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Extracellular activity of proteases from *Yarrowia lipolytica* IPS21 as a function of the carbon and nitrogen source

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Abstract
The yeast strain *Yarrowia lipolytica* IPS 21 was tested for its ability to produce the protease enzyme on analytically pure carbon sources as well as on waste carbon sources. It was confirmed that the yeast *Y. lipolytica* IPS21 can have a higher proteolytic activity in the presence of waste carbon sources in chrome-tanned leather shavings (CTLS) than on yeast extract alone. This is confirmed by the high concentration of amino acids in samples with CTLS, suggesting increased degradation of CTLS by *Y. lipolytica* or secretion of proteases into the medium. It was also confirmed that metals accumulate mainly in the biomass and not in the supernatant. The biomass was also found to contain high levels of Ca, K and P, which are essential for plant growth. These results show that *Y. lipolytica* strain IPS21 can be used for the production of extracellular alkaline proteases and for the degradation of protein waste.

Keywords
biopolymers, carbon source, *Yarrowia lipolytica*, waste, modification of materials.

1. Introduction

Y. lipolytica is one of the most extensively studied ‘’unconventional’’ yeasts, being a strictly aerobic microorganism capable of producing important metabolites and having an intense secretory activity, which justifies efforts to use it in industry (as a biocatalyst), molecular biology and genetic studies. *Yarrowia* assimilates hydrophilic substrates (glucose, glycerol, fructose, organic acids, and alcohol). However, it can also consume hydrophobic carbon sources (fatty acids, triglycerides, esters, and hydrocarbons). Their important role in various industries is based on producing many important chemical compounds, including lipids, citric acid, and erythritol”. The ability to synthesize many enzymes, including extracellular ones, is one of the most important characteristics of *Yarrowia* strains [1]. *Y. lipolytica* produces alkaline, neutral or acidic proteolytic enzymes. Proteases E.C.3.4 are considered one of the most important groups of enzymes produced on a commercial and industrial scale. They have a wide range of applications: in biotechnology as food additives [2], in bioremediation or biosurfactant production [3] for the recovery of biopolymers from waste, keratin, and collagen [4, 5], in pharmaceuticals and cosmetics as detergents [6], and in textile technology. The rapidly developing biotechnology industry is looking for new technologies to solve existing problems, and the global production of these enzymes was worth $5 billion in 2021, with a clear upward trend, making them an important subject for research [7]. Low investment costs and relatively simple unit operations are desirable characteristics of new technologies. A good example is the enzymatic treatment of textiles, which is gaining popularity due to the fact that it is an energy-efficient and environmentally friendly process [8,9]. It is believed that proteases only act on the surface of the fiber to give wool its anti-shrinkage properties when chemically modified to increase its weight [10].

The production of extracellular proteases with *Yarrowia* yeast is influenced by many factors including pH, temperature, availability and type of nutrients in the environment. The pH of the culture medium not only determines the ability of enzymes to be secreted outside the cell but also affects the function of secreted proteins. For example, at pH 8, extracellular alkaline proteases show esterase activity, and at pH 9 - caseinolytic activity towards substrates containing arginine, lysine or tyrosine [11]. An increase in temperature increases the rate of enzymatic reactions. This provides confirmation of those that are used to build microbial biomass. In the case of enzymes produced by strains of the genus *Yarrowia*, the temperature range is 25-45 ºC [12,13]. Carbon and nitrogen sources in a microbiological medium are limited; proteases secreted by the cells can also be used as a carbon source [14]. Nutrient availability also has a major effect on enzyme production with *Y. lipolytica*, e.g. low nitrogen (N) in the medium shifts the metabolism towards lipid accumulation and citric acid production, whereas a high N in the medium leads to increased biomass, resulting in increased enzyme biosynthesis [15]. Wild-type strains of *Y. lipolytica* are characterized by very different growth and colony appearances (dull or shiny, smooth or jagged), and occur in different morphological forms (yeast, hyphae) depending on environmental conditions. Their variability is related not only to the substrate but also to the genetic phenotype. This characteristic allows the genus *Yarrowia* to invade environments that are unstable or difficult to develop [16]. There is a strong relationship between the dimorphism of *Yarrowia* yeasts and their ability to assimilate

