Scientific paper

Release of Halophilic Extremozymes by Mechanical Cell Disruption

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Abstract

Trimatostroma salinum, Wallemia ichthyophaga, Hortaea werneckii and *Phaeotheca triangularis* are halophilic fungi, which can thrive in a wide range of salinity. They present a source of valuable bioactive compounds, enzymes and proteins interesting for food and pharmaceutical industry. To separate enzymes from halophilic fungi cells, the mechanical method was used. Obtained results and new findings are important from the biotechnological point of view, since the separation of in the form of cocktail from halophilic fungi is interesting for industrial applications, especially for cascade reactions. Enzymes from extremophiles namely possess improved properties and can be used at harsh conditions where non-extremophilic enzymes may deactivate.

Keywords: Activity; enzymes; halophilic fungi; mechanical method; proteins

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 physiological 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 microbial cells. 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 methods) 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] The mechanical disruption method is based on disrupting cells and tissues by applying a force not inherent to the sample. Lysates obtained by using mechanical forces and those produced by chemical lysis have different characteristics.

Various methods of mechanical cell disruption (such as bead mills, French press, high-pressure homogenizer, ultrasonification etc.) are currently commercially available for processing minute samples (<1 mL) to larger production quantities and are well documented as to the advantages and disadvantages of each method by Goldberg[3] and Flickinger.[4] Effective methods for disruption of the fungi or bacterial cell walls are fundamental to obtain higher intracellular compound extraction efficiencies. By using mechanical methods, non-selective destruction is usually achieved. These processes are easy to scale up and relatively cheaper to operate and are therefore often favoured

for large-scale cell disruption. Non-mechanical methods (enzymatic, physical or chemical) are more selective and mostly used on the laboratory scale owing to their operational and economic limitations.

Detergents and chaotropes can destabilize protein structures during the release of them from the cell. Therefore, they are not suitable tools for cell wall disruption for isolating proteins, while mechanical homogenization in combination with denaturing reagents is a useful tool for rapid cell and tissue disruption (especially during RNA isolation). When applying mechanical methods of cell disruption, the resulting stresses and strains disrupt the cell walls of the microorganisms. In mechanical disruption devices, load is transmitted to the cells. Cells are mainly stressed by shear and compressive stress and stress transmitted from the fluid medium.[4] Mechanical methods are most effective for the disintegration of yeast and filamentous fungi[5] and can lead to enzymatically active cell-free extracts. Moreover, intracellular enzymes of interest can be isolated and purified. A broad spectrum of experimental work on mechanical disruption of yeast cell,[3,6–8] *Escherichia coli, Bacillus subtilis*[9] and algae[10,11] using different methods in research and industry has been published. The purpose of these studies was recovering of the large volumes of intracellular substances inside cells. There are very few published studies on other organisms.

One reason for preferring mechanical methods to chemical and enzymatic methods for cell disruption on the industrial scale is to avoid the increase in unit operation steps during downstream processing.[12]

Bead mills represents the most effective ways for mechanical disruption of microbial cells with high disruption efficiency, good temperature control and easy scale-up procedure. High-pressure homogenizers are most commonly used for bacteria and yeast disruption in the pharmaceutical and biotechnological industries. The main drawback is the non-selective release of the products, which complicates the purification process, its price and operating costs. Ultrasonication is another common mechanical cell disruption method in which, because of high frequency ultrasound, high shear forces are created which cause cell disruption.

Halim et al.[10] studied the influence of varied mechanical methods for *Chlorococcum sp*. cell disruption expressed by disruption efficacy. They found that the highest disruption efficacy was achieved using high-pressure homogenization (73.8%); with the sulfuric acid treatment, 33.2% disruption efficacy was detected, and by usage of bead beating (33.2%) and ultrasonics (4.5%), the lowest disruption efficacies were detected.

A positive effect on protein activity was determined during ultrasonication of some commercially purified enzyme preparations. On the other hand, biological tissue denaturation at high intensity ultrasonication could appear.[13] Therefore, the selection of operational parameters during ultrasonication can strongly affect the activity and stability of bioactive components in the cell. Therefore, operational parameters (such as time and intensity) of the sonifier should be optimised specifically for each enzyme or biological tissue, since degradation of the released enzyme during the disruption of cells could appear.

However, there is no general rule regarding the use of a particular method for cell opening; the choice of disruption method is therefore dependent on the selected microorganism and the desired results.

Homogenization is a process where homogeneity throughout a product is achieved by particle size modification. It can be divided into three major categories: ultrasonic pressure homogenization, pressure homogenization and mechanical homogenization. Applications of homogenization are diverse in the food industry, pharmaceuticals and the chemical industry. Selection criteria for homogenizers depend on the particular application**.** High-pressure homogenizers and mechanical homogenizers have been for many years the most commonly used equipment for disruption of microbial cells.[14–17]

Organisms such as halophilic fungi that survive and thrive under conditions that are detrimental to the majority of other species have become an increasing scientific attention over the last few years.[18] The role of halophilic fungi in industrial and biotechnological areas become more and more important especially since they contain industrially important enzymes that are resistant to harsh conditions. Halophilic and halotolerant fungi can act as valuable. In the past, halophilic fungi have been used for production of fermentation-based foods such as soy sauce and fish sauce. Nowadays, their applications extend from bacteriorhodopsin production for optical and optoelectronic devices[19] to biopolymers such as biosurfactants and exopolysaccharides for oil recovery and compatible solutes as stress protectants.[20] Halotolerant fungi can act as valuable sources of transgenes for imparting tolerance to microbes such as *Saccharomyces cerevisiae* which present a very important microorganisms for industrially application, which are subjected to various abiotic stresses.

