Research Article

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Green "one-pot" fluorescent bis-indolizine synthesis with whole-cell plant biocatalysis

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Abstract: An efficient one-pot route leading to bis-indolizine symmetric compounds has been developed via a new approach from the dipyridinium heterocyclic compound, reactive halogenated derivative, and activated alkyne through biocatalysis. A set of local plants was evaluated for its catalytic potential in "one-pot" biocatalysis of these valuable fluorescent compound synthesis reactions. Most of these biocatalysts containing enzymes from the oxidoreductase class (peroxidase: 0.56–1.08 mmol purpurogallin·g^{-1} fresh weight·min^{-1}, polyphenol oxidase (PPO) : 27.19–48.95 PPO units·mg tissue^{-1}, CAT: 3.27–21.71 μmol O_2·g^{-1} fresh weight·min^{-1}), were used as green catalysts in the multi-component cycloaddition reaction, in an aqueous buffer solution, for the production of bis-indolizine compounds in moderate to excellent yields (45–85%). The horseradish root (*Armoracia rusticana*) has been selected as the most promising biocatalyst source among the evaluated plants, and the obtained yields were greater than in the conventional synthesis method. The structures of indolizine derivatives were confirmed by nuclear magnetic resonance spectra, elemental analyses, as well as Fourier transform-infrared spectra. The cytotoxicity of the latter obtained indolizine compounds on the growth of the model microorganism, *Saccharomyces cerevisiae* MIUG 3.6 yeast strain, was also evaluated. Various parameters (number of generations, growth rate, generation time, dry matter yield, the degree of the budding yeast cells, and the degree of yeast autolysis, fermentation intensity), which describe the yeast growth, suggest that the nutrient broth supplemented with different concentrations of bis-indolizine compounds (10 and 1 μM) had no toxic effect on the yeast strain growth, under submerged cultivation conditions.

Keywords: biocatalysis, green synthesis, enzyme, indolizine, cytotoxicity screening

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1 Introduction

The synthesis of N-fused heterocycles has been a topic of research interest for over a century because this class of compounds has been found in many natural products and synthetic drugs as their basic core structure which possesses a variety of important biological activities [1–3]. In recent years, numerous studies have been performed to obtain molecules for pharmaceutical purposes through biocatalysis which are remarkable because of their numerous advantages such as minimizing the use of toxic solvents, reducing the reaction time, and lowering the costs [4–7]. There is a growing need to use environmentally friendly synthetic methodologies to reduce the use of toxic solvents [8]. The development of "green" concepts has determined the orientation towards new ecological methods in organic chemistry [9–12]. Biocatalysis encompasses the use of biological systems, such as whole cells from microorganisms, microalgae, or plants, or isolated enzymes, as catalytic systems in chemical synthesis [13]. Biocatalyzed reactions by enzymes from whole-cell sources, which are the frame of "green chemistry," present many advantages such as high selectivity, mild reaction conditions, high yields, decreased reaction time, fewer steps, low toxicity, or production of

ecological waste [14–18]. It can often be difficult to identify optimized conditions for such reactions, as several undesirable effects may occur due to the reaction conditions. Therefore, these processes require designing the synthesis route, choosing biocatalyst sources, and establishing optimal reaction conditions such as solvent, temperature, and pH [5]. The use of enzymes in synthetic transformations is steadily increasing as an alternative source of catalysis [19–21]. Many studies have successfully used isolated pure enzymes in organic transformations but their uses require special materials and techniques, therefore they are often expensive and not economically advantageous [14,19,22].

Various approaches have been previously reported in the literature for indolizine synthesis, including conventional multi-step synthesis of bis-indolizines from 4,4′-bis-pyridinium salts [23–25] and one-step synthesis by other methods [24,26–28]. The principal methods for the synthesis of indolizine compounds refer to dipolar cycloaddition reactions of pyridinium and related heteroaromatic ylides with electron-deficient alkenes or alkynes [29], C–H functionalization reactions [30], cyclization of pyridine derivatives with alkenes, alkynes or carbonyl compounds, cycloisomerization transformation, Scholtz reaction, and Chichibabin reaction [24,27]. An efficient and economic synthesis of 3-benzoyl indolizine was recently reported through a reaction of picolinium salts and dimethylformamide-dimethyl acetal (DMF-DMA) with triethylamine base in DMF solvent by using microwave irradiation as a “greener” method with a better yield than the conventional method [24]. Indolizine derivatives were obtained by another green method through one-pot 1,3-dipolar cycloadditions in recyclable imidazolium ionic liquids ([Omim]Br) in good yields (78–87%) and broad substrate scope [27]. Indolizines were also obtained by irradiation of 2-benzoyl-*N*-benzylpyridinium derivatives and dimethyl acetylene dicarboxylate [31]. A catalyst-free annulation of 2-pyridylacetates and ynals under molecular oxygen was proposed to obtain 3-acylated indolizines in very good yields (up to 91%) in toluene at 100°C [28]. Pyrrolo[2,1,5-*cd*]indolizine derivatives were obtained by visible-light-induced intermolecular [3 + 2] annulations with internal alkynes in very good yields (58–91%) in dimethyl sulfoxide, in the presence of rose bengal and KI at room temperature for 10 h under 20 W blue LED [32]. In 2023, a mild and metal-free synthesis of indolizines using pyridinium salts and alkenes with ambient air as a sole oxidant to catalyze [3 + 2] annulation was reported [25]. Indolizine derivatives were obtained in good yields (up to 89%) in DMSO at 100°C by treatment of homopropargyl pryidines with cesium carbonate, involving α-C–H deprotonation, 5-*exo-dig* cyclization, and isomerization of the exocyclic double bond [33]. Pyridinium 1,4-zwitterionic thiolate reaction with a copper carbene was reported for the synthesis of indolizine scaffolds with yields of up to 90% [34]. Although studies with less toxic syntheses of indolizine compounds or without metal catalysts are presented in the specialized literature, to our knowledge, synthesis by biocatalysis with plant enzymes in aqueous buffer solution has not been reported so far.

Several indolizine derivatives have been reported to exhibit a wide spectrum of applications in many fields such as chemistry, biology, pharmaceutical, food industries, and medicine [35–37]. This class of bridgehead-*N*-fused heterocyclic compounds demonstrated valuable biological activities [38–40] such as antimicrobial [41–45], antioxidant [35], anticancer [41], enzyme inhibition [40,46–48], photoluminescence [49,50], calcium-entry blocking [51,52], and anti-inflammatory [53]. These compounds are also studied as molecules of interest in material science due to their fluorescence [37,50,54,55] or electric properties [56,57]. Therefore, the development of novel and efficacious methods for the design and synthesis of indolizine derivatives is of significant concern.

In order to be able to begin the selection of a possible drug candidate, the target compounds must be low or free of toxicity. In this context, numerous studies have evaluated the cytotoxicity of natural or synthetic chemical compounds by using biological models [58–60]. In several studies, the yeast *Saccharomyces cerevisiae* is noted for its remarkable and versatile resemblance to various eukaryotic species including human cells, in cellular molecular mechanisms related to basic cellular processes, and is used as a great eukaryotic model for exploring the effects of cytotoxic compounds due to its well-featured metabolic, genetic, and morphological characteristics [61–64].

Given the considerable increase in the importance of indolizine derivatives and also the essential role of natural sources as chemical catalysts, this work aimed to obtain indolizine derivatives by an attractive route of multi-enzymatic catalysis. In this direction, the synthesis of compounds with indolizine core was obtained by a one-pot, three-component procedure through a novel route by using whole-cell plant systems (Figure 1). The main benefit of this approach is that indolizine derivatives with valuable biological properties can be obtained straightforwardly in good yields by an accessible and environmentally friendly method, under mild reaction conditions, in high yields, and in short reaction times. Moreover, this approach for the effective implementation of synthetic transformations presents significant economic and environmental impacts. The cytotoxicity of these compounds was also investigated on the *Saccharomyces cerevisiae* model yeast cells, during the multiplication and alcoholic fermentation processes.

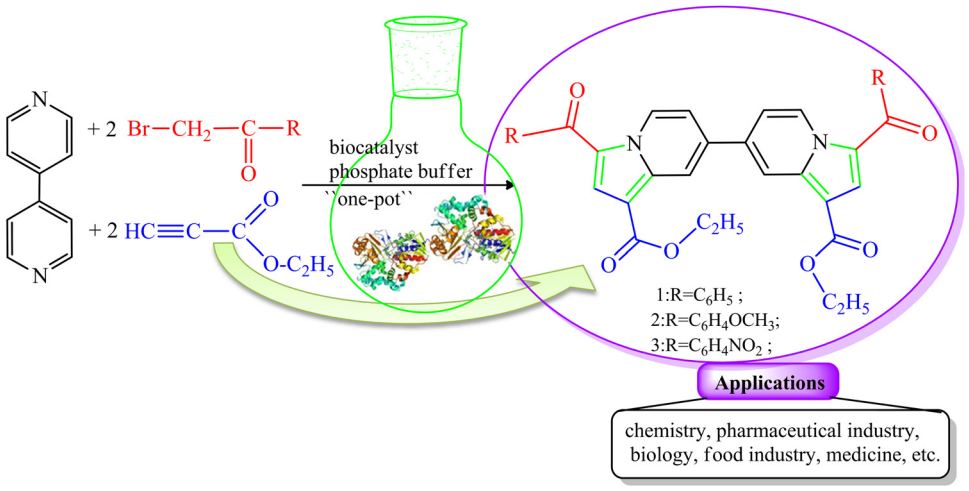


Figure 1: General pathway for the biocatalyzed synthesis of indolizine derivatives (**1**, **2**, **3**) in the presence of plant enzymes in buffer solution, at room temperature.

2 Materials and methods

2.1 Materials

All chemicals and solvents were of analytical grade and commercially available from Merck KGaA (Merck KGaA, Darmstadt, Germany) and Fluka (Honeywell Fluka™, Fluka, Germany).