selected nutrients, i.e. their response to different forms of nitrogen, carbon and metal ion concentrations [17]. The ability of *Y. lipolytica* yeast cells to biodegrade is various, and sometimes difficult, as it is influenced both by their ability to produce extracellular enzymes and by the strain's variability. Our previous research showed that the kinetics of biomass formation and the medium pH are increased in the *Y. lipolytica* IPS21 strain by adding CTLS (chrome-tanned leather shavings) to the modified YPG medium (containing 1.5 % (w/v) yeast extract and 0.5 % (w/v) sodium chloride). These studies indicate that the yeast used in the study can assimilate the carbon and nitrogen sources from the CTLS and can produce proteolytic enzymes on the CTLS [4]. The tanning industry produces large amounts of CTLS, the main compounds of which are trivalent chromium (1 to 3 %) and collagen (the content was estimated to be around 90 %). As the main component of solid leather waste is collagen, which is rich in C, O and N elements, the waste should not be managed solely by landfill and incineration. Two disposal methods are known and being developed for recycling leather waste containing chromium: the direct method and the indirect method (chromium recovery and de-chromed collagen). There are no known methods for the use of CTLS as substrates for the growth and production of selected enzymes by *Yarrowia* yeasts. Also, there are data indicating that the *Y. lipolytica* strain can produce high levels of proteolytic enzymes on the CTLS medium.

This work tested the ability of *Y. lipolytica* IPS 21y to produce protease enzymes on different carbon sources. We used not only analytically pure carbon sources but also waste carbon sources. The effect of changes in the amount of the nitrogen source in the medium on the potential for protease production with waste CTLS in the medium was evaluated. For the purpose of the investigation, changes in: the medium pH, biomass production, protein content, protease activity, the amino acid rate in the medium, and metal concentration in the biomass and supernatant were determined.

2. Material and methods

2.1. Biological material

The yeast was isolated from soil samples. The soil samples were collected in spring from the area of the former "BORUTA" dye factory in Lodz, Poland (51°50'35.4"N 19°23'33.5"E). The purified isolate was subjected to Sanger sequencing and identification at Nexbio in 2022 (Wroclaw, Poland). The yeast isolates were identified on the basis of the 18S rRNA gene sequence. The sequence obtained was aligned with nucleotide sequences available in the NCBI database using BLAST (Supplementary material 1). *Y. lipolytica* IPS21 from the pure culture collection of the Łukasiewicz Research Network - Lodz Institute of Technology was maintained on "modified YPG" medium: yeast extract – 15.0 g, sodium chloride – 5.0 g, agar – 25.0 g per litre of distilled water, stocks at 4 °C.

2.2a Carbon source material (substrates)

A "modified YPG liquid medium" containing: yeast extract – 15.0 g (BTL Sp. z o.o.) and NaCl – 5.0 g (Chempur) was used as the basic liquid medium in this study. Subsequently, one of the additional carbon sources was introduced into the medium: glucose (Chempur), saccharose (Chempur), oat (from the oat crop of 2022 from a farm in the Lodz province), glycerine (Eurochem BGD Sp. z o.o.), pectin (P.P.H.U Eccodin Sp. z o.o.), semolina (MW FOOD Sp. z o.o.), chrome-tanned leather shavings – CTLS (tannery in Lodz, Poland).

2.2b Inoculation of culture medium and production of inoculum

The inoculation liquid medium contained: yeast extract - 15.0 g; NaCl – 5.0 g; distilled water to 1 L, pH 7.0. Inoculation cultures were inoculated with yeast suspension from 'modified YPG' slants. A slant was used to prepare the inoculum medium, which was added with 10 mL of 0,85% saline. The whole was then transferred to Erlenmeyer flasks, comprising 30 ml of the 'modified YPG liquid medium'. Cultures were carried out in 100 mL Erlenmeyer flasks containing 40 mL of the inoculation medium at 140 rpm for 24 h at 30.0 °C using a Biosan ES-20 environmental shaker incubator (Biosan).

