^3 plumbous acetate. After centrifugation absorbance was measured at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that released one µmol of p-nytrophenol per minute under the assay conditions described.

Results and Discussion

By using Rhodamine B-agar plate method it was proved that the studied strain *Rhizopus arrhizus* was able to produce lipase (data not shown). Fluorescent zones were observed around active colonies. For development of the biosynthetic potential of the studied strain, the influence of fermentation medium composition was investigated.

Influence of carbon sources on lipase production

The highest activity, 42.9 $U.dm^{-3}$, was measured in the control sample in which there was only carbohydrate (glucose) as a carbon source (Figure 1). The presence of soybean oil, corn oil and tristearate led to almost fully inhibition of lipase biosynthesis. Chander et al. (1980) also reported that lipase production was reduced about 60% in the presence

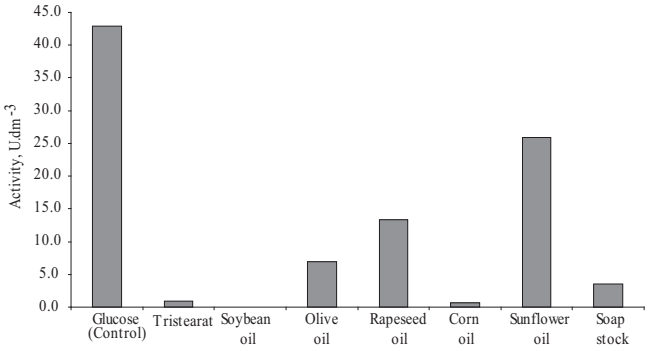

Fig. 1. Influence of different lipid inducers on lipase production

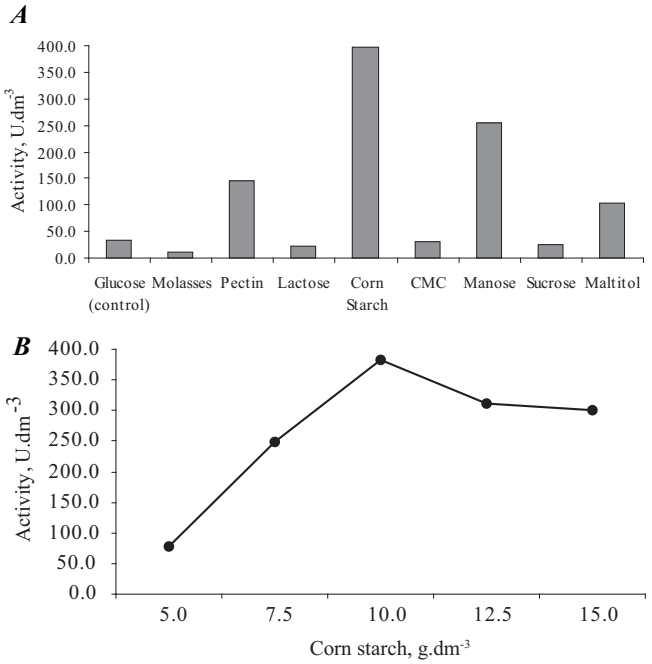

Fig. 2. Influence of carbon source on lipase production: *A* – component; *B* – concentration

of lipids during submerged cultivation of *Aspergillus wentii*.

Results for studying the influence of different carbon sources are shown on Figure 2a. The highest lipase activity was achieved by using corn starch as a carbon source – 396.7 U.dm^{-3}, which is about 7 times higher than the control with glucose (41.7 U.dm^{-3}). High lipase activity was measured when using manose, pectin and maltitol. The optimal concentration of corn starch was found to be 10.0 g.dm^{-3} (Figure 2b). Starch is used as carbon source for lipase production by submerged fermentation of *Serratia rubidaea* (Immanuel et al., 2008) and *Pseudomonas aeruginosa* (Bisht et al., 2012). The following experiments were prepared by using 10.0 g.dm^{-3} corn starches as carbon source.