The plant materials used in this study as biocatalyst sources were horseradish roots (*Armoracia rusticana*), celery roots (*Apium graveolens*), parsnip roots (*Pastinaca sativa*), parsley root (*Petroselinum crispum*), pumpkin sprouts (*Cucurbita pepo*), faba bean sprouts (*Vicia faba*), and soy sprouts (*Glycine max*). All plants were purchased from local markets and farms (Galati, Romania) so that they were as fresh as possible. These are common plant varieties that can be found in local markets in Romania. The sprouts were obtained by the previously described method [26].

The yeast strain used in the study, *Saccharomyces cerevisiae* MIUG 3.6, was provided by the Microorganisms Collection (acronym MIUG) of the Research and Education Platform (Bioaliment) of "Dunărea de Jos" University of Galati, Romania. The pure stock cultures were maintained by cultivation on the yeast extract peptone dextrose (YPD) agar medium and preserved at 4°C.

2.2 Experimental procedures

The activities of oxidant enzymes (catalase, peroxidase, and polyphenol oxidase [PPO]) were determined for each plant sample. The enzymatic assays were performed by microspectrophotometric adapted methods.

2.2.1 Peroxidase activity assay

The determination of peroxidase (EC 1.11.1.7) activity is based on the conversion of H_2O_2 to H_2O and O_2 in the presence of a hydrogen donor (pyrogallol) and an adapted microplate assay was used [65–67]. Pyrogallol is oxidized to purpurogaline, a colored compound whose absorbance was measured at λ = 430 nm. To prepare the enzymatic extract, 1 g of each plant was crushed by using a commercial machine (Tefal Frutelia ZE420D38, 11, 400 W, Groupe SEB, Romania), and 5 mL of 0.1 M phosphate buffer (pH 6.5) was added. The mixture was centrifuged at 9,000 rpm, for 30 min, at 4°C, and the centrifuged supernatant was further used as a crude enzymatic extract. The test samples were obtained by mixing 25 µL of 1% H_2O_2, 120 µL of 50 mM pyrogallol solution, and 5 µL of the enzymatic extract. The control sample was obtained by mixing 145 µL of 50 mM pyrogallol solution with 5 µL of the enzymatic extract, while the blank consisted of the solvent mixture. The samples were incubated at 25°C for 5 min, and the reaction was stopped by adding 50 µL of 1 M H_2SO_4 to the reaction mixture. The change in absorbance was measured in a 96-well plate with a multiplate reader at 430 nm (iTecan Pro 200, Tecan Trading AG, Männedorf, Switzerland). The peroxidase activity was determined as mmol purpurogallin formed per gram of fresh weight per min.

2.2.2 Catalase activity assay

The determination of catalase (CAT, EC 1.11.1.6) activity was performed by an adapted microplate method [68,69]. This method is based on the measurement of the UV absorption

of H_2O_2 degradation at λ = 240 nm. The decrease in absorbance occurs when hydrogen peroxide is degraded by catalase [70]. Enzymatic extracts were prepared by crushing 1 g of each plant with a commercial machine (Tefal Frutelia ZE420D38, 11, 400 W, Groupe SEB, Romania) and 5 mL of 67 mM phosphate buffer (pH 7.0) was added. The crude enzymatic extract was separated by centrifugation at 9,000 rpm, for 30 min at 4°C. The test sample was obtained by mixing 145 µL of 2 mM H_2O_2 and 5 µL of the enzyme extract. The control sample was obtained by mixing 145 µL of 0.067 M phosphate buffer (pH 7.0) with 5 µL of the enzymatic extract. The samples were analyzed in a 96-well plate with a multi-plate reader (Tecan Pro 200, Tecan Trading AG, Männedorf, Switzerland). One enzymatic unit represented the amount of enzyme needed to decrease the absorbance at λ = 240 nm by 0.05 units in 1 min, under the analyzed reaction conditions [70].

2.2.3 PPO activity assay

The determination of polyphenol-oxidase activity is based on proteins from polyphenol oxidases that catalyze the aerobic oxidation of phenolic substrates to quinones, which self-oxidizes and forms dark-brown pigments, known as melanin, which are spectrophotometrically determined at λ = 495 nm [71,72]. The enzymatic samples were prepared by mixing 1 g of plant with 5 mL of phosphate buffer (pH 6.8). After centrifugation at 9,000 rpm, for 30 min at 4°C, the supernatant was filtered through Whatman No. 1 filter paper and was further used as the crude enzymatic extract. The samples were obtained by mixing 50 µL of 0.1 M methyl catechol, 100 µL of 0.1 M phosphate buffer (pH 6.8), and 50 µL of crude enzymatic extracts. The absorbance was measured with a multi-plate reader (Tecan Pro 200, Tecan Trading AG, Männedorf, Switzerland) at 495 nm. The results were expressed as PPO units per mg of fresh weight.

2.3 Optimization of reaction conditions for whole-cell plant biocatalysis

The fresh plant materials of each selected species were washed with sterile water and crushed using a commercial blender (Tefal Frutelia ZE420D38, 11, 400 W, Groupe SEB, Romania). After that, 0.5–2 g of the plant (*A. rusticana*) were introduced into 25 mL sealed Erlenmeyer flasks with stoppers, containing 10 mL of phosphate buffer (pH = 6.0–8.0), and further used as whole-cell plant enzymatic systems. The preliminary optimization experiments were carried out by using the horseradish root as a biocatalyst source. The reactions were performed in 25 mL sealed Erlenmeyer flasks. The reaction mixture consisted of horseradish root enzymatic catalytic systems, 0.1 mmol dipyridinium heterocyclic compound (4,4′-dipyridyl), 0.25 mmol reactive halogenated derivative (ω-bromoacetophenone), and 0.25 mmol activated alkyne (ethyl propiolate). The reaction mixtures were continuously stirred (300 rpm) for up to 168 h. The effects of pH (6.0–8.0), the amount of plant (0.5–2.0 g), the reaction time, and temperatures (20–40°C) were monitored. The progress of each reaction was followed by thin-layer chromatography (TLC) analysis at various time intervals. The compounds were extracted and then purified from the reaction medium by extraction with chloroform and precipitated with methanol. Indolizine derivative structures and purities were verified by melting points, TLC, Fourier transform-infrared (FT-IR) spectroscopy and elemental analysis.

2.4 Indolizine “green-synthesis” reactions with whole-cell plant biocatalysts

The reaction mixture consisted of each enzymatic plant catalytic system, 0.1 mmol dipyridinium heterocyclic compound (4,4′-dipyridyl), 0.25 mmol reactive halogenated derivatives (ω-bromoacetophenone, 2-bromo-4′-methoxyacetophenone, 2-bromo-4′-nitroacetophenone), and 0.25 mmol activated alkynes (ethyl propiolate). The reaction mixtures were continuously stirred (300 rpm) at room temperature, for up to 120 h. The progress of each reaction was monitored by TLC and FT-IR analysis (Nicolet iS50, Thermo Scientific, United States) at various time intervals. For TLC analyses, the chromatography mixture was cyclohexane/ethyl acetate = 3:1 (*v*/*v*). The compounds were extracted and then purified from the reaction medium by extraction with chloroform. The results represent the average of three individual experiments.

2.5 Separation and purification of bioconversion products

Solvent extraction was used to separate the medium of the reaction and also for the separation of the reaction products. The reaction products were separated through liquid–liquid extraction with 10 mL of chloroform, three times. The organic layer was further washed with 10 mL of ultrapure water, three times, to remove any secondary products such as the intermediate bisquaternary pyridinium salts. TLC analyses were carried out for the chloroform extracts to

identify the reaction products. In the next step, the chloroform layer was dried over anhydrous sodium sulfate and filtered through Whatman No.1 filter paper. The solvent was removed under reduced pressure and the crude product was purified by recrystallization in a chloroform/methanol system to finally obtain pure reaction products, respectively, bis-indolizines in good yields for whole-cell plant biocatalysis. The purity of the obtained products was analyzed by TLC, melting points, FT-IR spectroscopy, and elemental analysis using as comparative standards the bis-indolizines obtained by the conventional method [23].

2.6 Analytical methods

Melting points were determined with a Büchi Melting Point B-540. FT-IR spectra were recorded from 4,000 to 400 cm^{-1} with 4 cm^{-1} resolution on a Nicolet iS50 FT-IR spectrometer (Thermo Scientific, USA), equipped with a KBr beamsplitter, a diamond crystal, built-in ATR accessory, a DTGS detector, and Omnic Software. Elemental analysis (C, H, N) was performed with a Fisons Instruments 1108 CHNS-O elemental analyzer. Reactions were monitored by TLC using silica gel 60 UV254 Merck pre-coated silica gel plates. In TLC studies, cyclohexane/ethyl acetate = 3:1 (*v*/*v*) was used as a chromatography developing mixture. A Bruker 400 Ultrashield (400 MHz) spectrometer, operating at room temperature, was used for ^{1}H NMR analysis and $CDCl_3$ was used as a solvent. Abbreviations for data quoted are m, multiplet; dd, doublet of doublets; q, quartet; t, triplet; d, doublet; s, singlet (Supplementary material, S1–S3).

Diethyl 1,1′-dibenzoyl-[7,7′-bisindolizine]-3,3′-dicarboxylate (**1**): yellow powder, mp 273–274°C; ^{1}H-NMR (400 MHz; $CDCl_3$; TMS) δ/ppm: 9.99 (d, J = 7.5, 2H); 8.78 (d, J = 1.2, 2H); 7.79–7.89 [m, 6H: 4H, 2H (7.83)]; 7.43–7.74 [m, 8H: 4H, 2H, 2H (7.53, J = 7.5, J = 1.2)]; 4.42 (q, J = 7.2, 4H); 1.45 (t, J = 7.2, 6H); IR (ATR, cm^{-1}): 3,063.43 (l, $CH_{arom.}$); 2,980.91 (l, CH_{alif}); 1,697.59 (s, C═O_{ester}); 1,641.57 (s, C═O); 1,527.71, 1,474.86, 1,447.50, 1,426.07 (s, C═N, C═C_{arom}); 1,340.41 (s, CO_{ester}) 1,228.37, 1,179.27 (s, C–O–C); 1,077.63 (s, C–N); anal. C 73.74%; H 5.06%; N 4.73%, calcd for $C_{36}H_{28}N_2O_6$, C 73.96%, H 4.83%, N 4.79%.