2.3a Selection of carbon sources for protease production

The liquid medium contained: yeast extract - 15.0 g; NaCl – 5.0 g; different carbon sources - equivalent to 15 g of carbon sources, distilled water up to 1 L, pH 7.0. The production cultures were inoculated with cell material taken from the inoculation medium. To 100 mL flasks filled with the production medium, 0.4 mL of inoculum was added at OD=1. Cultures were grown at 210 rpm for 72 h at 30.0 °C on a rotary shaker type Biosan ES-20 environmental shaker-incubator (Biosan) GmbH, Germany.

2.3b Selection of the amount of nitrogen sources in the CTLS (waste) medium for protease production

The liquid medium contained: 3 different amounts of yeast extract – 5.0 g, 10.0 g; 15.0 g, CTLS – 15.0 g, NaCl – 5.0 g; distilled water up to 1 L, pH 7.0. The production cultures were inoculated with cell material taken from the inoculation medium. To 100 mL flasks filled with the production medium, 0.4 mL of inoculum was added at OD=1. Cultures were grown at 210 rpm for 72 h at 30.0 °C on a rotary shaker type Biosan ES-20 environmental shaker-incubator (Biosan) GmbH, Germany.

2.4. Control parameters

The weight of dry yeast biomass was measured, after filtering the cells under pressure using Whatman GC/F filters (100 mm in diameter), and then washing the cells with acetone, hexane (3:1), and sterile water. The cells were dried to a constant weight at 80 °C. The results

are expressed in g g^{-1} of carbohydrate substrate.

The Lowry method for **protein quantitation** was used according to the literature method [18] .

The pH change was measured every 24 hours using an Elmetron CP411 pH meter. Measurements were made for a 5 ml post-culture liquid sample.

Proteolytic activity was assessed according to the literature [19, 20]. The reaction mixture contained 200 µL of medium supernatants and 200 µL of 5 % azocasein (w/v). The azocasein was dissolved in 0,1 M Tris HCl. The samples were then incubated at 40 °C for 40 min. To inhibit the reaction, 1 % (w/v) trichloroacetic acid (TCA) was added. The ratio of TCA to the reaction mixture was 3 to 1. After 10 min, samples were centrifuged (4000 rpm, 10 min). In the last step, the supernatant (1000 µL) was neutralized by adding 1000 µL of 1.0 mol L^{-1} NaOH. Absorbance at 440 nm was measured on a UV/Vis spectrophotometer (Rayleigh UV-9200). One unit of proteolytic activity was defined as an increase of 0.01 in 40 min at A440 absorbance.

Amino acids in the supernatants were determined by HPLC/DAD/FLD. All formulations tested were subjected to acid hydrolysis at 105 °C for 24 hours [21]. The derivatization procedure was applied. All samples were filtered through 0.45 µm membrane filters: Acrodisc 13 mm syringe filters, GHP (Waters, Milford, MA, USA). A Shimadzu Prominence-i LC-2030C, pump, autosampler injector, Nova-Pak C18, 4 µm column (150×3.9 mm, Shimadzu, Kyoto, Japan) was used for amino acid analysis. The column was thermostated at 37 °C with a flow rate of 1.0 cc/min and 10 µL (amino acid concentration 5-200 pmol) was injected. A gradient mobile phase was used for the chromatography. The mobile phase consisted of eluent A (prepared from Waters AccQ-Tag Eluent A concentrate by adding 200 cc of concentrate to 1 L of distilled water), eluent B (60 % acetonitrile).

Metals (Ca, Cr, Fe, K, Mg, Na, P, S) were determined in biomass and supernatants using atomic emission spectrometry with an inductively coupled plasma technique (ICP/AES). Samples of 0.2 g (biomass) and 5 g (supernatants) were placed in a Teflon vessel and 7 mL of HNO_3 (65 % wt, Chempur, Piekary Slaskie, Poland) was added. They were then mineralized using a Magnum II microwave mineralizer (Ertec, Wroclaw, Poland). The sample was then transferred to a 25 mL volumetric flask. The contents of the tested metals (Agilent ICP-OES 5110 spectrometer Santa Clara, USA) in the samples were read from the standard curves prepared from the ICP standard.