W. ichthyophaga is a halophilic basidiomycetous fungus,[21,22] *P. triangularis* is an obligate halophile, while *H. werneckii* and *T. salinum* are facultatively halotolerant. These fungi were first isolated from the hypersaline water of the Slovenian Sečovlje solar salterns, an environment characterised by high concentrations of NaCl, occasional rapid changes in water activity, low oxygen concentrations and high UV radiation.[21,23,24] Halophilic *T. salinum* was the first fungal species that was shown to thrive in the low water potential environment created by pure salt.[25,26] *H. werneckii* is the most salt-adaptable fungus known, as it can grow without NaCl and in almost saturated NaCl solutions, and its plasma membrane is rigid.[27] The saline and nonsaline conditions for growth enable the presence of the dark pigment melanin in their cell walls. Melanin plays a very important role not only in UV-protection, but also

in desiccation and radiation tolerance.[28–30] *W. ichthyophaga* has an obligate halophilic character, as it grows only in media with NaCl above 1.7 M and still shows growth to 5.2 M NaCl.[21,23]

To release enzymes from halophilic fungi cells, a mechanical homogenizer (rotor-stator homogenizer) was used. Homogenizing the black yeast cell suspensions allows the achievement of more rapid and effective results. It is an affordable, simple and reliable method, which enables the extraction of intracellular substances without needing to use solvents or other chemicals to break down the cell walls. During homogenization, protein deactivation could appear, owing to shearing at the molecular level or the thermal denaturation, caused by local overheating of the cell suspension. However, the degree of possible denaturation of proteins is dependent on the type of protein. Some proteins are easily deactivated and are very influenced by process parameters (e.g. homogenization time), whereas others are more stable.[31]

So far no publication regarding release of enzymes from studied halophilic fungi using homogenization method and the study of their activity was published. Since the studied halophilic fungi are adapted to the high salinity and their cells selectively accumulate compatible solutes such as glycerol, the cell wall of those fungi is more rigid and release of intracellular enzymes can be more difficult. These fungi can occur in stressful environments that are hostile to most eukaryotes and are therefore interesting and important for evolutionary processes. Extremophiles such as *T. salinum*, *W. ichthyophaga, H. werneckii* and *P. triangularis* are considered as a source of chemically diverse and often novel metabolites and proteins (e.g. enzymes such as protease, α-amylase, *β*-glucosidase and cellulase) which are interesting for food and pharmaceutical industry in a form of a cocktail, especially when the cascade reactions are required.

Therefore, the influence of homogenization time on various factors, such as the viability of black yeast cells, residual activity of enzymes (protease, *α*-amylase, *β*-glucosidase and cellulase), and the residual protein concentration was studied.

2. Experimental

2. 1. Materials

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). Peptone from meat, potassium phosphate, potassium dihydrogen phosphate, sodium carbonate, sodium bicarbonate and acetic acid were purchased from Merck (Darmstadt, Germany). Sodium pyrophosphate decahydrate (≥99.0%), sodium phosphate monobasic (≥99.0%), sodium phosphate dibasic (≥99.0%), albumin from bovine serum (BSA) (≥98.0%), malt extract, agar, D-(+)-glucose, sodium acetate, Sigmacell, glucose assay reagent, Casein, Hammarsten bovine, trichloroacetic acid (TCA), D-(−)-Salicin (≥ 99.0%), starch azure and sodium chloride were supplied by Sigma (Schnelldorf, Germany).

2. 2. Preparation of Halophilic Fungi Suspension

The microbial strains used in this study were *H. werneckii*, *P. triangularis, T. salinum,* and *W. ichthyophaga*. *H. werneckii, P. triangularis* and *T. salinum* were grown on solid malt extract agar (MEA) for 5–7 days at room temperature, while cells of *W. ichthyophaga* were grown on MEA with 17% NaCl under the same conditions. Black yeast cells were suspended in sterile saline solutions, incubated at 25 °C and stirred at 300 rpm for 10 min to achieve homogeneity of the cell suspension. All cultures were freshly prepared by the same procedure.

2. 3. Disintegration of Halophilic Fungi Cells

Rotor-stator homogenizers consist of two parts: a fast-spinning inner rotor and a stator (a stationary outer sheath. Their function is to homogenize samples based on the action of mechanical tearing, shear fluid forces, and/or cavitation (the rapid forming and collapsing of bubbles). Rotor-stator homogenizers are broadly capable of homogenizing a wide variety of tissues or cells.

The experiments were conducted in a 100 mL glass centrifuge tube. The sterile centrifuge tube 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) for volumes from 1 mL to 1 L 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.

2. 4. Survivor Colony Count

The number of colonies of *H. werneckii, T. salinum* and *P. triangularis* was determined before and after the defined homogenization time. The initial concentration (*N*o) was around 10^5 CFU mL^{-1} (colony forming units mL^{-1}) for each fungus. Parameters for determination of CFU mL^{-1} were volume of sample 100 μL, an incubation temperature of 25 °C and an incubation time of 5 to 7 days.

2. 5. Bradford Assay

A Bradford assay is a highly sensitive colorimetric method, with perceived linearity, and it presents high

speed of analysis.[32–35] Bovine serum albumin (BSA) was used as the standard protein.

2. 6. Determination of Enzymatic Activity

The optical density of halophilic fungi cell suspensions was determined at 600 nm by the UV-Vis spectrophotometer and were between 0.47 and 0.54 before homogenization. Samples of halophilic fungi cell suspensions after homogenization were centrifuged for 2 min, and the supernatant without cells and debris was analyzed spectrophotometrically. The amount of total proteins was determined with the Bradford method.[32] Activities of the cellular enzymes of halophilic fungi, α-amylase, cellulase, β-glucosidase and protease, which are the most commonly used enzymes in applied biocatalysis were determined by the UV-Vis spectrophotometer at wavelengths of 595 nm, 340 nm and 280 nm, before and after homogenization of the black yeast culture.

2. 6. 1. Assay of Cellulase Enzyme Activity

Cellulase activity was defined at 340 nm (25 °C) with a UV-Vis spectrophotometer using the method with a Sigmacell solution as a substrate.[36]

2. 6. 2. Assay of α-amylase Enzyme Activity

α-Amylase activity was defined spectrophotometrically at 595 nm, using starch azure as a substrate. The activity was expressed as the change in absorbance at 595 nm per minute per mL of cell suspension.[36]

2. 6. 3. Assay of Protease Enzyme Activity

Protease enzyme activity was determined at 280 nm on a UV-Vis spectrophotometer, using the TCA method.[36]

2. 6. 4. Assay of β-glucosidase Enzyme Activity

β-glucosidase enzyme activity was determined at 340 nm with a UV-Vis spectrophotometer, using salicin as a substrate.[36]

2. 6. 5. Statistical Method

For the statistical evaluation of the deviation of individual measurements, the standard deviation method was used, and for regression analysis, the coefficient of determination (R^2) for linear regression was determined.