Diethyl 1,1′-bis(4-methoxybenzoyl)-[7,7′-bisindolizine]-3,3′-dicarboxylate (**2**): yellow powder, mp 290–292°C; ^{1}H-NMR (400 MHz; $CDCl_3$; TMS) δ/ppm: 9.97 (d, J = 7.5, 2H); 8.89 (d, J = 1.2, 2H); 7.88 (d, J = 7.5, 2H); 7.70 (s, 2H); 7.30 (d, J = 8.8, 4H); 7.05 (d, J = 8.8, 4H); 4.48 (q, J = 7.2, 4H); 3.87 (s, 6H); 1.45 (t, J = 7.2, 6H); IR (ATR, cm^{-1}): 3,118.38, 3,076.01 (l, $CH_{arom.}$); 2,982.48 (l, CH_{alif}); 1,697.24 (s, C═O_{ester}); 1,638.65 (s, C═O); 1,596.24, 1,512.05, 1,475.58 (s, C═N, C═C_{arom}); 1,341.30 (s, CO_{ester}), 1,264.44, 1,234.54, 1,197.89 (s, C–O–C); 1,084.03 (s, C–N); anal. C 70.94%; H 4.86%; N 4.26%, calcd for $C_{38}H_{32}N_2O_8$, C 70.80%, H 5.00%, N 4.35%.

Diethyl 1,1′-bis(4-nitrobenzoyl)-[7,7′-bisindolizine]-3,3′-dicarboxylate (**3**): dark-yellow brownish powder, mp 271–273°C; ^{1}H-NMR (400 MHz; $CDCl_3$; TMS) δ/ppm: 10.12 (d, J = 7.5, 2H); 8.98 (d, J = 1.2, 2H); 8.01 (d, J = 8.8, 4H); 7.78 (d, J = 8.8, 4H); 7.46 (d, J = 7.5, 2H); 7.40 (s, 2H); 4.42 (q, J = 7.2, 4H); 1.45 (t, J = 7.2, 6H); IR (ATR, cm^{-1}): 3,354.78 (l, $CH_{arom.}$); 2,979.66 (l, CH_{alif}); 1,697.86 (s, C═O_{ester}); 1,602.25 (s, C═O); 1,443.94, 1,402.49 (s, C═N, C═C_{arom}); 1,526.42, 1,347.14 (s, NO_2); 1,224.99, 1,163.33 (s, C–O–C); 1,100 (s, C–N); anal. C 64.16%; H 4.10%; N 8.25%, calcd for $C_{36}H_{26}N_4O_{10}$, C 64.09%, H 3.88%, N 8.31%.

2.7 Cytotoxicity test: evaluation of yeast multiplication by submerged cultivation in the presence of indolizine derivatives

To determine the cytotoxicity of the indolizine derivatives, the *Saccharomyces cerevisiae* MIUG 3.6 yeast strain was selected as a eukaryotic cell model. The pure culture of *Saccharomyces cerevisiae* was reactivated on the tilted malt agar culture medium for 48 h at 25°C [73]. To obtain the inoculum, a cell suspension was made and the sizing of the inoculum was performed by direct counting using the Thoma chamber. The *Saccharomyces cerevisiae* yeast strain was cultivated in submerged conditions in 300 mL Erlenmeyer flasks with cotton stoppers, containing sterile YPD broth liquid medium (20 g·L^{-1} peptone; 10 g·L^{-1} yeast extract; 10 g·L^{-1} glucose) pH = 5.5, with 20 g·L^{-1} agar, supplemented with indolizine compounds at different concentrations (10^{-5} M, 10^{-6} M). After homogenization, the medium was inoculated with the inoculum (10^6 CFU·mL^{-1}). Control samples were obtained by yeast cultivation in a medium without organic compounds. The samples were incubated on an orbital shaker (Companion Comecta S.A., Spain) at 250 rpm and 25°C, for 48 h. All the experiments were done in triplicate.

The parameters evaluated to describe the yeast multiplication were the kinetic parameters that describe cell multiplication (number of generations; growth rate; generation time), dry matter yield, the degree of the budding yeast cells, and the degree of yeast autolysis [74,75] as follows:

$$n = (\log N - \log N_0)/\log 2 \quad (1)$$

where n is the number of generations, N is the number of living cells·mL^{-1} after 48 h of aerobic culture with stirring, and N_0 is the number of living cells·mL^{-1} after 24 h of aerobic culture with stirring.

$$\mu = n/t[h^{-1}] \quad (2)$$

where μ is the growth rate, n is the number of generations, and t is the cultivation time.

$$t_g = 1/\mu[h] \quad (3)$$

where t_g is the generation time.

The dry matter yield was evaluated after 48 h by placing the cultures in a stationary system for 3 days at 25°C, and the active biomass was separated by centrifugation at 6,000 rpm for 15 min at 4°C. To remove the components from the medium, the wet biomass was washed twice with sterile saline solution, when the cells were suspended in a volume of solution equal to the initial volume of the culture medium. After each wash, the cell suspension was centrifuged at 6,000 rpm for 15 min. The wet biomass thus obtained was thermobalance-dried at 105°C, resulting in a dry matter yield.

To assess the degree of budding yeast cells, wet preparations were made at 24 and 48 h, and from ten different fields, the total number of cells and the number of budded cells were determined. The average percentage of budding cells was calculated. All the experiments were done in triplicate.

The degree of autolysis was determined by mixing a suspension of cells in equal volumes with a solution of methylene blue. After 5 min, a wet microscopic preparation was performed. Autolyzed cells appeared as intensely blue-colored cells, compared to living uncolored cells. The number of living and autolyzed cells from ten different fields was determined and averages were calculated. After 48 h of cultivating under stirring conditions, the vials were kept stationary for 48 h, after which the degree of autolysis was assessed [76]. The degree of autolysis was calculated as follows:

$$\%\ \text{Autolysed cells} = N_a/N_t \times 100 \quad (4)$$

where N_a represents the number of autolyzed cells and N_t is the total number of cells.

2.8 Effect of synthesized compounds on alcoholic fermentation

For the fermentation experiment, pure yeast cultures, *Saccharomyces cerevisiae*, were used to evaluate the effect of the synthesized compounds on alcoholic fermentation [77]. The fermentation experiment was carried out in Erlenmeyer flasks equipped with fermentation valves, 200 mL of fermentation culture medium was introduced into each flask before inoculation, and the culture medium was supplemented with the tested compounds **1**, **2**, and **3** at two different concentrations (10 and 1 µM). After the installation of the fermentation valves, concentrated sulfuric acid was introduced through the upper hole, which allowed the resulting carbon dioxide to be removed during the fermentation and to retain water vapors and other volatile substances. The samples were incubated at 25°C for 4 days. At various time intervals, 24, 48, 72, and 96 h, the flasks were gently shaken to remove the formed CO_2, weighed, and the amounts of CO_2 released were calculated by difference [77]. The fermentation rate was assessed according to the amount of CO_2 released per time unit, based on the unit volume of the medium (g of CO_2 released by 1 L of medium per h). The amount of fermented sugar was calculated according to the global alcoholic fermentation reaction:

$$C_6H_{12}O_6 \rightarrow 2CH_3–CH_2–OH + 2CO_2$$

From this balance, Z was calculated as follows:

$$Z = \text{Qt} \times 180/88 = \text{Qt} \times 2.045\ \text{g fermented sugar} \quad (5)$$

The fermentation intensity, expressed as the percentage of fermented sugar that correlated to the initial amount of sugar, was calculated after a certain fermentation interval:

$$R = Zf/\text{Day} \times 100 \quad (6)$$

For the fermentation experiment, the samples were incubated at 25°C for 4 days at 250 rpm. For the negative control sample preparation, there was no addition of compounds.

2.9 Statistical analysis

The data presented here represent mean ± standard deviation (SD) of three replicates. Data were subjected to statistical analysis in the Microsoft Excel program. Data were shown as mean values ± SD (n = 3).

3 Results

3.1 Extraction and characterization of enzymes (peroxidase, catalase, and PPO) from plant tissues

The greater peroxidase activity, among the analyzed plant varieties, was obtained for horseradish roots (1.08 ± 0.019 mmol purpurogallin·g^{-1} fresh weight·min^{-1}), while the parsley roots recorded the lowest enzymatic activity of the analyzed samples

(0.56 ± 0.024 mmol purpurogallin·g^{-1} fresh weight·min^{-1}) (Figure 2a). The best catalase activities were found for horseradish and pumpkin sprouts (21.71 ± 1.3 and 18.86 ± 1.6 µmol O_2·g^{-1} fresh weight·min^{-1}). The highest PPO activity was found for pumpkin sprouts (48.95 ± 1.74). Enzymatic activities are known from the literature data to depend very much on the plant species and their cultivation conditions [71,78]. Crude plant enzymes are valuable and versatile tools for organic synthesis [14,79–81]. Therefore, in our study, several local plant species rich in oxidoreductase enzymes were evaluated for their ability to catalyze reactions to obtain N-heterocyclic compounds.