2.5. Statistica

Statistica 10.0 (13) was used for mathematical and statistical analyses. Differences were determined by analysis of variance (ANOVA) and the Tukey-Kramer test, also with ± standard errors (SD). The probability level was set at 0.05 for three determinations.

3. Result and discussions

3.1. Characteristics of the biomass amount, proteases activity and pH medium on different carbon sources.

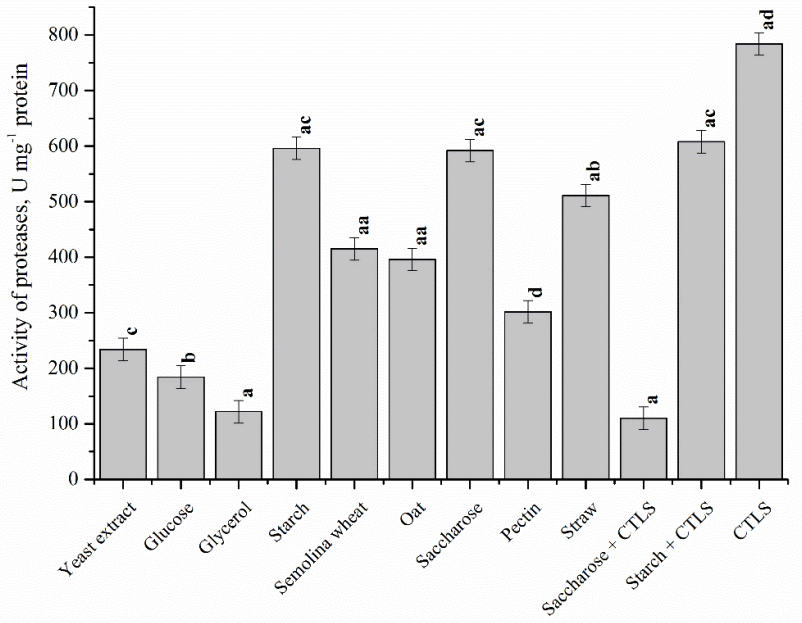
The results for *Y. lipolytica* IPS21 biomass production and total protein obtained from different carbon sources are shown in Table 1. The highest biomass production was observed on glycerol (0.465±0.3 g g^{-1} of carbohydrate substrate) and glucose (0.452±0.11 g g^{-1} of carbohydrate substrate). These data are consistent with literature reports that *Yarrowia* yeast prefers glycerol and glucose for growth [22,23]. The lowest biomass production was obtained with CTLS as a carbon source (0.204±0.09 g g^{-1} carbohydrate substrate). The lower biomass production is due to the more complex structure of the protein waste, in which the availability of carbon atoms for efficient biomass formation is greatly reduced. The CTLS used in the study contains collagen cross-linked with a chromium complex [24]. The effect of the change in the carbon source on the increase in biomass was observed, but not on the amount of protein determined.

The amount of total protein, ranging from 109±1.99 to 130±2.33 µg mL^{-1} (Table 1), is not statistically different among the variants tested. This is probably due to the fact that biomass is a complex mixture of different components such as polysaccharides, lipids and polypeptides [25]. Other studies have shown that high levels of extracellular proteins (proteolytic enzymes) are not necessarily associated with high biomass production [25]. Getting the right amount of extracellular protein secreted by the cell is more important. This is consistent with what we observed with protease activity - Figure 1. The highest value of protease activity was obtained for the CTLS variant as a carbon source of 800 U mg^{-1} of protein (Fig. 1); when the production of biomass was low (Table 1). CTLS waste proved to be the most favorable substrate for enzyme biosynthesis among the carbon sources used in the experiments. The lowest enzyme activity was observed in the variant with glycerol or saccharose and CTLS. For both variants, it was slightly below 100 U mg^{-1} protein. In this case, the metabolism was “switched” to polyol production due to the presence of glycerol as a carbon source in the medium. [26]. The ability of *Y. lipolytica* to produce erythritol on glycerol-containing media has been extensively described in the literature [27]. When saccharose was added to the medium with CTLS (Fig. 1), the protease activity decreased too. Similar data were obtained by Akpınar et al. (2011). They examined media supplemented with skimmed milk, glutamine and glucose. Adding glucose or glutamine to skimmed milk had a negative effect on the overall metabolic transformations of the *Yarrowia* strain, which was reflected in decreased synthesis of proteolytic enzymes [28]. Similar results were reported by Ogrydziak et al. (2003). They showed that the secretion of proteases and ribonucleases from yeast cells of the genus *Yarrowia* can be inhibited by some