For the calculation of standard deviation, the following equation was used:

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \quad (1)$$

Symbols: x – each value in the population; $\bar{x}$ – the mean value of the sample; n-1 – the number of values in the sample minus 1.

3. Results and Discussion

3. 1. Viability of Halophilic Fungi Exposed to Mechanical Forces

Structural damage or physiological dysfunction of the cell are often associated with the reason for its death. Other effects that influence or lead to cell death are disruption of the envelopes, DNA conformational changes, ribosome alterations or protein aggregation, as well as physiological disorders (membrane selective permeability alteration or loss of key enzyme function).[37] In addition, elevated temperatures can also affect the cellular structure; therefore, it is very difficult to determine which parameter contributes most to cell death. For that reason, in our study a low temperature (35 °C) was chosen for exposure of the fungi to mechanical forces. The influence of mechanical forces on the viability of *H. werneckii*, *P. triangularis, T. salinum* and *W. ichthyophaga* cells in the cell suspension was a subject of interest (Figure 1).

Cell division, growth and morphogenesis are phases during which the fungal cell structure dynamic is changing. No similarity in the composition and mechanical properties of the cell wall is expected either between genera, or even within closely related species. Moreover, growth conditions and genetic modifications could affect the fungal cell structure.[38,39]

Therefore, it was interesting to study the influence of homogenization on cell death of these four halophilic fungi.

Before homogenization of the halophilic fungi, the number of viable cells was cca. 10^5 CFU mL^{-1} for *H. werneckii*, *T. salinum* and *W. ichthyophaga*, and 10^2 CFU mL^{-1} for *P. triangularis*.

The viability of all four treated halophilic fungi after 10 minutes of homogenization was relatively high, from

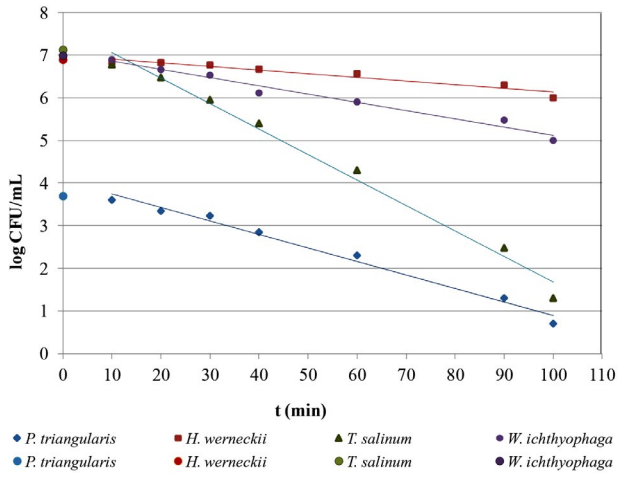

Figure 1: Survivor lines for *H. werneckii*, *P. triangularis, T. salinum,* and *W. ichthyophaga* after homogenization of cell suspension at 35 °C in relation to homogenization time. Experiments were repeated three times. Coefficients of determination (R^2) for linear regression are 0.985 (for *P. triangularis*); 0.942 (for *H. werneckii*); 0.987 (for *T. salinum*); and 0.974 (for *W. ichthyophaga*).

88% (corresponding to the $68 \cdot 10^5$ CFU mL^{-1} for *H. werneckii*), to 99% (corresponding to the $79 \cdot 10^5$ CFU mL^{-1} for *W. ichthyophaga*). With further increases in homogenization, a rapid decline in the viability of *T. salinum* and *P. triangularis* cells was observed. In comparison to *T. salinum* and *P. triangularis*, *W. ichthyophaga* and *H. werneckii* cells are more resistant to the influence of mechanical forces. After 100 minutes of homogenization of the *W. ichthyophaga* cell suspension, 1% of the cells (corresponding to the $1 \cdot 10^5$ CFU mL^{-1} for *W. ichthyophaga*) had survived, and for the same time of treatment, 13% (corresponding to the $10 \cdot 10^5$ CFU mL^{-1} for *H. werneckii*) of the *H. werneckii* cells had survived. In our earlier studies, it was also found that the black yeasts *W. ichthyophaga* and *H. werncekii* were highly resistant to high pressure.[36,40] On the basis of the survivor lines constructed for these halophilic fungi, decimal reduction times (D-values) were determined. The times required to kill 90% of tested microorganisms exposed to mechanical forces at 35 °C, expressed as D-values. The greatest resistance to mechanical forces was observed for the *H. werneckii* cells (D-value = 115.0 min), while the least resistant were the *T. salinum* cells (D-value = 17.0 min). D-values for *W. ichthyophaga* and *P. triangularis* were following; 51.5 min and 31.7 min.

After treatment of the halophilic fungi cell suspensions by mechanical forces, the shape of the cells was examined under an environmental scanning electron microscope (ESEM).

Kralj Kunčič et al.[41] reported that at low salinity (15% NaCl), the cell walls of *W. ichthyophaga* cells were threefold thicker than the cell walls of the hyphae of both *W. muriae* and *W. sebi* (both belong also to the halophilic fungal genus *Wallemia*). This could be the reason for the very high D-values for *W. ichthyophaga* and *H. werneckii* that were obtained in our study.

Figure 2 shows the ESEM figures for these fungi before (Figures 2 a, c, e, g) and after (Figures 2 b, d, f, h) exposure to mechanical forces, using the homogenization method. As can be seen from Figure 2, untreated halophilic fungi cells had a smooth surface and typical shape. Homogenized halophilic fungi cells were damaged and deformed. Slightly wrinkled cell walls of dead halophilic fungi cells were detected, especially among the treated cells of *T. salinum* (Figure 2d). Ruptures and broken pieces of the cell walls were also detected. All these morphological changes indicate damage to the cell walls of the treated fungi. Therefore, the release of intracellular proteins could be predicted.

