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Halophilic Fungi - Alternative Raw Materials for Extremozymes Production

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Abstract *Trimatostroma salinum, Wallemia ichthyophaga, Hortaea werneckii* and *Phaeotheca triangularis* are halophilic fungi, which can thrive in a range of salinity from 0% to 32% NaCl. They present a source of valuable bio-active compounds, enzymes and proteins interesting for food, textile and pharmaceutical industry. Enzymes from these fungi, e.g. α-amylase, cellulase, lipase, and protease, which are thermostable, tolerant to a wide range of pH, less susceptible to denaturation and tolerant to high salt concentrations, present a novel catalytic alternative for biotechnological applications. Different methods (such as homogenization and supercritical treatment) for the separation of enzymes from halophilic fungi cells were used. When SCF is used for enzyme release from fungi cells, additional benefits regarding performance of a biochemical reaction and bioseparation in the same medium leading in integration of all three processes into a single step is offered and presents, from an economic point of view, important advantage in industrial processes. The influence of operation conditions and used separation methods of extremozymes from fungi cells on residual activities of enzymes (protease, α-amylase, β-glucosidase and cellulase) was studied.

Keywords: • Halophilic fungi • Enzymes• Proteins • Enzyme activity • Supercritical treatment •

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1 Introduction

The great importance of the biotechnology industry is shown in the use of microbial intracellular proteins that possess catalytic or biological activity. For the manufacture of recombinant proteins, the release of catalytic active proteins and enzymes from living cells presents a key unit operation. Any living organism contains biologically active enzymes that can be extracted from them. The importance of microbial cells as a source of commercially useful chemicals, antibiotics and enzymes has been recognized for a very long time. They have several advantages over plant and animal cells with many good physiologic characteristics, such as high growth rate, capability to grow on simple media, no requirements of expensive additives, generation of high yields on the chosen carbon substrate, ability to grow at high cell densities and stable growth in continuous culture [1]. The most common source of industrial enzymes are microbes. The production of microbial enzymes takes place inside their cells (intracellular enzymes), although some may be secreted from outside the cell (extracellular enzymes). Extracellular enzymes are often soluble in water, which facilitates their extraction from the culture medium and purification. Obtaining an intracellular enzyme from microbial cells consists of two steps: harvesting microbial cells (by physical, chemical or enzymatic) from the culture and breaking the microbial cells to release the enzymes [2]. Several intracellular enzymes are produced industrially. The necessity of harvesting the producing cells, in order to subsequently extract an intracellular product, is a major economic disadvantage. Simultaneous isolation of intracellular products following cell disruption could lead to cost reduction [2]. Extremozymes, enzymes derived from extremophilic microorganisms, are an attractive alternative to tuning a given biocatalyst for a specific industrial application, since they can catalyze reactions in non-aqueous environments, water/solvent mixtures, at extremely high pressures, acidic and alkaline pH and also at very high temperatures. These enzymes already contain properties that are usually created in synthetically tailored enzymes by genetic engineering [3]. Extremophilic microorganisms are a rich source of naturally tailored enzymes with great potential for applications at extreme conditions. Especially lignocellulolytic, amylolytic, and other biomass processing extremozymes with unique properties are very interesting as biocatalysts for industrial processes. Extremophiles such as *T. salinum*, *W. ichthyophaga, H. werneckii* and *P. triangularis* present a source of chemically diverse and novel metabolites and proteins (e.g. enzymes such as protease, α-amylase, *β*-

glucosidase and cellulase) which are interesting for food, waste treatment, textile and pharmaceutical industry and offer new catalytic alternatives for industrial applications.