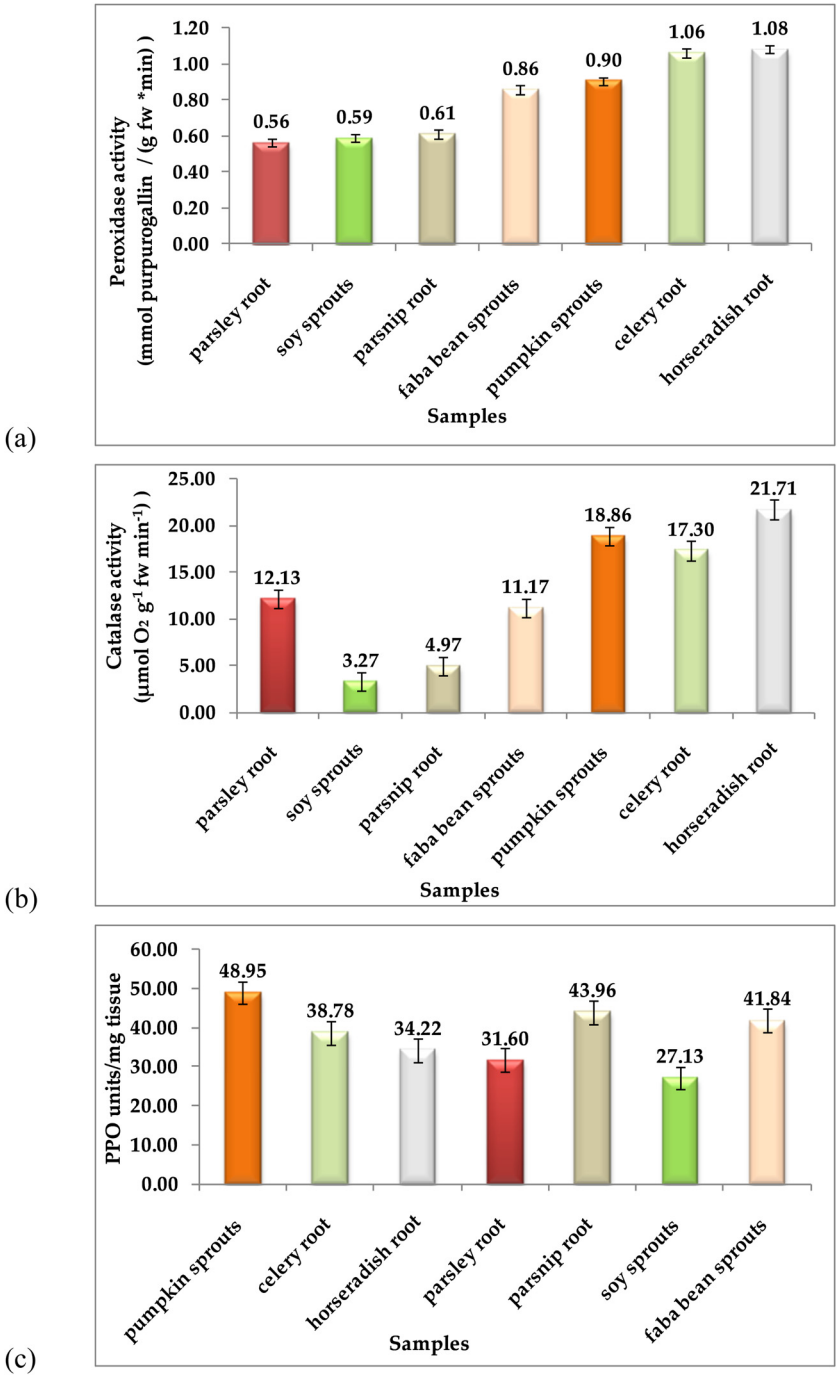


Figure 2: Activities of oxidoreductase enzymes: (a) peroxidase activity, (b) catalase activity, (c) PPO activity in tissue extracts for each plant species: horseradish roots (*A. rusticana*), celery roots (*A. graveolens*), parsnip roots (*P. sativa*), parsley roots (*P. crispum*), pumpkin sprouts (*C. pepo*), faba bean sprouts (*V. faba*), and soy sprouts (*G. max*).

3.2 Optimization of reaction conditions for whole-cell plant biocatalysis

Aiming to optimize the synthetic yield, the influence of the biocatalytic medium and conditions were investigated (Table 1). The first experiments were performed with 1 g of horseradish roots in phosphate buffer at pH 6.0, with continuous stirring at room temperature for 168 h. By monitoring the reaction by TLC, it was found that the reactants were consumed in time and the final reaction product (compound 1) was achieved with a reaction time of more than 120 h. The reaction time was considered to be the time required to consume the 4,4′-dipyridyl reactant, meaning the hours after which its corresponding spot was no longer chromatographically identified in the reaction mixture. Further optimization experiments were made for the synthesis of compound **1**, and the results are shown in Table 1. As small amounts of reactants were used in the synthesis, the increase in the plant weight made it difficult to extract the final reaction product, and therefore, these reactions resulted in smaller amounts of the desired compound. The most promising results were obtained at pH 7.0 and with 2 g of horseradish roots, when product **1** was obtained in 84% yield, at room temperature, and with a reaction time of 84 h.

3.3 Indolizine derivative synthesis reactions with whole-cell plant biocatalysts

Further reactions were performed at room temperature (25 ± 3°C), phosphate buffer pH 7.0, 2 g of the fresh plant as the biocatalytic medium, 0.1 mmol 4,4′-dipyridyl, 0.25 mmol reactive halogenated derivatives (ω-bromoacetophenone, 2-bromo-4′-methoxyacetophenone, 2-bromo-4′-nitroacetophenone), and 0.25 mmol ethyl propiolate. Subsequently, the work was extended to the seven plants taken in this study and to the use of various substituted halogenated reactive derivatives as reactants (Figure 3). When different halogenated derivatives (ω-bromoacetophenone, 2-bromo-4′-methoxyacetophenone, 2-bromo-4′-nitroacetophenone) were used, the corresponding products were obtained with reaction times between 84 and 168 h, in moderate to very good yields (45–85%). The lowest yields were obtained for the reactions where 2-bromo-4′-nitroacetophenone was used in the biocatalyzed reactions.

3.4 Cytotoxic activity: evaluation of indolizine derivative toxicity on yeast multiplication by submerged cultivation

The present study sought an application of alternative approaches that present a perspective on the minimal use of animals in scientific experiments and aimed to evaluate the cytotoxicity of the compounds on *Saccharomyces cerevisiae* yeast cells. The values of the kinetic parameters determined in the time interval of 24–48 h, for the samples supplemented with compound **2**, are the closest to those of the control sample (Table 2).

The dry matter yield is close to the control compared to the samples to which the analyzed compounds were added: **1**, **2**, and **3** at concentrations of 1 and 10 μM. The best yield was obtained for the supplemented medium

Table 1: Optimization of reaction conditions for the synthesis of compound **1**

Catalyst	Fresh weight (g)	pH of the phosphate buffer	Reaction time (h)		
			20 ± 3°C	25 ± 3°C (room temperature)	37 ± 3°C
Horseradish roots	0.5	6.0	>120	>120	>120
	0.5	7.0	>120	>120	>120
	0.5	8.0	>120	>120	>120
	1	6.0	>120	>120	>120
	1	7.0	>120	>120	>120
	1	8.0	>120	>120	>120
	1.5	6.0	117	112	113
	1.5	7.0	114	106	109
	1.5	8.0	119	124	110
	2	6.0	96	88	94
	2	7.0	87	84	86
	2	8.0	93	87	88

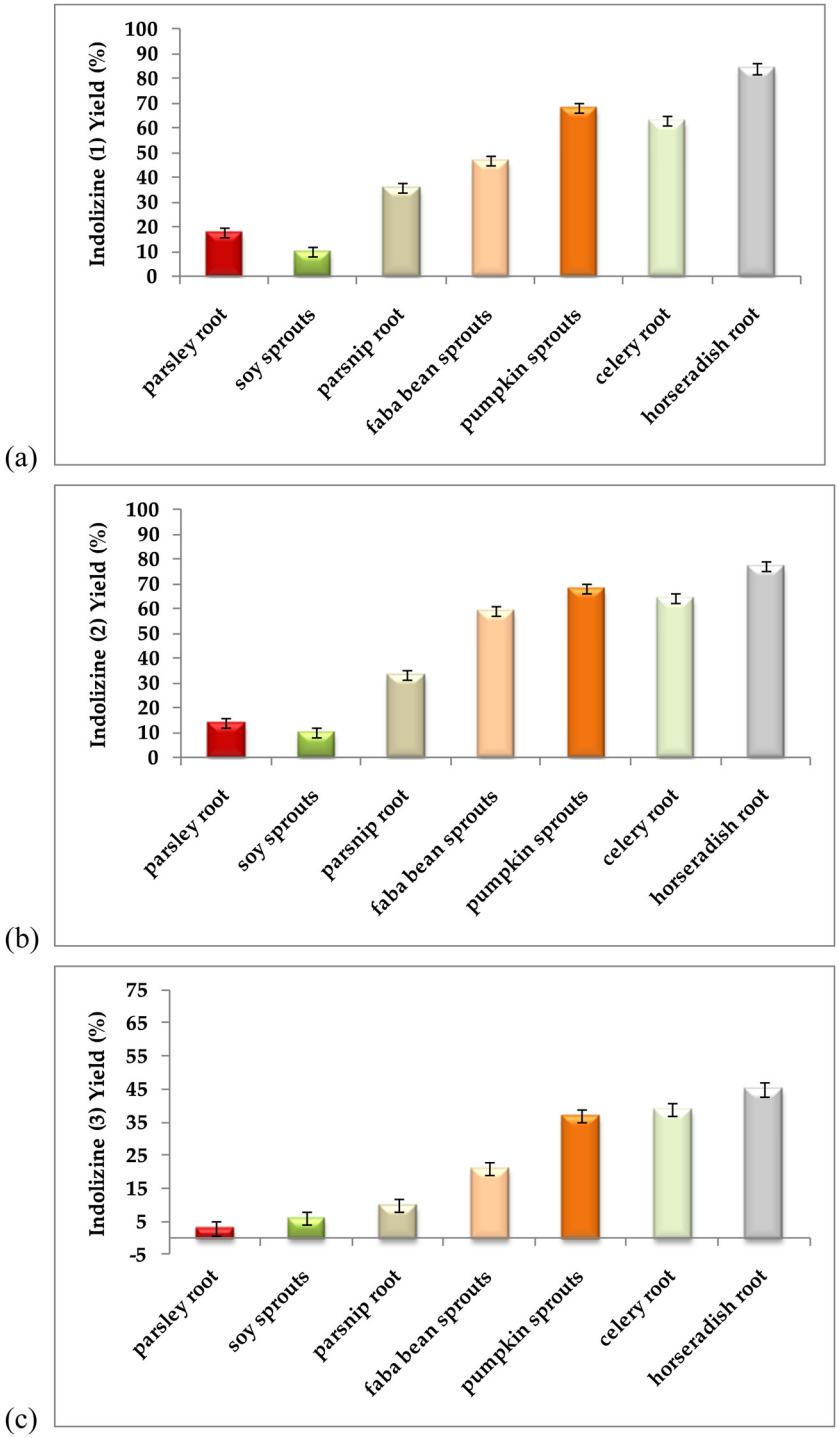


Figure 3: Yields of final indolizine products: (a) **1**, (b) **2**, and (c) **3**, achieved by using whole plant-cell systems as biocatalysts.

with compound **1** at a concentration of 10 µM, followed by the samples to which the analyzed chemical compounds were added: 1 µM compound **3** and 10 µM compound **2**. A lower biomass yield compared to that of the control sample shows the samples to which compounds **2** and **3** were added at a concentration of 1 and 10 µM, respectively (Figure 4).