3. 2. Residual Protein Concentration

Besides the cell types, the appropriate time of exposure of the biological material to homogenization is also a critical parameter influencing the activity of the intracellular compounds. The residual protein concentration in the selected halophilic fungi suspensions increased linearly with the increase in homogenization time (Figure 3). The highest residual protein concentration (330%), which is equivalent to 8.7780 $mg_{proteins}$ mL^{-1} $_{of\ cell\ suspension}$, was detected after 100 min of homogenization in the *P. triangularis* cell suspension. The lowest protein concentration after 100 min of homogenization was observed in the *H. werneckii* cell suspension.

These results coincide with those obtained by determination of the survivor curves in selected halophilic fungi. The lowest D-value was obtained for *P. triangularis*,

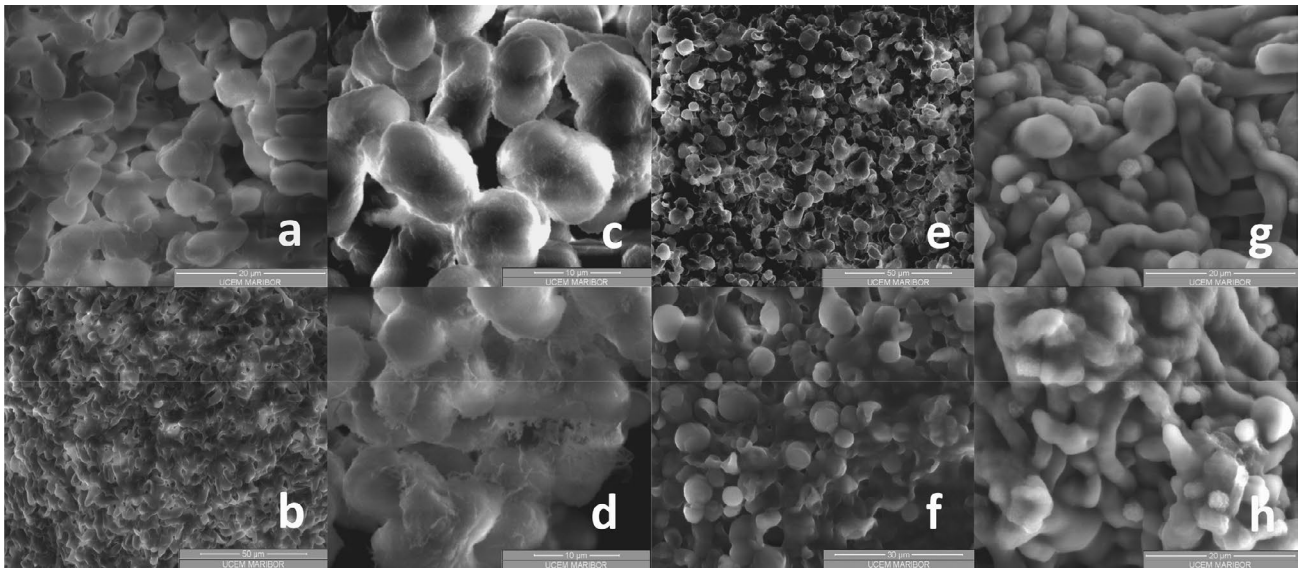

Figure 2: Halophilic fungi cells observed under an environmental scanning electron microscope (ESEM). *H. werneckii* cells a) before (magnification: 3000 X) and b) after 100 min of homogenization, *T. salinum* cells (magnification: 1000 X) c) before (magnification: 4000 X) and d) after 100 min of homogenization, *P. triangularis* cells (magnification: 4000 X) e) before (magnification: 1000 X) and f) after 100 min of homogenization and *W. ichthyophaga* (magnification: 2000 X) g) before (magnification: 3500 X) and h) after 100 min of homogenization (magnification: 3500 X).

where the highest protein concentration in the cell suspension was also detected. In contrast, the highest D-value and greatest viability was observed for *H. werneckii*, where the residual concentration of proteins in the cell suspension was the lowest. Obviously, intracellular proteins were extracted from the cells and consequently, the total protein concentration in selected halophilic fungi cell suspensions was increased.

The strength of the fungal cell wall is defined by its structure, which comprises glycoproteins and polysaccharides, mainly glucan and chitin. Several studies suggest that chitin is a primary effector for melanin deposition within the fungal cell wall.[42] It has an enormous tensile strength and significantly contributes to the overall integrity of the cell wall. However, chitin microfibrils account for only 1–2% of the yeast cell walls by dry weight.[43] This could be the reason for the very high level of proteins released under variable conditions during the homogenization disruption procedure.

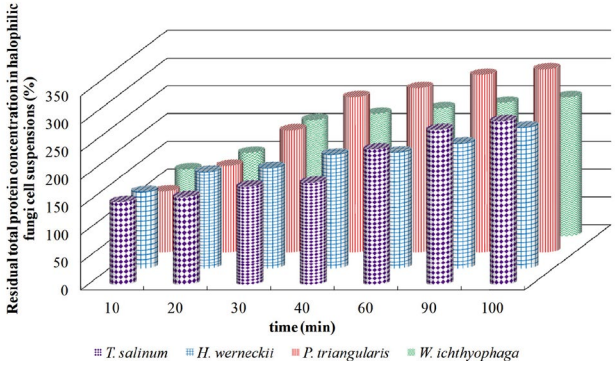

Figure 3: Residual total protein concentration in halophilic fungi cell suspensions in relation to homogenization time at 35 °C and a homogenization speed of 25,000 rpm. Experiments were repeated three times. Standard deviation for all measurements was less than ± 3%.

Initial concentration of the proteins in the halophilic fungi cell suspensions before homogenization are presented in Table 1 and were set to a value of 100%.

Table 1: Initial concentration of proteins in halophilic fungi cell suspensions before homogenization and standard deviation of measurements (SD).