Carbon source	Biomass production, g g^{-1} of carbohydrate substrate	Total protein, µg mL^{-1}
Yeast extract	0.385 ± 0.02[d]	120 ± 5.20[a]
Glucose	0.452 ± 0.11[aa]	124 ± 4.73[a]
Glycerol	0.465 ± 0.03[aa]	120 ± 6.55[a]
Starch	0.401 ± 0.02[d]	114 ± 4.66[a]
Semolina wheat	0.225 ± 0.09[a]	111 ± 7.22[a]
Oat	0.317 ± 0.12[b]	109 ± 1.99[a]
Saccharose	0.364 ± 0.09[c]	130 ± 2.33[a]
Pectin	0.299 ± 0.16[b]	128 ± 2.98[a]
Straw	0.364 ± 0.04[c]	112 ± 7.66[a]
Saccharose + CTLS*	0.354 ± 0.04[c]	124 ± 8.13[a]
Starch + CTLS	0.320 ± 0.08[b]	120 ± 5.78[a]
CTLS	0.204 ± 0.09[a]	110 ± 2.59[a]

**CTLS -chrome-tanned leather shavings*

Table 1. Influence of the carbon source on the biomass and yeast Y. lipolytica IPS21 protein concentration. Values for single elements are not significantly different (P < 0.05) according to Tukey's Kramer test for multiple comparisons of the biomass or total protein marked with the same letter



**CTLS - chrome-tanned leather shavings*

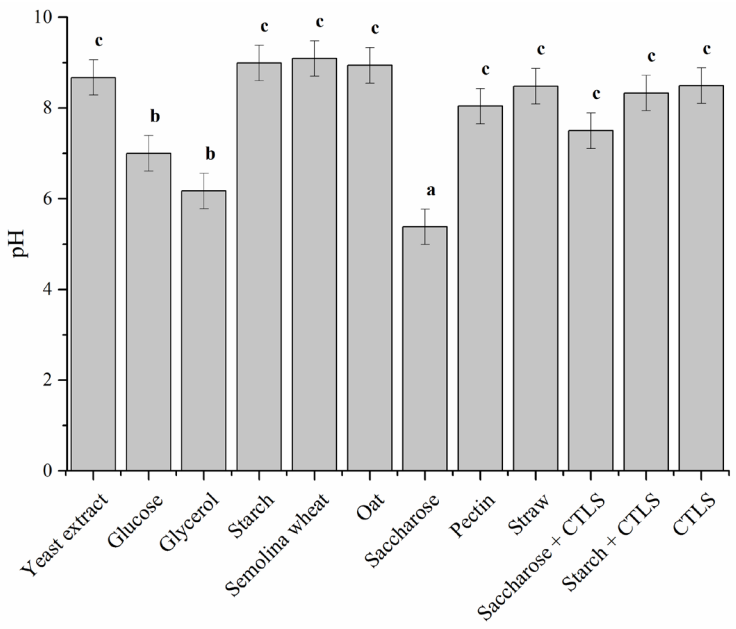
Fig. 1. Influence of the carbon source on the activity of the proteases of the yeast Y. lipolytica IPS21. Values for single elements are not significantly different (P < 0.05) according to Tukey's Kramer test for multiple comparisons of supernatants marked with the same letter

additional sources of carbon and nitrogen in the medium [29].