From Figure 5a, it can be seen that the presence of compound **1** at a concentration of 1 µM determined a lower degree of autolysis of *Saccharomyces cerevisiae* yeast cells in the multiplication process (2.27%, 0.87%, 1.07%) than that of the control sample after 24, 48, and 120 h of cultivation (1.60%, 2.34%, 2.68%). Also, the presence of compound

Table 2: Kinetic parameters determined after 48 h of cultivation under submerged conditions, with stirring and aeration: *n* – number of generations; μ – growth rate; tg – generation time

Compound	Concentration	*n*	μ (h^{-1})	tg (h)
Control		18.6 ± 0.4	0.38 ± 0.06	2.58 ± 0.26
1	1 µM	15.4 ± 0.1	0.32 ± 0.12	3.12 ± 0.28
1	10 µM	13.2 ± 0.3	0.27 ± 0.14	3.63 ± 0.14
2	1 µM	19.4 ± 0.1	0.40 ± 0.08	2.47 ± 0.36
2	10 µM	21.4 ± 0.0	0.44 ± 0.04	2.24 ± 0.28
3	1 µM	11.4 ± 0.4	0.24 ± 0.13	4.21 ± 0.16
3	10 µM	11.8 ± 0.3	0.25 ± 0.17	4.07 ± 0.24

1 at a concentration of 10 µM determined a lower degree of autolysis after 24 and 48 h of cultivation (0.82%, 1.04%) than in the case of the control sample; however, after 120 h of cultivation, it can be seen that the degree of autolysis has a higher value than that of the control sample. Figure 5b shows that the presence of compound **2** in the cultivation medium of *Saccharomyces cerevisiae* yeast cells, at a concentration of 1 µM, determined lower values of the degree of autolysis than those presented by the control sample after 24, 48, and 120 h of cultivation. Also, the presence of compound **2** at a concentration of 10 µM in the cultivation medium, determined lower values of the degree of autolysis (1.09%, 1.36%) than those of the control sample (2.34%, 2.68%) after 48 and 120 h of cultivation. The presence of compound **3** in the growth medium, at both tested concentrations, determined higher degrees of autolysis after 24 h with approximately 0.6% than that of the control sample. From Figure 5c, it can be seen that after 48 and 120 h of cultivation, the values of the degree of autolysis for the

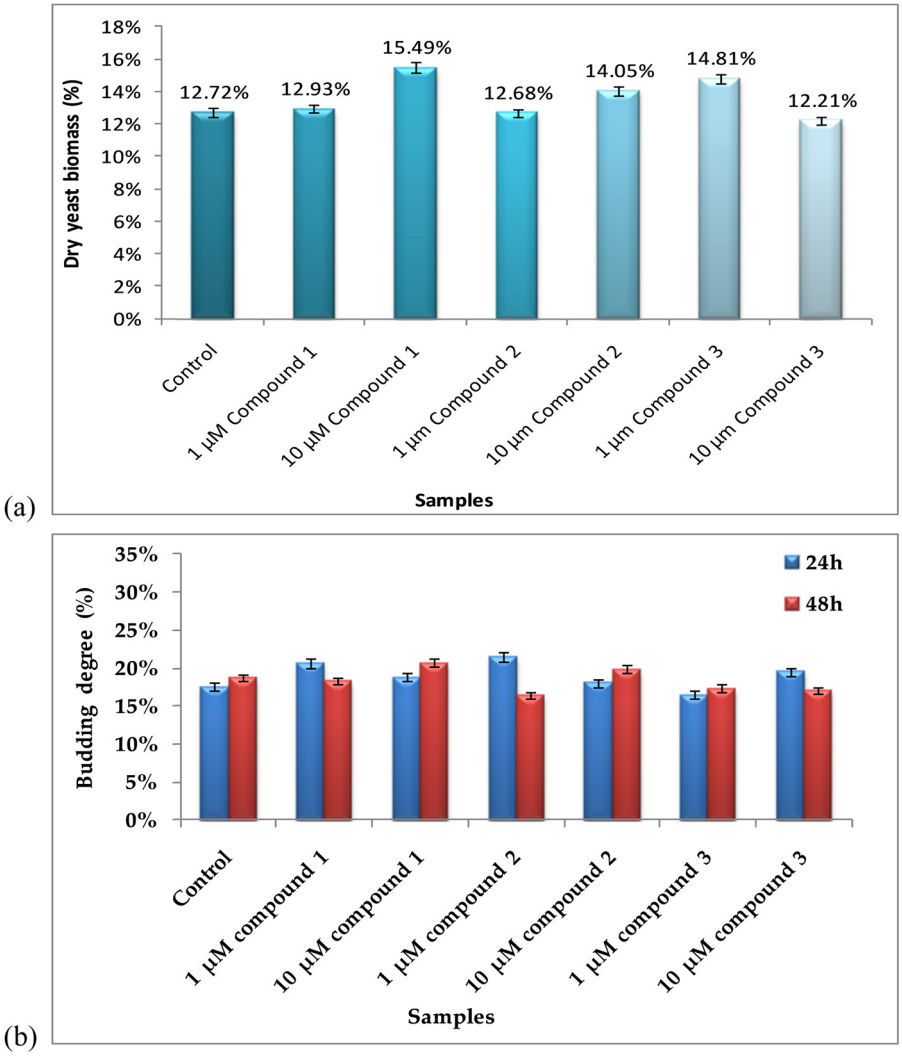
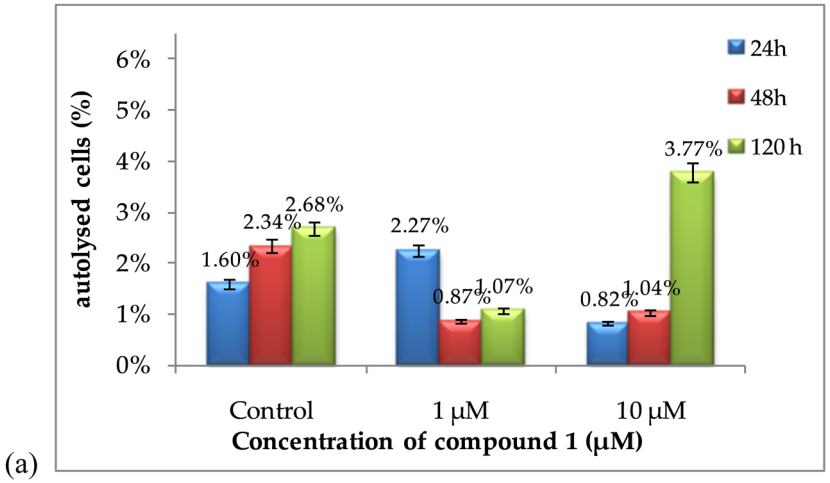
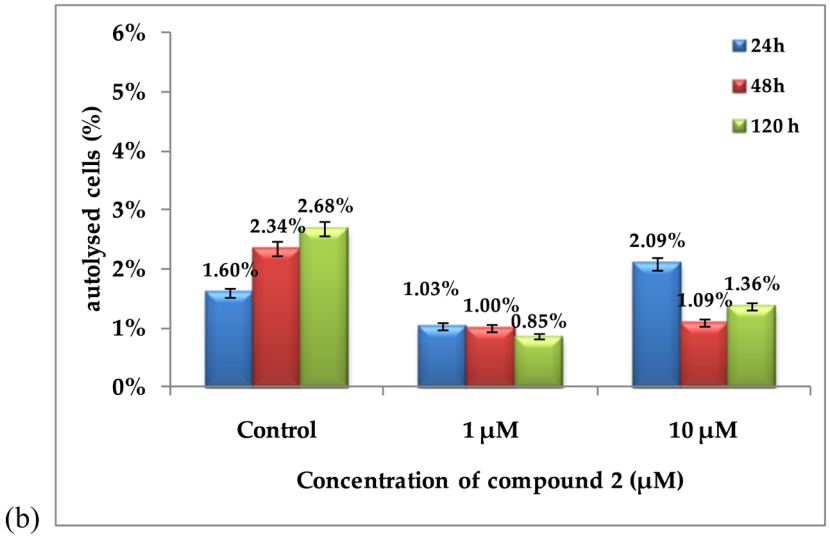


Figure 4: (a) Dry *S. cerevisiae* yeast biomass. (b) Budding degree of *S. cerevisiae* yeast cells, in the presence of indolizine compounds at different concentrations. The control sample was considered without the indolizine compounds in the yeast cell growth medium.



(a)



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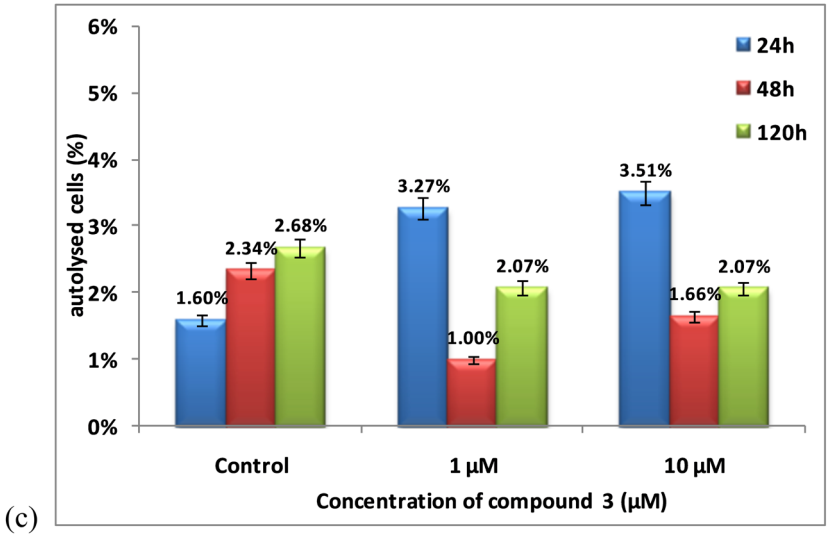

(c)

Figure 5: The degree of autolysis of yeast cells for the cultivation medium supplemented with compounds: (a) **1**, (b) **2**, and (c) **3** at different concentrations. The control sample was considered without the indolizine compound in the yeast cell growth medium.

samples supplemented with compound **3** at both tested concentrations show values very close to those presented by the control sample after 48 and 120 h of cultivation.