Influence of nitrogen sources on lipase production

The most appropriate component as an organic nitrogen source was found to be casein trypsic peptone (tryptone). In this case (Figure 3a) 658.3 U.dm^{-3} lipase activities were achieved which is almost 3 times higher than the control (with peptone – 253.1 U.dm^{-3}). The optimal concentration of tryptone was 5.0 g.dm^{-3} (Figure 3b) which were chosen for the following experiments. Fickers et al. (2004) got a similar result for lipase production by *Yarrowia lipolytica*. In this case maximum activity was achieved by using 10.0 g.dm^{-3} casein trypsic peptone. It is known that tryptone is characterized by higher hydrolysis rate compared with the other studied organic nitrogen sources used. Also it contains large amounts of arginine and tryptophan. These characteristics could be a key factor for the lipase biosynthesis.

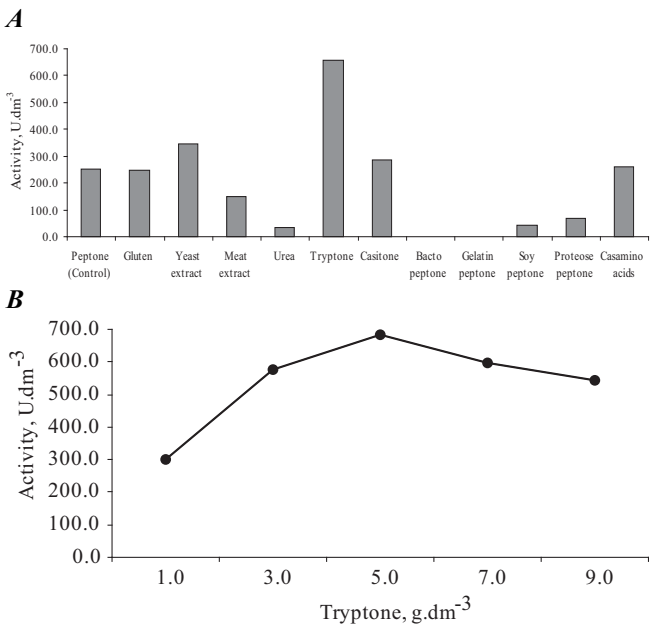

Fig. 3. Influence of organic nitrogen source on lipase production: *A* – component; *B* – concentration

Inorganic nitrogen source significantly affects biosynthesis of lipase. The effect of different inorganic nitrogen sources was investigated (Figure 4a).

It was established that highest lipase activity (1192.2 U.dm^{-3}) was obtained by using 0.5 g.dm^{-3} $(NH_4)_2C_2O_4$ as a source of inorganic nitrogen (Figure 4b) that's why it was chosen for further experiments. Ammonium oxalate is unconventional component used in microbial production even though Iftikhar et al. (2010) reported that 8 g.dm^{-3} $(NH_4)_2C_2O_4$ was the most appropriate nitrogen salt for lipase production by *Rhizopus oligosporous* var. *microsporous*.

Influence of phosphorus source on lipase production

The source of phosphorus is very important for various metabolic pathways including enzyme biosynthesis (Lu and Li, 2011). That's why the effect of different phosphates on lipase production was studied (Figure 5a).

The most effective salt as a phosphorus source was $NH_4H_2PO_4$ when added in a concentration of 3.0 g.dm^{-3} (Figure 5b). $NH_4H_2PO_4$ is a combined source of nitrogen and phosphorus. In this case lipase biosynthesis reached 1109.0 U.dm^{-3}. The