For the disruption of microbial cell walls, different methods can be used. They can be divided into two main groups: mechanical and (such as bead mills, French press, high-pressure homogenizer, ultrasonification etc.) and non-mechanical methods (enzymatic, physical or chemical). Various methods for cell disruption are currently commercially available from small to larger production quantities [4,5]. Nowadays, an interesting alternative to mechanical methods for cell lysis is usage of supercritical technology. Supercritical fluids (SCFs) can also serve as a solvent for the extraction of intracellular components from microbial cells or for isolation of products from the reaction mixture in the production of biomass. When the SCFs (such as supercritical carbon dioxide (SC CO_2)) is used for cell disruption, a sudden release of the pressure resulting in its penetration into cells and after expansion of gas within the cells and decompression of pressure forces the cell walls and causes cell disruption. It is a relatively simple method, can easily be scaled up and it is also comparable from efficiency, as well as economical points of view with a mechanical methods. Additional benefits of using supercritical technology for cell lysis are shown in the possibility of carrying out the release of enzymes from microbial cells, biochemical reaction(s) and bioseparation into a single step. The comparison of using mechanical method – homogenization and non-mechanical method – SC CO_2 treatment for black yeast cell disruption and influence of used method on activity of secreted enzymes was studied.

2 Materials and methods

The halophilic fungi *H. werneckii* EXF- 225, *P. triangularis* EXF-206, *T. salinum* EXF-295 and *W. ichthyophaga* EXF-5676 were kindly donated by the University of Ljubljana, Biotechnical Faculty, Department of Biology (Ljubljana, Slovenia). Carbon dioxide 2.5 (purity 99.5%) was supplied by Messer MG (Ruše, Slovenia). Peptone from meat (pancreatic) granulated, K_2HPO_4 (≥98.0%), Na_2CO_3 (anhydrous, ≥99.5%), $NaHCO_3$ (Ph Eur) and CH_3COOH (p.a.) were supplied by Merck (Darmstadt, Germany). $Na_4P_2O_7 \cdot 10H_2O$ (BioUltra, ≥99.5%), NaH_2PO_4 (≥99.0%), Na_2HPO_4 (BioReagent, suitable for cell culture, ≥99.0%), albumin from bovine serum (BSA) (lyophilized powder, ≥98.0%), malt extract

(for microbiology), agar, D-(+)-glucose (BioReagent, suitable for cell culture, ≥99.5%),), CH_3COONa (anhydrous, for molecular biology, ≥99.0%,), Sigmacell, glucose assay reagent, Casein, Hammarsten bovine, CCl_3COOH (TCA), 2-(Hydroxymethyl)phenyl-β-D-glucopyranosid (≥99.0%), starch azure, KH_2PO_4 (for molecular biology, ≥98.0%) and NaCl were purchased from Sigma (Schnelldorf, Germany).

2.1 Cultivation of black yeast and cell disruption

The procedure for black yeast cultivation is documented by Čolnik et al. [6]. Cell lysis was performed using two methods: SC CO_2 treatment [7] and homogenization. The homogenization was performed in sterile centrifuge tube, which was filled with a fresh cell suspension of selected black yeasts, mixed and then placed into a water bath at a fixed temperature of 35 °C. For black yeast cell disruption, a rotor-stator homogenizer (Homogenizer, Polytron Pt1200, Kinematica AG, Switzerland) was used. The cell suspension was homogenized from 10 to 100 min at 25,000 rpm, and at a predetermined time, samples were taken for subsequent analysis. The experiments were repeated three times.

The activities of selected extremozymes from *T. salinum*, *W. ichthyophaga*, *H. werneckii* and *P. triangularis* were defined spectrometric using activity assay for protease [8] at 280 nm, α-amylase [9] at 595 nm, β-glucosidase [10] at 340 nm and cellulase [10] at 340 nm.

3 Results and discussion

An essential first step in the enzyme extraction process from a microbial cell is its rupture. Two different methods were used for black yeast cell wall disruption – SC CO_2 treatment and homogenization.

Some studies of cell wall disintegration and extraction of intracellular components privilege the use of non-mechanical methods but it has been demonstrated that even using mechanical methods such as homogenization, some intracellular enzymes from the halophilic fungal cells can be released while their activity is maintained.