Figure 6 shows that the multiplication dynamics of yeast cells for the control sample were similar to those of the samples containing the studied compounds, meaning that these compounds do not significantly influence the behavior of yeast growth.

Regarding the effect of the synthesized compounds on alcoholic fermentation, from Figure 7a, it can be seen that

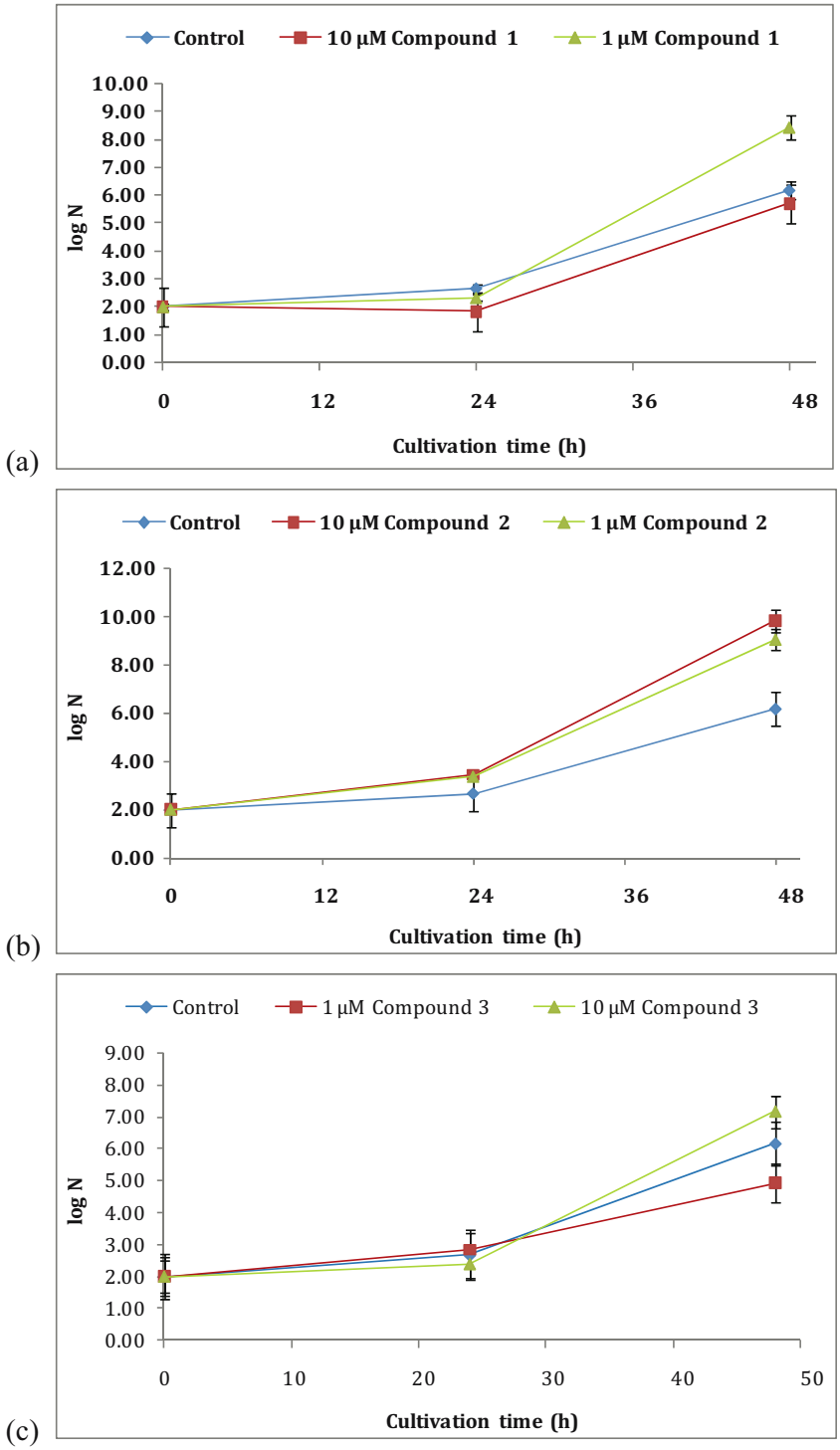

Figure 6: The multiplication dynamics of yeast cells for the cultivation medium supplemented with compounds: (a) **1**, (b) **2**, and (c) **3** at different concentrations. The control sample was considered without the indolizine compound in the yeast cell growth medium.

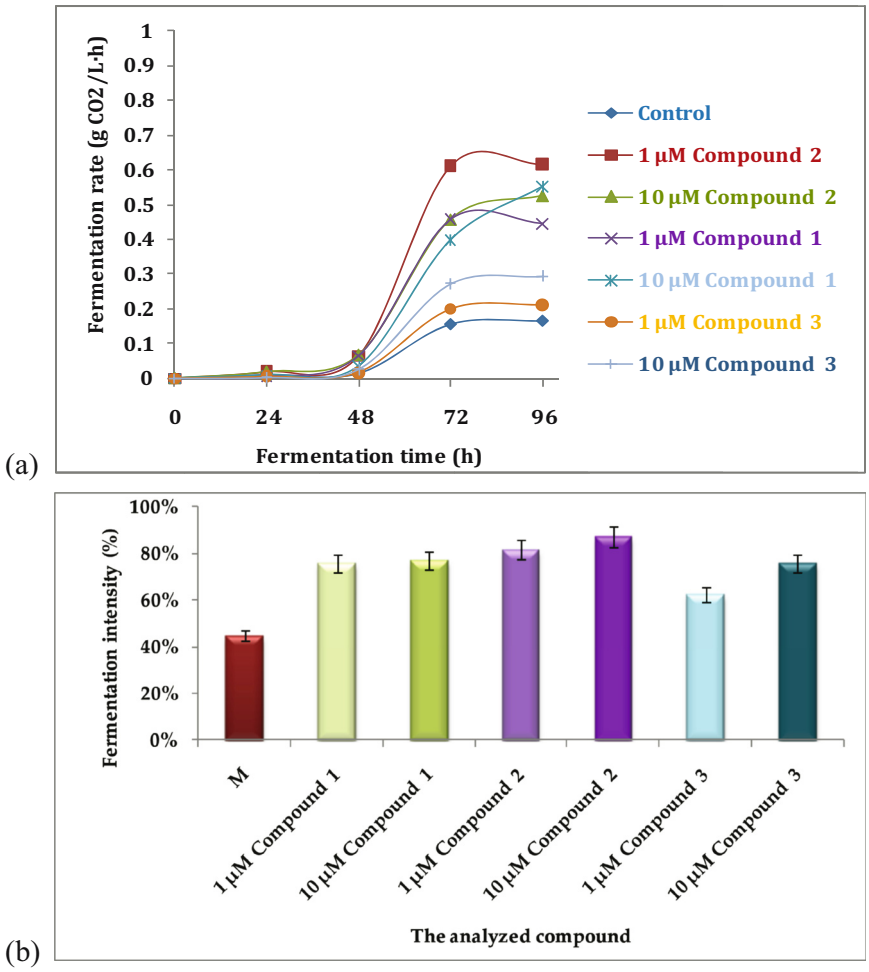

Figure 7: Fermentation (a) rate and (b) intensity of alcoholic fermentation in the presence of compounds: **1**, **2**, and **3** at different concentrations. The control sample was considered without the indolizine compound in the yeast cell growth medium.

the grams of CO_2 released during the fermentation process, related to the volume of 1 L of the fermentative medium, shows lower values in the case of the control sample after 96 h of cultivation compared to the samples supplemented with the studied compounds. The highest amounts of CO_2 are shown by compound **2**, at the tested concentrations of 10 and 1 µM, followed by those of compound **1**. Therefore, these results indicate that the compounds stimulate the fermentative capacity of the yeast *Saccharomyces cerevisiae*.

Figure 7b shows that the fermentation intensity of the control sample has the lowest value compared to the samples that were supplemented with the analyzed compounds: **1**, **2**, or **3** at concentrations of 1 and 10 µM. The sample containing compound **2** at the minimum analyzed concentration, 1 µM, has the highest value of fermentation intensity (22.77%), being approximately 3.7 times higher than that of the control. Compound **3** at the minimum analyzed concentration, 1 µM, shows a value of fermentation intensity close to that of the control. Samples containing compound **1**, at both tested concentrations, also showed fermentation intensity higher values than the control sample.

4 Discussion

In recent years, the need to use environmentally friendly synthetic methodologies has emerged, especially in the chemical and pharmaceutical industries that arise from the large-scale use of solvents [3,82,83]. The use of polar aprotic solvents has become a major focus of green chemistry because of their impact on the environment and human health. The search for solvents with reduced environmental impact is one of the major goals identified in chemical synthesis [84,85]. In this research, this concern prompted the use of green solvents, such as water, to improve the safety of synthetic processes to obtain indolizine derivatives.

Seven plants were screened as enzymatic sources for the catalysis of one-pot indolizine derivatives synthesis. The initial experiments demonstrated the synthesis of the desired product in the presence of the enzymatic extract of horseradish roots. As a result, the research was expanded by testing other six local plants for reaction optimization purposes. The multi-step reaction was performed in a one-pot system by cascading biocatalysis, with multiple enzymes from whole plant tissues to obtain reaction products with good isolated yields (45–85%) to minimize the number of unwanted by-products and also to avoid the use of toxic solvents. After the separation and purification of the synthesized bis-indolizines, it was found that they have the expected structures and purities (Figure 8), the results of the present analysis being consistent with those of control bis-indolizines previously obtained by classical chemical synthesis [86]. Future studies can benefit from these results considering that this approach could easily be extended to screen larger libraries of potential biocatalysts from plant sources.