Halophilic fungi	c ($mg_{proteins}$ $mL^{-1}_{of\ cell\ suspension}$)	SD ($mg_{proteins}$ $mL^{-1}_{of\ cell\ suspension}$)
T. salinum	0.0341	± 0.0010
H. werneckii	0.0472	± 0.0014
P. triangularis	0.0266	± 0.0007
W. ichthyophaga	0.0114	± 0.0003

Nucleic acids and their related compounds, such as pyrimidines and purines, are known to absorb UV light at a wavelength of 260 nm. Nucleic acids and proteins have absorbance maxima at 260 and 280 nm, respectively. The ratio of absorbances at 260 nm and at 280 nm could be used as a measure of purity in both nucleic acid and protein extractions. A 260/280 ratio is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. A ratio lower than ~1.8 may indicate the presence of protein, phenol or other compounds, which are characterized by an absorption maximum at 280 nm.[44] The absorbic ratio of the UV-absorbing materials was measured after the mechanical treatment of the halophilic fungi cell suspensions. Figure 4 shows the absorbic ratio of the absorbance 260 nm and 280 nm. The highest absorbic ratio was determined for *T. salinum* cell suspension after 100 min of homogenization (A 260/280 = 1.9). Obviously, during the homogenization of the *T. salinum* cell suspension, pure DNA was also extracted. Absorbic ratios A260/280 for the other halophilic fungi (*P. triangularis*, *W. ichthyophaga* and *H. werneckii*) were lower than 1.8. Regarding the trend

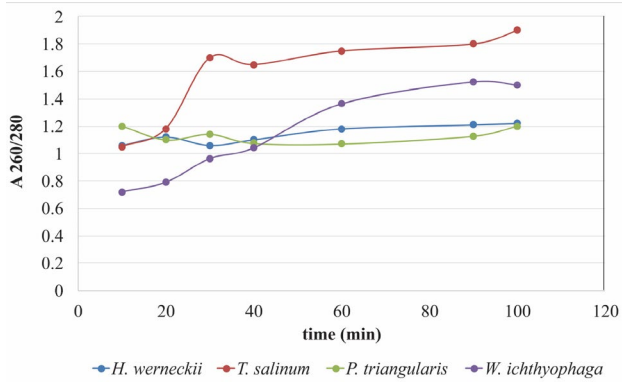

Figure 4: Absorbic ratio (A 260/280) after homogenization of halophilic fungi cell suspensions at 35 °C and a homogenization speed of 25,000 rpm. Experiments were repeated three times. Standard deviation for all measurements was less than ± 0.004.

curves for other studied halophilic fungi, it is expected that with prolongation of homogenization time, pure DNA will also be extracted from other halophilic fungi cells.

3. 3. Influence of Homogenization Time on Residual Activity of Different Enzymes from the *T. Salinum* Cell Suspension

T. salinum is a melanized meristematic fungus that was isolated from saltern water along the Adriatic coast.[25] The influence of homogenization time on the residual activity of a range of extracellular and intracellular enzymes, α-amylase, β-glucosidase, protease and cellulose, from the *T. salinum* cell suspension was studied.

As can be seen from Figure 5, the highest residual enzyme activity (440%) in the *T. salinum* cell suspension, which is equivalent to the enzyme activity of 1.6720 U mL^{-1} of cell suspension, was determined for α-amyl-

ase after 60 min of homogenization time. α-Amylase belongs to the group of endoamylases that catalyse initial hydrolysis of starch into shorter oligosaccharides through the cleavage of interval α-D-(1-4) glycosidic bonds, resulting in α-anomeric product.[45,46] Therefore, it is a very important enzyme in industrial processes such as starch saccharification, the textile, paper and food industries and pharmaceuticals.[47–49] Since α-amylase from *T. Salinum* is an intracellular enzyme [50], an increase in its residual activity was expected after the defined time of homogenization. By prolonging the exposure of the *T. salinum* cell suspension to mechanical forces, the cell walls were damaged and intracellular enzymes were released in the cell suspension. A similar trend was detected for protease (maximal residual activity was determined after 30 min of homogenization), which is also an intracellular enzyme of *T. salinum*.[50] Prolongation of homogenization time results in a decrease of α-amylase and protease activity. Probably, the overly long exposure to shear forces caused disruption in the enzyme tertiary structure and, consequently, its deactivation.

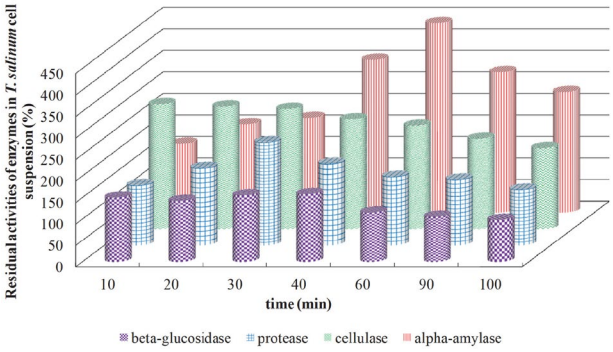

Figure 5: Residual activity of various enzymes in the *T. salinum* cell suspension in relation to homogenization time at 35 °C and a homogenization speed of 25,000 rpm. Experiments were repeated three times. Standard deviation for all measurements was less than ± 3%.

Initial activity of the studied enzymes in the *T. salinum* cell suspension before homogenization are presented in Table 2 and were set to the value of 100%.

Table 2: Initial activity of the studied enzymes in the *T. salinum* cell suspension before homogenization and standard deviation of measurements (SD).

Enzyme	Enzyme activity ($U\ mL^{-1}_{of\ cell\ suspension}$)	SD ($U\ mL^{-1}_{of\ cell\ suspension}$)
β-glucosidase	0.0035	± 0.0001
protease	0.0108	± 0.0003
cellulase	0.0350	± 0.0010
α-amylase	0.0038	± 0.0001

β-Glucosidase and cellulase are extracellular enzymes of *T. salinum*. The residual activity of β-glucosidase in the *T. salinum* cell suspension after 10 min of homogenization was detected to be 150% (the enzyme activity was 0.5250 $U\ mL^{-1}_{of\ cell\ suspension}$), and it remained unchanged until 40 min of homogenization time, when by prolonging the homogenization time, a decrease in β-glucosidase activity was detected. A similar trend was observed for cellulase. Cellulases are of great interest in the field of organic acids and biotechnology industries. They have remarkable applications in areas such as alternate energy, detergent, textile, food, the pharmaceutical industry, nutrition and the agriculture industry.[51] The reason for a decline in the residual activity of β-glucosidase and cellulase after a longer homogenization time could lie in the denaturation of both enzymes as a result of longer exposure to shear forces.