A number of environmental factors are known to influence the process of protease biosynthesis [13,30]. One of the most important factors in the production of proteolytic enzymes is the pH of the medium [31]. In many of the variants tested, a pH above 8.00 was achieved (Figure 2). Our results confirm that a pH above 7.00 may be optimal for alkaline protease synthesis (Figure 1 and Figure 2) [28]. The lowest pH of the medium was determined for saccharose, glucose, and glycerol variants. The yeast *Y. lipolytica* hydrolyses saccharose to D-glucose and D-fructose. These sugars are incorporated into the glycolysis pathway. The by-products of glycolysis lower the pH of the medium. In turn, the metabolism of glycerol can lead to the formation of citric acid, which also lowers the pH of the medium. Our results confirm previous reports that multifactorial optimization of enzyme biosynthesis conditions is necessary to produce high levels of proteases in culture supernatants [32]. It is not sufficient to check only one of the parameters. We showed that changing the sugar source had a greater effect on the proteolytic activity of *Y. lipolytica* IPS21 than the pH. This may be due to the fact that the pH of the medium was not under control during the process, which is normally used to produce industrial enzymes [31].

3.2. Concentrations of the amino acids in supernatant on waste or lack of carbon sources (only yeast extract or yeast extract with CTLS)

In the research, the highest proteolytic activity was found for the CTLS variant and much lower for the yeast extract variant, thus it was decided to compare the amounts of amino acids in the respective supernatants. Casein or yeast extract was reported as the main source of nitrogen and carbon for maximum protease production by yeast [13]. By comparing the retention time with a standard sample, 22 amino acids were identified. The results of the experiments are presented in Table 2. The total amount of amino acids identified in the supernatant was the highest in the CTLS medium with *Y. lipolytica* IPS21 – 2.78 g 100 g^{-1} of supernatant. The increase in selected amino acids in these samples may indicate the intensification of the degradation of CTLS by *Y. lipolytica* or the secretion of proteases into the medium [33, 34, 35]. The lowest total amount of amino acids identified were found in a variant with the yeast extract medium with *Y. lipolytica* IPS21 – 0.12 g 100 g^{-1} of supernatant. The yeast *Y. lipolytica* IPS21 can use amino acids as a substrate for biomass formation [36]. Compared to the literature data [13,31], the amount of extract in our medium was lower than in other studies. Almost all amino acids were incorporated into the biomass.



**CTLS -chrome-tanned leather shavings*

Fig. 2. Influence of the carbon source on the pH of the medium during the biological process with the yeast Y. lipolytica IPS21. Values for single elements are not significantly different (P < 0.05) according to Tukey's Kramer test for multiple comparisons of the medium marked with the same letter

3.3. Characteristics of the biomass, activity of proteases with different concentrations of yeast extract (nitrogen sources) and the same concentration of waste CTLS in the medium

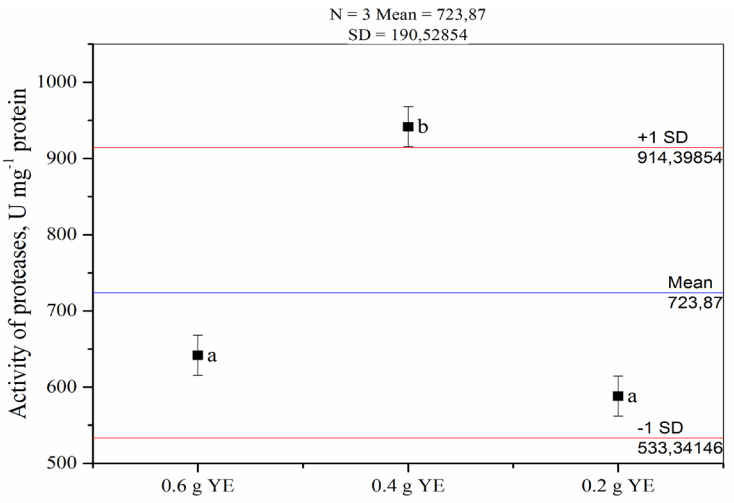
In the second set of studies, it was decided to test the effect of reducing the amount of yeast extract in the CTLS medium. In the first series, the highest protease activity was obtained in the medium containing CTLS and yeast extract. Yeast extract is the product of yeast cells, containing amino acids, lipids, vitamins, minerals, and other soluble components. It is considered to be a good source of nitrogen. The biomass production of the yeast *Y. lipolytica* IPS21 and the total protein at different levels of yeast extract in the CTLS medium are shown in Table 3.