The highest obtained residual activities of cellulase, *α*-amylase, *β*-glucosidase and protease from the studied black yeasts; *T. salinum*, *W. ichthyophaga, H. werneckii* and *P. triangularis*, at defined conditions using homogenizer or SC CO_2 for cell disruption, are presented in table 1.

The initial activity of selected enzymes in the black yeast cell suspensions before homogenization or treatment with SC CO_2 were set to a value of 100 %. Residual activity of enzymes are presented as an increase or decrease in a value after treatment at defined conditions in comparison with initial value.

As can be seen from table 1, the residual activities of enzymes regardless the used method for cell disruption, increase after the treatment. The reason for increase in residual activities is in secretion of intracellular enzymes in cell suspension after damaging or breaking down the cell walls. The highest residual activities for all studied enzymes were detected after the treatment with homogenizer. However, more intracellular enzymes with high residual activities were secreted from the black yeast cells using SC CO_2 treatment.

For release of the high-active enzymes from the black yeast cells, shorter time (from 30 min to 60 min) was needed when the cell suspensions were treated with homogenizer versus SC CO_2 treatment, where the cell suspension were exposed to high pressure from 30 min to 24 h. Each of these methods has its own advantages and disadvantages.

Since it is known, that SCFs can improve enzyme activity and that the release of enzymes from microbial cells, biochemical reaction(s) and bioseparation can be united into a single step (when the SCF is used as reaction medium), the use of SC CO_2 can be an alternative for performance of biochemical reactions in this medium with fungi cells as source of biocatalysts. Anyway, no uniform estimate of the activity of each enzyme after using different method for cell lysis can be given in advance.

Extremophilic microorganisms are a rich source of naturally tailored enzymes with great potential for applications at extreme conditions. Especially lignocellulolytic, amylolytic, and other biomass processing extremozymes with unique properties are very interesting as biocatalysts for industrial processes. Extremophiles such as *T. salinum*, *W. ichthyophaga, H. werneckii* and *P. triangularis*

present a source of chemically diverse and novel metabolites and proteins (e.g. enzymes such as protease, α-amylase, *β*-glucosidase and cellulase) which are interesting for food, waste treatment, textile and pharmaceutical industry and offer new catalytic alternatives for industrial applications.

Table 1: Influence of used method and conditions for cell lysis on residual activity of secreted enzymes. Symbol: H – homogenization.

Enzymes	*The maximum residual activity of extremozyme in black yeasts cell suspensions (%)*		*Conditions*		*Black yeasts cell suspensions*	
	SC CO_2	**H**	**SC CO_2**	**H**	**SC CO_2**	**H**
cellulase	300	290	p = 30 MPa t = 24 h T = 35 °C	t = 30 min υ = 25,000 rpm T = 35 °C	*W. ichthyophaga* (intracellular)	*T. salinum* (extracellular)
***α*-amylase**	400	610	p = 30 MPa t = 5 h T = 35 °C	t = 60 min υ = 25,000 rpm T = 35 °C	*T. salinum* (intracellular)	*W. ichthyophaga* (intracellular)
***β*-glucosidase**	370	509	p = 10 MPa t = 30 min T = 35 °C	t = 60 min υ = 25,000 rpm T = 35 °C	*W. ichthyophaga* (extracellular)	*P. triangularis* (extracellular)
protease	230	800	p = 30 MPa t = 2 h T = 35 °C	t = 60 min υ = 25,000 rpm T = 35 °C	*T. salinum* (intracellular)	*H. werneckii* (intracellular)

4 Conclusion

From the biotechnological point of view, the separation of extremozymes (intracellular and extracellular) in the form of cocktail from halophilic fungi is interesting for industrial applications especially for cascade reactions. Different methods can be used to secrete enzymes from fungi cells, and the most

appropriate method for a specific microorganism should be determined experimentally. Enzymes from extremophiles possess improved properties and can be used at harsh conditions where non-extremophilic enzymes may deactivate.

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