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Isolation of bioactive peptides from tryptone that modulate lipase production in *Yarrowia lipolytica*

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ABSTRACT

In this work the effect of several organic nitrogen sources on lipase production in *Yarrowia lipolytica* LgX64.81 overproducing mutant was studied. Among them, tryptone and peptone showed the most prominent stimulatory effect. Interestingly, only tryptic and peptic casein digest were found to highly induce lipase biosynthesis while lipase production was very limited in the presence of casein digest from papain and pronase-catalysed hydrolysis and absent in case of chymotryptic digest. It was also demonstrated that the stimulatory peptides should be present in the culture medium at specific proportions and molecular size to match the physiological requirement of *Yarrowia lipolytica* strain for lipase biosynthesis.

Herein, the lipase-production stimulatory peptides were isolated by ion exchange chromatography for the first time. These results had contributed to gain an insight on tryptone role in lipase production by *Yarrowia lipolytica*. Moreover the use of a chemically defined medium supplemented with the isolated peptides, will improve the efficiency of the process for lipase production in this yeast.

1. Introduction

Currently, protein hydrolysates are commercially available from various sources like animal tissues, milk products, microorganisms, plants, etc. These compounds are commonly considered for various microorganisms, as a potential source of nutrients including amino-acids, oligopeptides and trace elements. They are often used as an excellent culture medium supplement for the enhancement of cell growth, productivity and product quality (Chun et al., 2007).

Supplementation of culture media with protein hydrolysates such as peptones, yeast extract or casein digests has been studied for many years in a wide range of applications. A cheap medium supplemented with peptone and casein hydrolysate was developed by Saha and Sen (1989) for the production of foot and mouth-disease vaccine in a continuous culture of BHK-21 cells. Furthermore, peptones derived from bovine milk were described for their cell growth support of many different cell lines in the absence of serum; they were shown to exert anti-apoptotic activity (Schlaeger, 1996).

Casein pancreatic digest has been also used as an essential nutritional support in bacterial growth media for the production of tetanus toxin by *Clostridium tetani* (Porfirio et al., 1997). Similarly, enzymatic casein hydrolysates were found to significantly stimulate the ß-galactosidase activity in *Kluyveromyces bulgaricus* (Decleire et al., 1996).

In some cases, the presence of these compounds in the culture media greatly affects the productivity of the process so that drawbacks related to their inconsistency are neglected.

Lipase production by some bacterial and fungal species is a good example of such processes. Indeed, organic nitrogen source such as yeast extract and peptone were found to increase the synthesis of lipase by *Candida rugosa* (Fadiloglu and Erkmen, 2002), *Candida deformans* and *Yarrowia lipolytica* (Novotný et al., 1988) when olive oil was used as a carbon source and even in the absence of olive oil in *Hansenula anomala* (Banerjee et al., 1985). By contrast, mineral nitrogen source exhibited an inhibitory effect on lipase production in many fungal species such as *Yarrowia lipolytica* (Corzo and Revah, 1999; Pereira-Meirelles et al., 1997), *Geotrichum candidum* (Ginalska et al., 2007) and *Rhizopus homothallicus* (Rodriguez et al., 2006).

In the non conventional yeast *Yarrowia lipolytica,* a 166-fold increase of lipase activity level was observed when casein hydrolysates were added to the culture medium. The presence of mineral nitrogen in the culture medium, as a unique source, did not result in any production of lipase (Fickers et al., 2004).

In an attempt to explain lipase production enhancement upon the addition of organic nitrogen to the culture medium, the hypothesis of a regulation by specific peptides present in casein hydrolysate was formulated (Fickers et al., 2004; Makhzoum et al., 1995). However, various aspects of this regulation are not

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yet well understood; the existence of these lipase-production stimulatory peptides still needs to be confirmed.

The aim of this work is to demonstrate that casein enzymatic digest contains specific peptides that induce lipase production in the overproducing mutant LgX64.81 of *Yarrowia lipolytica*.

To isolate these bioactive peptides different chromatographic methods were applied to fractionate peptide mixtures from commercial tryptone. Lipase production experiments were then performed separately on each isolated peptidic fraction in order to ascertain their physiological role in the enhancement of lipase production. Stimulatory fractions were finally analysed for their amino acid content.

2. Methods

2.1. Chemicals and enzymes

Methyloleate was provided by Cognis (Saint Forgean Ponthierry, France). Potassium phosphate monobasic and potassium phosphate dibasic were purchased from USB Corporation (Cleveland, OH, USA). Thiamin was from Merck (Darmastadt, Germany) whereas Myo-inositol was from Calibiochem (La Jolla, Canada). All other chemicals unless stated otherwise, were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Trypsin from porcine pancreas (Difco, USA), papain from *Carica papaya* (Fluka, USA) pepsin from porcine gastric mucosa, pronase from *Streptomyces griseus* and chymotrypsin from bovine pancreas, were used in this study.

2.2. Micro organism

Yarrowia lipolytica LgX64.81 overproducing mutant isolated at the Centre Wallon de Biologie Industrielle, Unité de Technologie Microbienne (Liège, Belgium) was used through out this study.