Name of acid	Yeast extract medium with *Yarrowia*	CTLS medium with *Yarrowia*	Yeast extract medium without *Yarrowia*	CTLS medium without *Yarrowia*
	g 100 g^{-1} of supernatant			
Aspartic acid	0.03	0.22	0.07	0.13
Threonine	<0.02	0.05	0.07	0.04
Serine	<0.02	0.10	<0.02	0.06
Glutamic acid	0.09	0.29	0.09	0.26
Proline	<0.02	0.33	0.03	0.25
Glycine	<0.02	0.55	0.02	0.37
Alanine	<0.02	0.21	0.06	0.20
Valine	<0.02	0.05	0.05	0.06
Methionine	<0.02	<0.02	<0.02	<0.02
Isoleucine	<0.02	0.19	0.03	0.05
Leucine	<0.02	0.08	0.06	0.08
Tyrosine	<0.02	0.03	0.03	0.03
Phenylalanine	<0.02	0.07	0.04	0.05
Ornithine	<0.02	<0.02	<0.02	<0.02
Gamma-Aminobutyric acid	<0.02	<0.02	<0.02	<0.02
Lysine	<0.02	0.09	0.05	0.12
Histidine	<0.02	0.03	<0.04	<0.02
Arginine	<0.02	0.17	0.03	0.11
Taurine, 2-aminoethanesulfonic acid	<0.02	<0.02	<0.02	<0.02
Hydroxyproline	<0.02	0.30	<0.02	0.17
Cysteine	<0.02	<0.02	<0.02	<0.02
Hydroxylysine	<0.02	0.04	<0.02	<0.02
Sum of amino acid content (mean value)	**0.12**	**2.78**	**0.63**	**1.98**

Table 2. Composition of amino acids in the supernatant after 48 hours of biological treatment with Y. lipolytica IPS21

Carbon sources	Biomass production, g g^{-1} of carbohydrate substrate	Total protein, µg m^{L-1}
0.6 g yeast extract	0.205 ± 0.14[a]	112.4 ± 6.55[c]
0.4 g yeast extract	0.375 ± 0.16[c]	89.2 ± 4.22[b]
0.2 g yeast extract	0.316 ± 0.09[b]	64.2 ± 4.33[a]

Table 3. Influence of the amount of yeast extract in the medium with 0.6 g of CTLS on the biomass and protein concentration of the yeast Y. lipolytica IPS21. Values for single elements are not significantly different (P<0.05) according to Tukey's Kramer test for multiple comparisons of biomass or the total protein marked with the same letter



**YE – yeast extract*

Fig. 3. Influence of the amount of yeast extract in a medium with 0.6 g of CTLS on the activity of proteases of Y. lipolytica IPS21 yeast. Values for single elements are not significantly different (P < 0.05) according to Tukey's Kramer test for multiple comparisons

The highest biomass production was determined after the addition of 0.4 g of yeast extract – 0.375±0.16 g g^{-1} as a carbohydrate substrate. However, the test with 0.6 g of yeast extract in the CTLS medium showed the highest total protein 112.4±6.55 µg mL^{-1} (Table 3). The results obtained are in line with previous work [37,38], showing the different adaptation processes in yeast and bacteria exposed to environmental stress, such as changes in carbon sources. The protease activities of the IPS21 yeast were also determined – Figure 3. The highest proteolytic activity of 941.0±21.55 U mg^{-1} protein was observed in the variant with 0.4 g of yeast extract in the CTLS medium. The potential of *Yarrowia* to produce proteolytic enzymes was affected by changing the amount of yeast extract. A factor in the reduction of protease production may have been too little or too much yeast extract in the CTLS medium.

The differentiation of these results shows the importance of the carbon source in the process of the formation and secretion of proteolytic enzymes from the cell, which is consistent with the literature [29]. The data obtained show how important it is to have a ratio of carbon and nitrogen sources in a medium with *Y. lipolytica* IPS21. A small change can significantly slow down the secretion of enzymes.