3. 4. Influence of Homogenization Time on Residual Activity of Various Enzymes from the *W. Ichthyophaga* Cell Suspension

Wallemia belongs to the cosmopolitan xerophilic fungi and can be found in habitats with low a_w and high concentrations of toxic inorganic ions.[50] When a fungal cell is exposed to a high salinity environment, it uses common molecular mechanisms as well as specific molecular mechanisms to react rapidly to the consequent loss of water. In *W. ichthyophaga* and *H. werneckii*, this is achieved by the synthesis of glycerol.[52]

The *W. ichthyophaga* cell suspension was homogenized from 10 to 100 min, and the residual activity of α-amylase, β-glucosidase, protease and cellulase vs. the time of homogenization was studied (Figure 6).

Initial activity of the studied enzymes in *W. ichthyophaga* cell suspension before homogenization are presented in Table 3 and were set to the value of 100%.

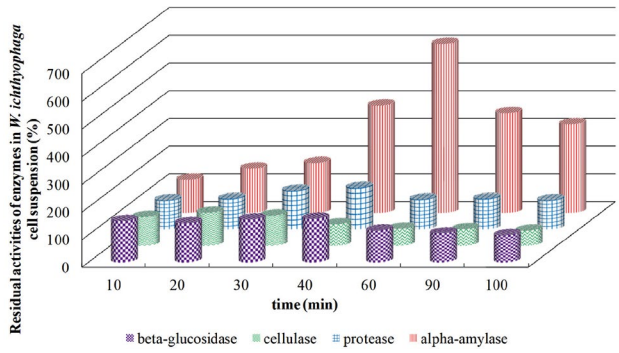

Figure 6: Residual activity of various enzymes in the *W. ichthyophaga* cell suspension in relation to homogenization time at 35 °C and a homogenization speed of 25,000 rpm. Experiments were repeated three times. Standard deviation for all measurements was less than ± 3%.

Table 3: Initial activity of various enzymes in the *W. ichthyophaga* cell suspension before homogenization and standard deviation of measurements (SD).

Enzyme	Enzyme activity ($U\ mL^{-1}_{of\ cell\ suspension}$)	SD ($U\ mL^{-1}_{of\ cell\ suspension}$)
β-glucosidase	0.0279	± 0.0008
protease	0.0082	± 0.0002
cellulase	1.2200	± 0.0317
α-amylase	0.0074	± 0.0002

With the increase in homogenization time, the activity of the intracellular enzymes α-amylase, cellulase and protease also increased. The highest residual activity of α-amylase (610%), which is equivalent to an enzyme activity of 4.5140 U mL^{-1} $_{of\ cell\ suspension}$, was detected after 60 min of homogenization, and with a further increase in homogenization time, its residual activity decreased. A slight increase in the activity of protease from 0 to 40 min homogenization time and cellulase from 0 to 20 min was also observed. *W. ichthyophaga* cells are obviously rich in α-amylase, while concentrations of the other enzymes were much lower. An increase in homogenization time up to 100 min caused a rapid decrease in all enzyme activity. Longer exposure to shear forces resulted in conformation changes to the proteins' molecular structure, which led to their activity loss.

3. 5. Influence of Homogenization Time on Residual Activity of Various Enzymes from the *H. Werneckii* Cell Suspension

The living environments of *H. werneckii* are seawater and natural or man-made saltpans with reduced water activity[53], but it can also be present in house dust.[54] It is a halophilic species with the capacity to survive in environments with high salt concentrations (3–30% NaCl). This microorganism has been used as a model for conditions of extremotolerance studies (e.g. oxidative stress, osmotic adaptation and melanisation).[55–57] The influence of homogenization time on residual activity of selected enzymes in the *H. werneckii* cell suspension was studied (Figure 7).

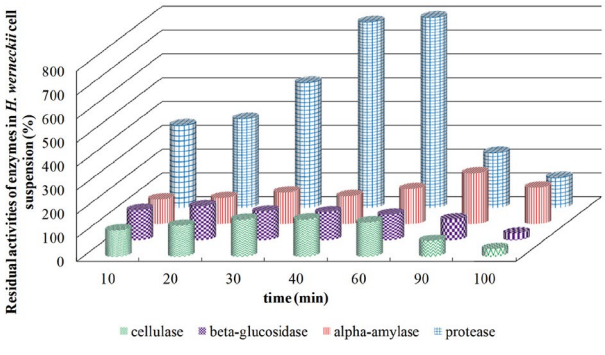

Figure 7: Residual activity of various enzymes in the *H. werneckii* cell suspension in relation to homogenization time at 35 °C and a homogenization speed of 25,000 rpm. Experiments were repeated three times. Standard deviation for all measurements was less than ± 3%.

Initial activity of the enzymes in the *H. werneckii* cell suspension before homogenization are presented in Table 4 and were set to the value of 100%.

Table 4: Initial activity of various enzymes in the *H. werneckii* cell suspension before homogenization and standard deviation of measurements (SD).