By comparing the reaction conditions in classical and biocatalyzed synthesis and the yields in final products, it was found that the use of multi-enzyme systems to catalyze the reactions to obtain bis-indolizines was the most effective among the studied methods (Table 3). The advantages of using a multi-enzyme system and the low cost compared to the use of pure enzymes, which require purification and high costs, have also been found. These whole-cell systems demonstrated essential advantages such as reducing the consumption of toxic solvents, performing multi-step reactions in "one-pot," reducing the reaction by-products, and decreasing reaction costs [87,88]. Application of cheap and green catalysts, environmentally benign reaction conditions, and good to high yields are salient features of this method.

The proposed reaction mechanism implies the formation of the pyridinium salts from pyridine derivatives and halogenated-carbonyl compounds (Figure 4). The free radical products generated by oxidases are highly reactive species that can, in some cases, covalently alter the heme group of the enzyme [80]. For example, for horseradish peroxidase (HRP), the substrates for which this type of reaction has been noted cover alkyl and aryl hydrazines, nitromethane, or cyclopropanone hydrate. Low reactivity radicals can be added to vinyl heme groups, and these changes in the prosthetic heme also occur in some cases with two-electron oxidation products formed by peroxidases when they oxidize halide and pseudohalide ions. Therefore, the oxidation of bromide by HRP results in the addition of HOBr to the vinyl groups in the enzyme heme [89]. Thus, this could explain the uptake of the bromine ion from the reaction medium and its addition in the form of HOBr to the vinyl groups in the heme oxidases. Subsequently, the pyridinium-*N*-ylides were generated. The Fe–N coordination between the prosthetic heme and pyridinium-*N*-ylides enhanced the reaction rate with the activated alkyne and showed an overall improvement in catalytic efficiency to give the corresponding dihydro-indolizines as the primary cycloadducts. Finally, by rearrangement and *in situ* dehydrogenation of the primary cycloadduct, the indolizine derivatives were obtained (Figure 9). The mechanism was proposed according to other studies from the literature [90,91].

The cytotoxicity of the studied compounds was previously tested by the *Triticum* test, and it was found that the presence of the compounds in the germination process of wheat seeds (*Triticum aestivum*) had no toxic effects [26]. This study continued to evaluate the cytotoxicity of these compounds on model microorganisms to assess the impact of compounds on the environment. In recent years, attempts have been made to reduce the number of animal tests due to the ethical issues associated with them, but also disadvantages such as high costs and the long time required to perform such experiments [92]. In this process, the direction of reduction, refining, and replacement of laboratory tests on animals was followed in many types of research, which involves the search for alternative methods and model organisms [93,94]. Yeast cells are often used as a model organism in laboratory testing of chemicals because they react to stress by triggering a response to it as well as adapting to the new environment. If the stress is too strong, it can even cause cell death [94]. Following these results, it was found that the presence of indolizine compounds in the yeast cultivation medium of *Saccharomyces cerevisiae* does not negatively influence the behavior of the yeast cultures by cultivation under aerobic submerged conditions, with continuous stirring. By supplementing the fermentation medium with these compounds, a stimulation of alcoholic fermentation was found for all analyzed samples compared to the control sample, where no synthetic compound was added. The maximum fermentation intensity was observed when the culture medium was supplemented with compound **2** at a concentration of 1 μM. It can be concluded that these compounds do not have negative effects on the multiplication and fermentation processes of the *Saccharomyces cerevisiae* yeast cells. This kind of *in vivo* assay on model microorganisms is valuable to pursuing various applications of synthetic and natural compounds in chemical and pharmaceutical industries [59,60,95]. The current state of research in response to cellular stress and cell death shows that although there are differences between the response pathways of yeasts and higher eukaryotes, they reinforce the idea that many aspects are phylogenetically

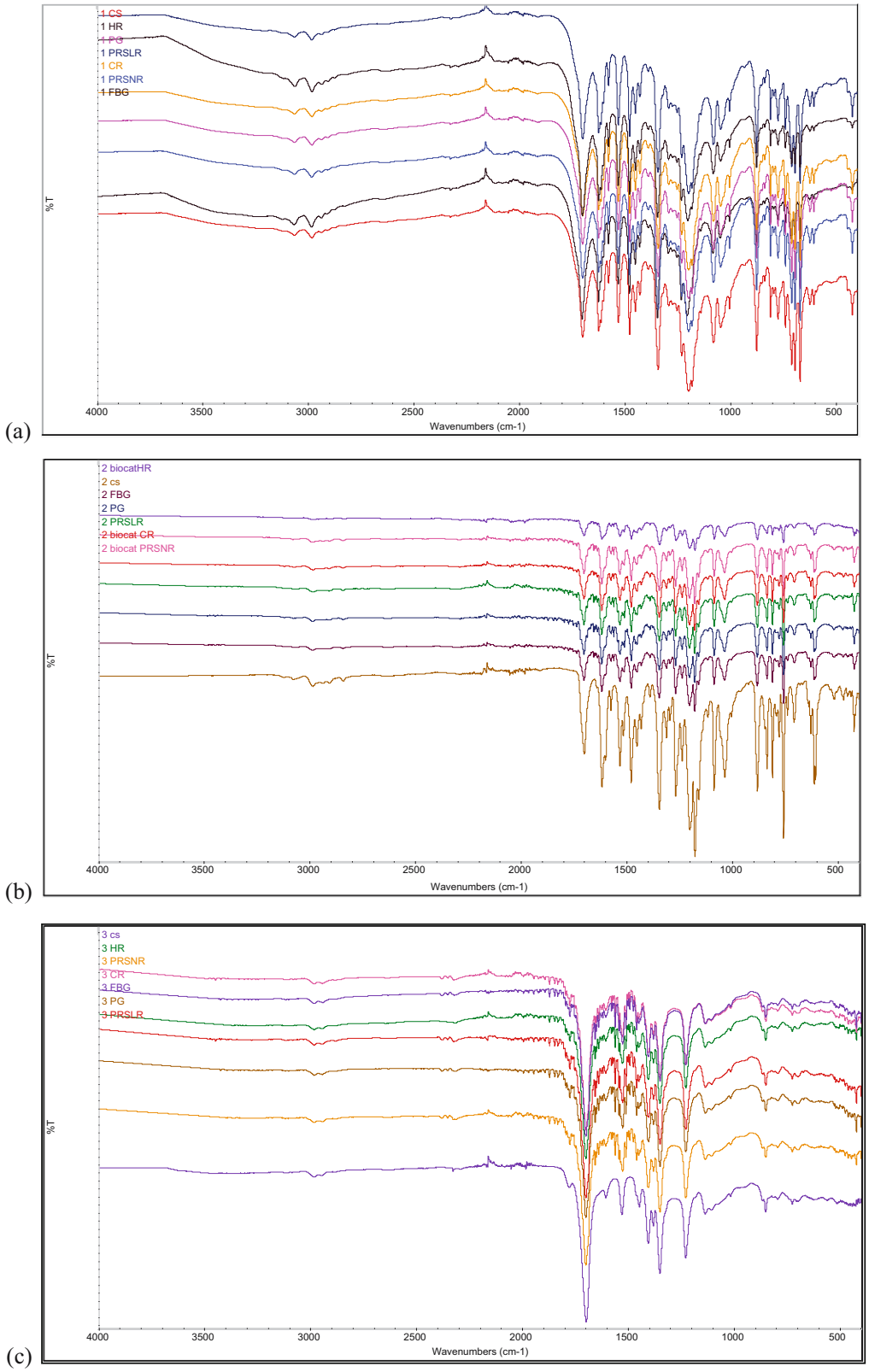

Figure 8: FT-IR analysis of compounds: (a) **1**, (b) **2**, and (c) **3** obtained through conventional catalysis (CS) and plant biocatalyzed reactions: HR – horseradish root, PRSNR – parsnip root, CR – celery root, FBG – faba bean sprouts, PG – pumpkin sprouts, and PRSLP – parsley root.

Table 3: Yields, melting points, and reaction conditions for the synthesis of Compounds **1–3**

Compound	m_p (°C)	Classical synthesis [86]		Plant whole-cell biocatalysis (horseradish root experiments)		Whole cells of *Y. lipolytica* biocatalysis [26]	
		Time (min)	Yield (%)	Time (h)	Yield (%)	Time (min)	Yield (%)
1	273–274	360	58	88	85	144	77
2	236–237	180	58	84	77	144	47
3	229–230	240	56	87	45	144	48

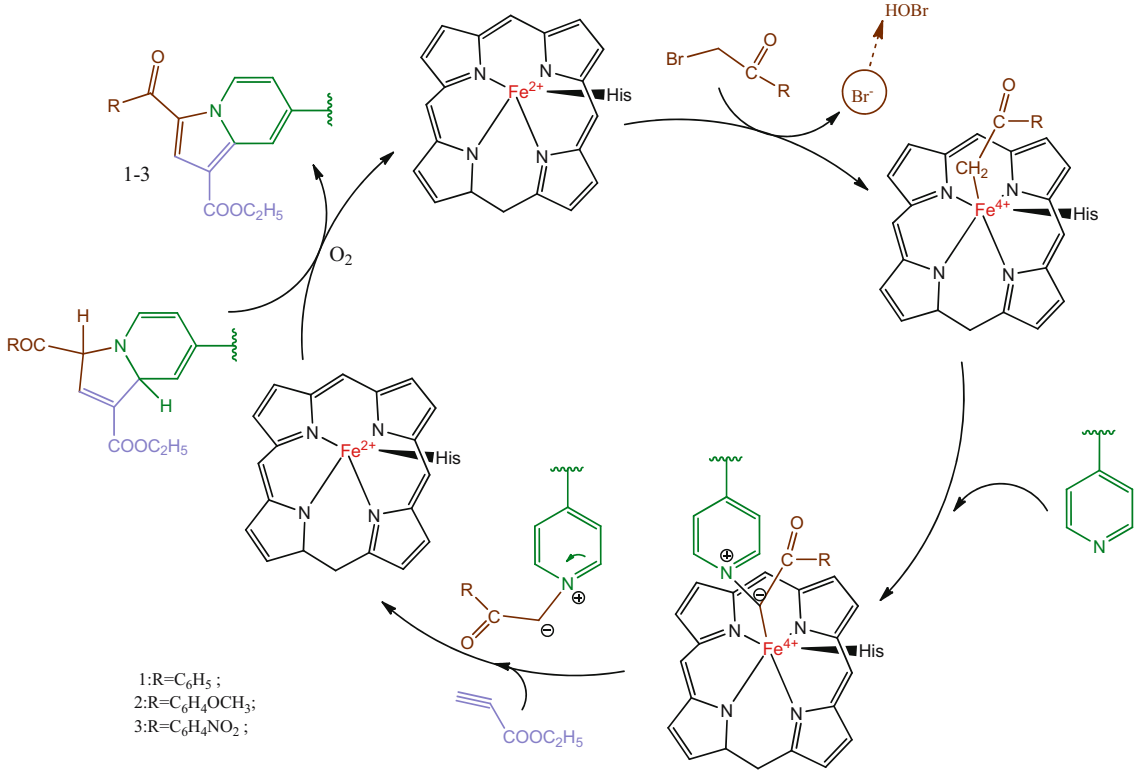

Figure 9: The proposed mechanism pathway for the substrate conversion in pyridinium-ylide intermediates and further 1,3-dipolar cycloaddition to form indolizine derivatives **1**, **2**, and **3** with enzymatic catalysts from plant sources.

conserved and therefore yeasts are ideal versatile models for evaluating the basic molecular pathways of these processes [61,63,96].