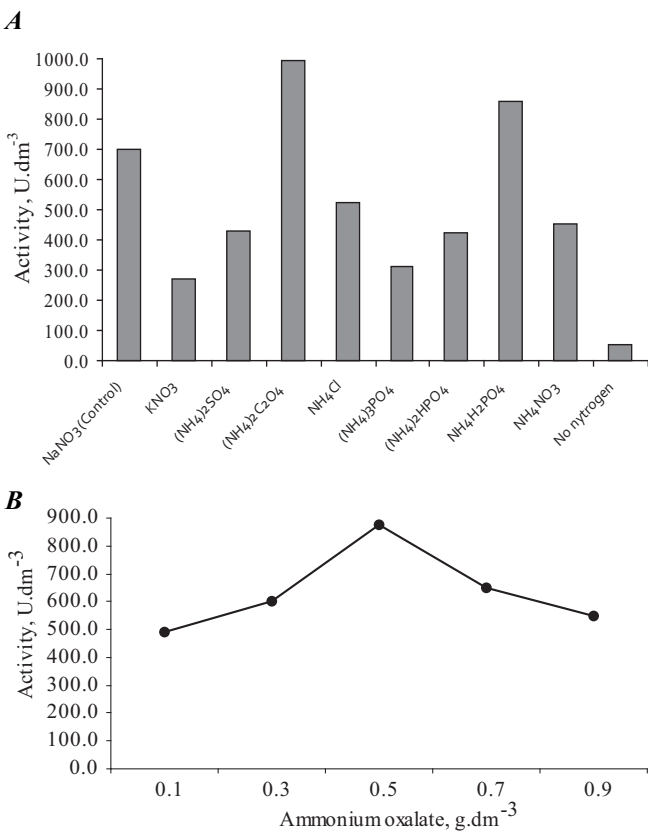

Fig. 4. Influence of inorganic nitrogen source on lipase production: *A* – component; *B* – concentration

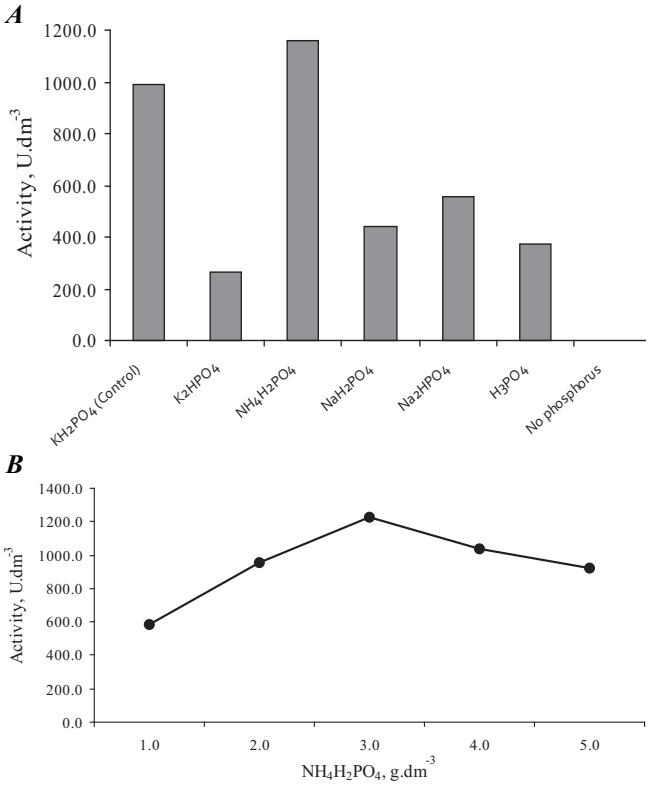

Fig. 5. Influence of phosphorus source on lipase production by: *A* – component; *B* – concentration

presence of a phosphorus source is of great importance for lipase production. This was proved by a sample with no phosphorus in which there was no lipolytical activity.

Influence of additional salts on lipase production

It is known that various microbial lipase producers require different ions in the fermentation medium. In some cases they are necessary for the catalytic action of lipases while in other cases they affect the producer growth and enzyme biosynthesis. That's why the effect of additional inorganic salts on the lipolytical activity was investigated (data not shown). The results revealed that by adding $MgSO_4$ and KCl, both in concentration 0.5 g.dm^{-3}, lipase activity was increased to 1265.0 U.dm^{-3}. The same salts in 0.05% concentration were used by Kamini et al. (1997), for reaching maximum lipolytical activity in submerged fermentation of *Aspergillus niger*.

Conclusion

It was proved that the studied strain *Rhizopus arrhizus* was a producer of extracellular lipase. According to the obtained results it can be concluded that there is no need of lipid inducer for lipase production in submerged fermentation of *Rhizopus arrhizus* and corn starch was the most appropriate carbon source. Tryptone and ammonium oxalate (which is not traditional component for microbial production) were found to be the most proper organic and inorganic nitrogen sources. Increasing lipase biosynthesis was achieved by adding $NH_4H_2PO_4$, $MgSO_4$ and KCl when the lipase activity reached 1265.0 U.dm^{-3}. It was about 30 times higher than the activity measured with the starting medium (42.9 U.dm^{-3}). Those components will be used for further optimization by using response surface methodology.

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