Enzyme	Enzyme activity ($U\ mL^{-1}_{of\ cell\ suspension}$)	SD ($U\ mL^{-1}_{of\ cell\ suspension}$)
β-glucosidase	0.0042	± 0.0001
protease	0.0164	± 0.0004
cellulase	0.0320	± 0.0008
α-amylase	0.0029	± 0.0001

An immense increase in residual activity of protease was detected in the *H. werneckii* cell suspension (800%), which is equivalent to the enzyme activity of 13.1200 U mL^{-1} $_{of\ cell\ suspension}$, after 60 min of homogenization. As expected, with the increase in homogenization time, the viability of *H. werneckii* cells decreased, and intracellular enzymes were released in the cell suspension. Among the selected enzymes, *H. werneckii* cells contain mostly protease (intracellular enzyme).[50] Proteases are among the three largest groups of industrial enzymes and represent about 60% of the total worldwide sale of enzymes, where approximately 40% of sales comprise microbial proteases.[58]

A slight increase in the residual activity of α-amylase in the *H. werneckii* cell suspension in relation to homogenization time was also detected. The highest residual activity of α-amylase was observed after 90 min of homogenization, when the viability of the *H. werneckii* cell was also low (less than 25%, corresponding to the $20 \cdot 10^5$ CFU mL^{-1}). Since α-amylase is an intracellular enzyme,[50] sufficient destruction of the cell walls was needed to release α-amylase in the cell suspension and, consequently, a higher residual activity was reached after 90 min of homogenization. Obviously, enzymes released in the suspension (α-amylase, as well as protease) were less resistant to mechanical forces, since a rapid decrease in residual activity was determined after only an additional 10 min of homogenization. The highest residual activity of β-glucosidase and cellulase was observed after 10 min and 30 min of homogenization, and with an additional increase in homogenization time, a decrease in their residual activity was also determined.

3. 6. Influence of Homogenization Time on Residual Activity of Various Enzymes from the *P. Triangularis* Cell Suspension

P. triangularis is an obligate halophile meristematic black yeast that can live in humidifiers.[59] Meristematic growth of the *P. triangularis* cell is slow, and it possesses

very thick, darkly pigmented cell walls.[30] The *P. triangularis* cell suspension was treated with a rotor-stator homogenizer to disintegrate the halophilic fungus cells. Residual activity of various enzymes from *P. triangularis* was studied in relation to homogenization time (Figure 8).

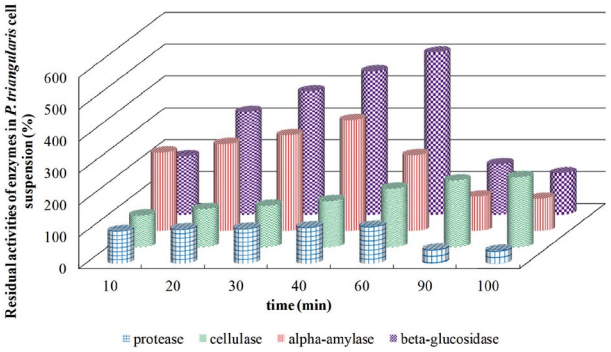

Figure 8: Residual activity of various enzymes in the *P. triangularis* cell suspension in relation to homogenization time at 35 °C and a homogenization speed of 25,000 rpm. Experiments were repeated three times. Standard deviation for all measurements was less than ± 3%.

Initial activity of the enzymes in the *P. triangularis* cell suspension before homogenization is presented in Table 5 and was set to the value of 100%.

Table 5: Initial activity of various enzymes in the *P. triangularis* cell suspension before homogenization and standard deviation of measurements (SD).

Enzyme	Enzyme activity ($U\ mL^{-1}_{of\ cell\ suspension}$)	SD ($U\ mL^{-1}_{of\ cell\ suspension}$)
β-glucosidase	0.0040	± 0.0001
protease	0.0648	± 0.0019
cellulase	0.0237	± 0.0007
α-amylase	0.0064	± 0.0002

As can be seen from Figure 10, the highest residual activity was detected for β-glucosidase. By prolonging the homogenization time from 10 min to 60 min, an increase in β-glucosidase residual activity was observed, while additional prolongation of the homogenization yielded a decrease in its residual activity. Many bacteria, yeasts and filamentous fungi can produce β-glucosidas,[60] which is an essential component of the cellulose complex, since it relieves the glucanases from product inhibition by hydrolysing cellobiose to glucose and thus is crucial for rapid and efficient saccharification of cellulose.[61]

High residual activity of α-amylase, which is an intracellular enzyme of *P. triangularis*, after only a short time of homogenization indicates that *P. triangularis* has low resistance to mechanical forces (D-value *P. triangularis* was 31.7 min). The cell walls were damaged, resulting in the release of intracellular enzymes into the cell suspension. The influence of mechanical forces on the released enzymes in the suspension was high, since with an increase in homogenization time, a rapid decrease in their residual activity was detected, except for cellulose, where a slight increase in its residual activity with increase in homogenization time was observed. No significant increase was detected in the residual activity for protease (an extracellular enzyme) after 60 min of homogenization.

Too long exposure of enzymes in the cell suspension to mechanical force may result in enzyme inactivation.

4. Conclusion

The influence of a mechanical cell-disruption method (use of a rotor-stator homogenizer) on the viability of the halophilic fungi *T. salinum, W. ichthyophaga*, *H. werneckii* and *P. triangularis* and residual activity of selected enzymes were studied. The homogenization method was successfully used for the destruction of selected halophilic fungi cell walls. The greatest resistance to mechanical forces was observed for the *H. werneckii* cells (D-value = 115.0 min), while the least resistant were the *T. salinum* cells (D-value = 17.0 min). Due to the damage of cell walls, the residual concentration of proteins and residual activity of the intracellular enzymes from extremophilic fungi were increased.

McMillan et al.[11] reported about 92.95% efficiency of algae cell disruption using mechanical solid shear, while ultrasonication was less efficient.

Recovery of intracellular enzymes using homogenization is a challenging problem because the released enzymes can be inactivated in the homogenizer. Another challenge is to keep the activity of extracellular enzymes unchanged during the homogenization method, while at the same time obtaining a high level of active intracellular enzymes.

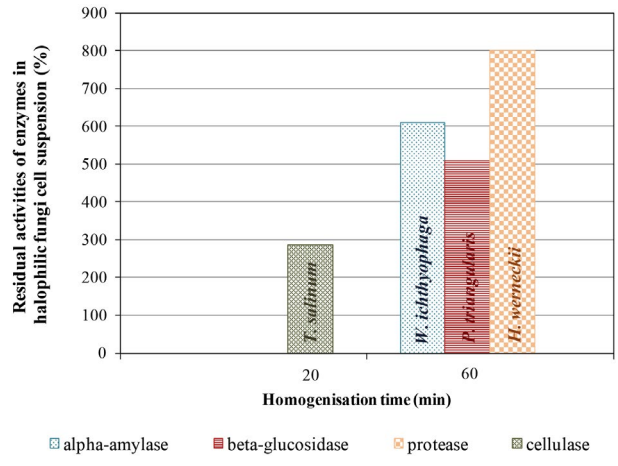

Figure 9: The highest residual activity of α-amylase, β-glucosidase, cellulase and protease obtained in various halophilic fungi cell suspensions vs. optimal homogenization time.