3.4. The concentration of metals in the biomass and supernatant after the biological process with different concentrations of yeast extract (nitrogen sources) and the same concentration of waste CTLS in the medium

Yeast cells, through their inherent mechanisms, can easily adapt to, tolerate, and detoxify metal-contaminated environments. *Yarrowia* strains can be used to accumulate different metals [39, 40]. It was therefore decided to determine the concentrations of selected metals in biomass and cells. The presence of the metals in the samples was related to that of metals in the medium substrates. The results for the metal content of selected samples regardless of the amount of yeast extract in the medium indicate that metals accumulate primarily in the biomass and not in the supernatant (Table 4).

Significantly different amounts of elements were found in the biomass. The biomass of the yeast *Yarrowia* IPS21 seems to be a useful waste from the point of view of the elemental composition due to the high content of Ca 3.16 g kg^{-1}, K 5.16 g $kg^{-1,}$ and P 16.02 g kg^{-1}. These elements are essential for proper plant growth and development [43]. The biomass obtained in the process of protease production could also be used as a biostimulator or soil conditioner [44]. The literature also describes the possibility of the absorption of dyes from textile effluents by yeast. Adsorption on biomass may be one of the basic mechanisms for removing pollutants from textile wastewater in aerobic biological treatment systems [41, 42]. In future research biomass of yeast IPS21 could be used to adsorb dyes from textile effluents.

4. Summary

This article shows the effect of different carbon and nitrogen sources on proteolytic activity. It was confirmed that the yeast *Y. lipolytica* IPS21 can have a higher proteolytic activity in the presence of waste carbon sources (CTLS) than on the yeast extract alone. This is confirmed by the high concentration of amino acids in samples with CTLS, suggesting increased degradation of CTLS by *Y. lipolytica* or the secretion of proteases into the medium. It was also confirmed that metals accumulate mainly in the biomass and not in the supernatant. The biomass was also found to contain high levels of Ca, K, and P, which are essential for plant growth. Therefore, the results obtained lead to the following conclusions:

1. *Y. lipolytica* IPS21 yeast can be used for the degradation of protein waste or for the biotransformation of chrome-tanned leather shavings.

Metal / Variant	Ca	Cr	Fe	K	Mg	Na	P	S
				g kg^{-1}				
0.2 g YE biomass	3.16 ± 0.21^{a}	3.68 ± 0.30^{a}	0.55 ± 0.02^{a}	5.16 ± 0.55^{a}	2.44 ± 0.13^{a}	15.44 ± 1.22^{a}	16.02 ± 1.45^{a}	3.36 ± 1.24^{a}
0.4 g YE biomass	1.92 ± 0.11^{b}	3.33 ± 0.22^{a}	0.51 ± 0.02^{a}	4.06 ± 0.45^{b}	2.12 ± 0.18^{b}	9.81 ± 1.18^{b}	19.89 ± 2.66^{b}	5.15 ± 0.12^{b}
0.2 g YE supernatant	0.10 ± 0.01^{a}	0.54 ± 0.01^{a}	0.09 ± 0.01^{a}	0.25 ± 0.08^{a}	0.05 ± 0.32^{a}	3.0 ± 0.54^{a}	0.09 ± 0.02^{a}	0.52 ± 0.13^{a}
0.4 YE supernatant	0.10 ± 0.01^{a}	0.53 ± 0.02^{a}	0.10 ± 0.04^{a}	0.26 ± 0.02^{a}	0.06 ± 0.01^{a}	3.3 ± 0.03^{a}	0.08 ± 0.01^{a}	0.57 ± 0.03^{a}

Table 4. Metal concentrations in selected samples of biomass with Y. lipolytica IPS21 yeast or a supernatant after the process of protease production in a medium with 0.6 g of CTLS and different amounts of yeast extract. Values for single elements are not significantly different (P<0.05) according to Tukey's Kramer test for multiple comparisons of the biomass or supernatant marked with the same letter

2. *Y. lipolytica* IPS21 yeast may have future applications in the production of extracellular alkaline proteases.

Further studies are needed to investigate the effect of different mechanisms at work in *Y. lipolityca* IPS21 and biological products obtained with the inclusion of IPS21 yeast. There is a need to define their beneficial effects and identify product formulations and application methods that will optimize their benefits under different applications.

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