2.3. Growth media

The yeast strain was isolated on YPG-agar medium containing 20 g/L glucose; 10 g/L yeast extract; 10 g/L peptone and 16 g/L agar.

The mineral medium was prepared as described by Gordillo et al. (1998). It has the following composition: 15 g/L KH_2PO_4; 5.5 g/L K_2HPO_4; 1 g/L $MgSO_4 \cdot 7H_2O$; 4 g/L $(NH_4)_2SO_4$; 10 mg/L $FeCl_3 \cdot 6H_2O$; 4 μg/L myo-inositol; 8 μg/L biotin and 200 μg/L thiamine HCl.

2.4. Culture conditions

Pre-cultures were carried out in 250 mL shake flask containing 50 mL of YPG medium. *Yarrowia lipolytica* pre-cultures were inoculated at an initial OD 600 nm of 1–1.5 and incubated at 29 °C and 140 rpm for 16 h.

Shake-flask cultures were carried out at 29 °C and 140 rpm in either 500 mL Erlenmeyers with 100 mL growth medium or 125 mL Erlenmeyers with 10 mL working volume.

Cultures were also performed in 6-well microplates (Nunc, Denmark) with 5 mL working volume; they were incubated at 29 °C in a plate shaker at 140 rpm and in humidified atmosphere to avoid medium evaporation.

Hydrolysates were sterilized by filtration using 0.22 μm-size pore filter (Sartorius, Goettingen, Germany).

2.5. Preparation of casein hydrolysates

Casein derived from bovine milk was dissolved at a concentration of 10 g/L in NaOH 1 M according to the manufacturer recommendations. Buffer exchange was then performed using Sephadex G25 prepacked PD10 column (Amersham Biosciences, Uppsala, Sweden) in order to transfer the casein substrate to a pH environment suitable for enzymatic hydrolysis. Peptic hydrolysis was performed in acidic condition in 0.05 M Tris–HCl buffer at pH 2. Hydrolysis with papain was performed at pH 6.2 in 0.02 M Sodium phosphate buffer. Whereas, hydrolysis with trypsin, pronase and chymotrypsine were carried out in the presence of 10 mM of $CaCl_2$ in 0.02 M Tris–HCl pH 8, 7.5 and 7.8, respectively. The enzyme to substrate ratio (E/S) were of 5:100 (pepsin), 1:100 (papain), 1:250 (trypsin), 1:100 (pronase) and 1: 60 (chymotrypsin).

Casein substrate was digested at 37 °C under agitation. The hydrolysis reaction was stopped by heating up to 98 °C for 10 min except for peptic digestion where the reaction was first stopped by adjusting the pH at 8 before heating.

In all cases, the pH was finally adjusted to 6.5 before addition of the hydrolysates to the culture medium.

2.6. Size exclusion chromatography

Size exclusion chromatography was carried out using an XK16/20 column (Amersham Biosciences, Uppsala, Sweden) packed with Sephadex G10 (Amersham Biosciences). The column was pre-equilibrated with phosphate buffer at 1 mL/min. Elution was performed at a flow rate of 0.5 mL/min. Fractions of 1 mL were collected and the absorbance was monitored at 214 nm. Fractions of interest containing the size excluded peptides were lyophilized and resuspended in the mineral medium so that their concentration in the culture medium will be equivalent to that present in 10 g/L of tryptone.

2.7. Ion exchange chromatography

Tryptone solution (10 g/L) was loaded on 1 mL HiTrap SP or 1 mL HiTrap Q columns (Amersham Biosciences, Uppsala, Sweden). SP column was equilibrated by either 10 column volumes of 50 mM HEPES buffer pH 8 or 50 mM Lactic acid buffer pH 5. Whereas MonoQ column was equilibrated with 10 column volumes of 50 mM Piperazine buffer or 50 mM Tris HCl buffer at pH 5 and 8, respectively. Elution was carried out with stepwise gradient 0.1–1 M of NaCl at a flow rate of 0.5 mL/min. Absorbance was monitored at 214 nm. Eluted fractions were then concentrated by speed vacuum (Integrated Speed Vac Concentrator System, Savant, USA).

2.8. Analytical methods

Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600}) of culture samples. Lipids from culture samples were first extracted with 2/5 (V/V) of propanol/butanol solution before OD_{600} determination. Correlation between OD 600 nm and dry cell weight was determined according to standard protocols. One unit of OD was found to be equivalent to 0.18 g/L dry cell weight (DCW).

Lipase activity was determined by the titremitric method using olive oil as substrate as described by Destain et al. (2005). One lipase unit was defined as the amount of enzyme required to liberate 1 μ mol of fatty acid per minute at 37 °C and pH 7.

Protease activity was measured by the colorimetric method using azoalbumin as a substrate. Each assay was run in duplicate. 40 μL of a 2.5% azoalbumin solution, 60 μL of phosphate-citrate buffer (0.1 M pH 6.5) and 10 μl of culture supernatant fraction were mixed and incubated at 37 °C for 18 h. The reaction was stopped by the addition of 10% TCA (W/V) and samples were centrifuged at 12,000 rpm for 10 min then the absorbance of the supernatant fraction was read at 440 nm against the blank. The