5 Conclusions

We have demonstrated the potential of using crude plant enzymatic extracts as biocatalysts with excellent improvements such as reduced reaction time, selectivity, increased productivity, and low costs. In this article, we report the synthesis of fluorescent indolizine compounds, recognized as powerful tools in many research fields, using a "green chemistry" method by using whole plant-cell biocatalysis, making it more attractive for industrial possible drug production. The results highlighted in this research encourage the use of reproducible and cheap techniques for application in industry to optimize drug production processes with the utilization of biocatalysts from natural sources. This multi-component reaction, which takes place under mild conditions, has provided an interesting new pathway to obtain fluorescent indolizines with reduced costs and good yields in metal-free catalytic systems. In the worldwide market for the production of a great variety of drugs, where companies try to improve their processes to reduce waste and make them more reliable in terms of environmental concerns, this might be considered a competitive

process. Furthermore, the final products were evaluated for their cytotoxicity on the model microorganism *Saccharomyces cerevisiae* and no significant toxicity was found by evaluating the values of the kinetic parameters that describe the growth of this yeast. The present work provides potentially new avenues for the use of enzymes from plant sources in organic compound synthesis and drug development in environmentally friendly conditions.

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Data availability statement: The data used to support the findings of this study are included in the article. If further data or information are required, these are available from the corresponding author upon request.

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Appendix

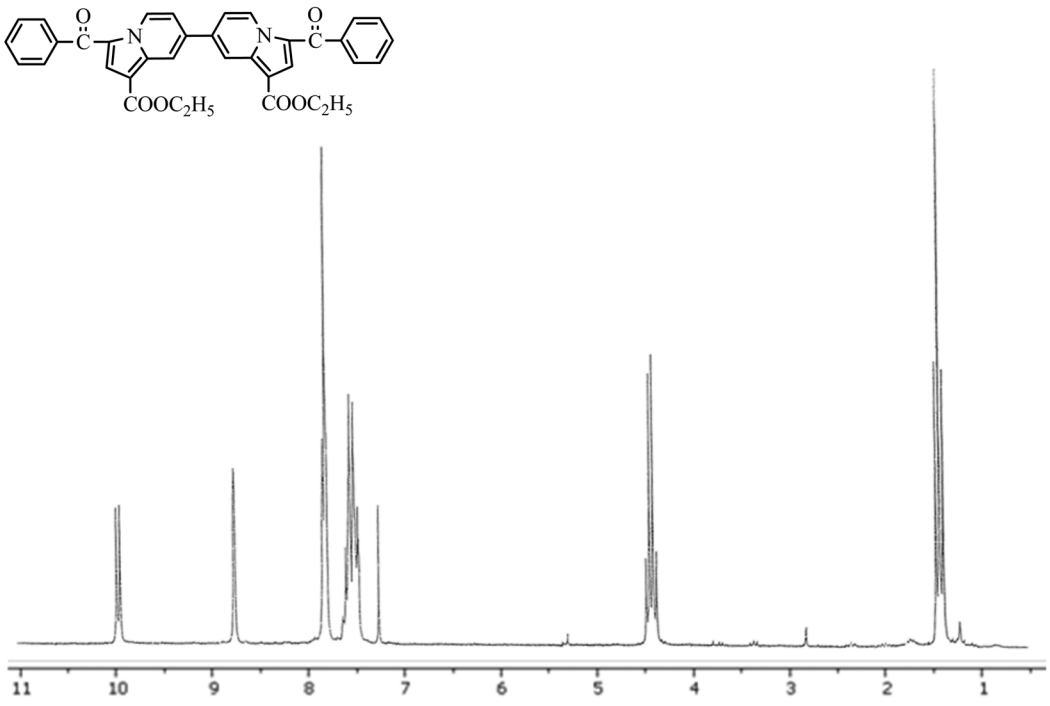

Figure A1: ^{1}H NMR spectrum of indolizine compound **1**.

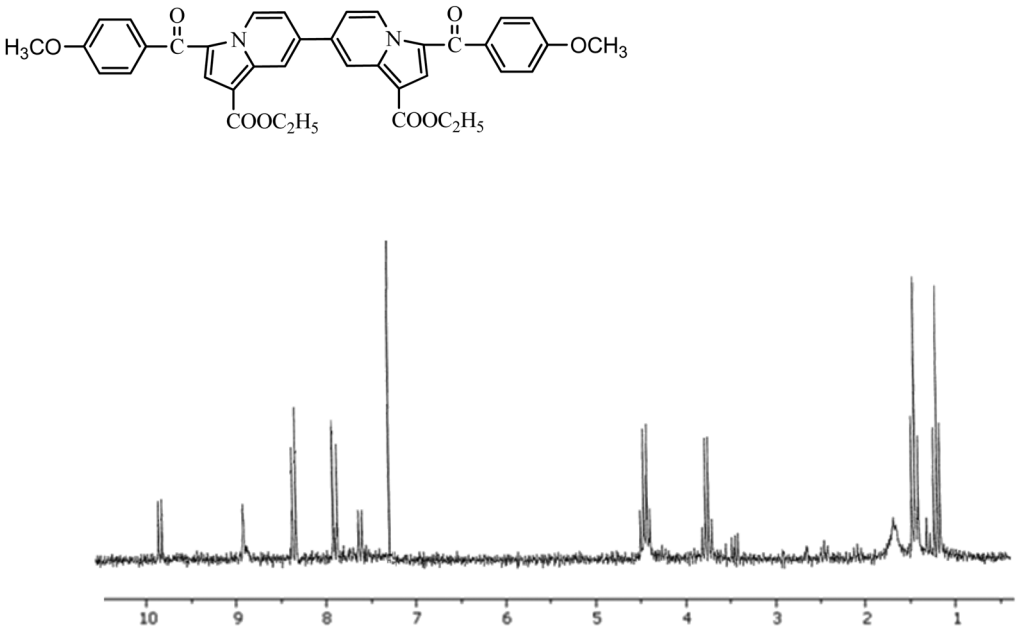

Figure A2: ^{1}H NMR spectrum of indolizine compound **2**.

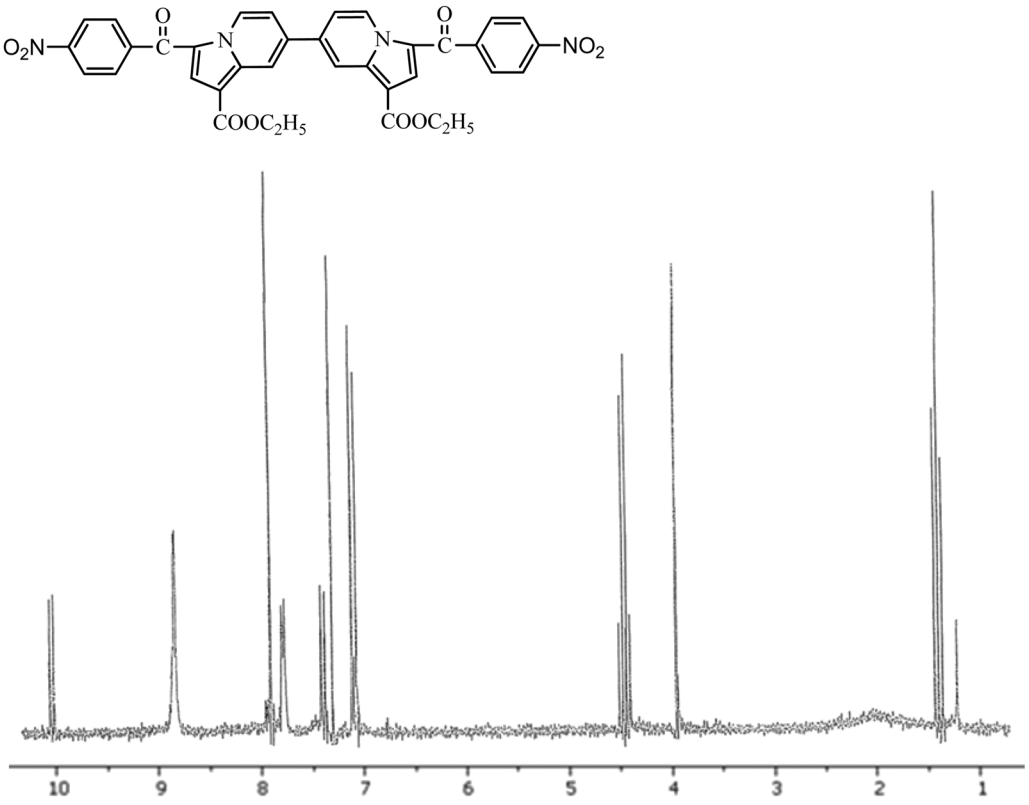

Figure A3: ^{1}H NMR spectrum of indolizine compound **3**.