The highest residual activity among the selected enzymes was detected for protease in the *H. werneckii* cell suspension (800%), with 13.1200 U mL^{-1} $_{of\ cell\ suspension}$, after 60 min of homogenisation time (Figure 9).

Furthermore, the highest residual activity of *β*-glucosidase in the *P. triangularis* cell suspension (509%) of 2.0360 U mL^{-1} $_{of\ cell\ suspension}$, and *α*-amylase in *W. ichthyophaga* cell suspension (610%) of 4.5140 U mL^{-1} $_{of\ cell\ suspension}$, was also detected after 60 min of cell suspension treatment with the rotor-stator homogenizer. *T. salinum* cells are rich in cellulase, since after 20 min of homogenization, the highest residual activity of cellulose among the tested halophilic fungi was detected in the *T. salinum* cell suspension.

The level of proteins released under variable conditions and with these specific disruption method is usually higher in the case of yeast cells than fungi. Different strengths and structures of the fungal cell walls, which comprise glycoproteins and polysaccharides, mainly glucan and chitin, are the reason for the lower levels of protein released from fungi in comparison to those from yeast.

Due to the diversity of environmental sources of isolation and the cultivation conditions for these individual halophilic fungi, the content of individual enzymes may vary. Therefore, the results of our study did not turn out as expected. Moreover, no standard trend of enzyme release from the cells that would apply to all microbial cells could be determined.

The appropriate time of exposure of the biological material to certain forces causing cell lysis is a critical parameter that could influence the activity of the intracellular compounds. Klimek-Ochab et al.[5] reported that the prolongation of sonication cycles from 11 to 30s admittedly resulted in a decrease in the amount of proteins released from *P. citrinum*, but allowed a higher level of specific activity of G6P dehydrogenase. In contrast, for the *A. fumigatus* cells, the short period of sonication enables the release of a high amount of protein, and a high specific activity of G6P dehydrogenase was detected.

Table 6 shows the highest residual activity of the enzymes related to viability of the halophilic fungi and residual protein concentration obtained in the cell suspensions.

While the α-amylase in these four halophilic fungi is an intracellular enzyme, whose residual activity increased after exposure to mechanical forces, regardless of the treated fungi, *β*-glucosidase belongs to the group of extracellular enzymes not exhibiting the expected significant increase in its residual activity after homogenization. Nevertheless, its residual activity significantly increased when the cells from *P. triangularis* were disrupted by the homogenization method. Most likely, the *β*-glucosidase in this fungus is located on the inner edge of the cell wall, and only after exposure to shear forces is released from the cell wall, which is reflected in the increase in its residual activity. Consequently, no uniform estimate of the content and activity of each enzyme can be given, and results thus did not turn out as mostly expected.

An essential first step in the enzyme extraction process from a microbial cell is its rupture. Homogenization is an important and widespread method for acquiring intracellular enzymes and organelles from microbial, plant and animal sources. It enables extraction of intracellular substances without the presence of solvents or other chemicals to break down the cell wall.

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

Extremophiles such as *T. salinum*, *W. ichthyophaga*, *H. werneckii* and *P. triangularis* present a source of chemically diverse and often novel metabolites and proteins (e.g. enzymes such as protease, α-amylase, *β*-glucosidase and cellulase) which are interesting for food and pharmaceutical industry and offer new catalytic alternatives for industrial applications and represent the basis for the development of environmentally friendly, efficient, sustainable and cleaner industrial technologies.

Therefore, obtained results represent significant new findings, which are of broad biotechnological importance and are especially interesting for industrial application and useful also for a wider scientific sphere working in bio-

Table 6: Correlation between the viability of halophilic fungi, residual protein concentration and residual enzyme activity for selected enzymes (α-amylase, *β*-glucosidase, cellulase and protease) in the halophilic fungi cell suspensions.

Halophilic fungi	α-amylase			*β*-glucosidase			cellulase			protease		
	RPC (%)	VHP (%)	REA (%)	RPC (%)	VHP (%)	REA (%)	RPC (%)	VHP (%)	REA (%)	RPC (%)	VHP (%)	REA (%)
T. salinum	245	0.15	440	185	2	158	150	44	290	178	7	240
W. ichityophaga	230	8	610	100	100	100	150	47	120	220	13	150
H. werneckii	226	25	213	175	87	143	206	61	156	210	47	800
P. triangularis	280	14	348	296	4	509	330	0.1	220	296	4	116

Legend: RPC (%) – Residual protein concentration (%); VHP (%) – Viability of halophilic fungi (%); REA (%) – Residual enzyme activity (%). Red color – intracellular enzyme; Blue color – extracellular enzyme

technology on protein purification. Based on the obtained results, studied halophilic fungi contain industrial important enzymes with high catalytic activity which can be released from the cells with a cheap and simple method and are suitable for different industrial applications as batch or continuous operations e.g. reduction of mixed wastes, leading in reduction of environmental impacts.

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Povzetek

T*rimatostroma salinum, Wallemia ichthyophaga, Hortaea werneckii* in *Phaeotheca triangularis* so halofilne glive, ki lahko uspevajo v širokem razponu slanosti. Predstavljajo vir dragocenih bioaktivnih spojin, encimov in beljakovin, ki so zanimivi za živilsko in farmacevtsko industrijo. Za ločevanje encimov iz celic halofilnih gliv smo uporabili mehansko metodo. Dobljeni rezultati in nova spoznanja so pomembna z biotehnološkega vidika, saj je ločevanje encimov v obliki koktajla iz halofilnih gliv zanimivo za industrijsko uporabo, zlasti za kaskadne reakcije. Encimi iz ekstremofilov imajo namreč izboljšane lastnosti in se lahko uporabljajo v težkih pogojih, pri katerih se lahko neekstremofilni encimi deaktivirajo.