Acces to the indolizine core: statistical modelling of a whole-cell biocatalyst

UNIVERSITAS GALATIENSIS

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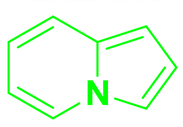
Introduction

Major target: use of plant enzymes to catalyze **cycloadditions for N-heterocyclic compounds synthesis: indolizines**

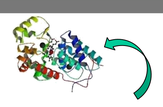
Interest for indolizines compounds

- ***Strong fluorescent properties***
- ***Enzyme inhibitors***
  - ✓ Phosphatases inhibitors
  - ✓ 4-Phosphodiesterase inhibitors
  - ✓ Aromatase inhibitors
  - ✓ 15-Lipooxygenase inhibitors
  - ✓ Acetylcholinesterase inhibitor
  - ✓ Tyrosine-phosphatase inhibitors
- ***Histamine antagonist***
- ***Anticancer, anti-tuberculosis, analgesic, antioxidant agents***

Indolizine nucleus

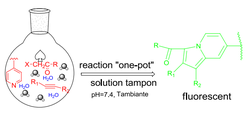


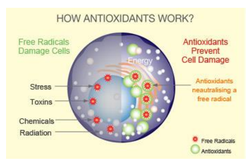
Objectives



- ❖ Extraction of enzymes from microorganisms for use in biocatalytic reactions.
- ✓ Selected microorganism: *Yarrowia lipolytica* RD14 MIUG yeast
- ❖ *One pot* synthesis of heterocyclic indolizine using whole cells of the yeast
- ✓ The challenges was to test the biocatalytic power of intra- and extracellular enzymes produced by *Yarrowia lipolytica* from the Dunarea de Jos University collection upon cycloaddition reactions, in aqueous media, and analyze the reaction products-indolizines.

- ❖ Determination of antioxidant activity through ***two methods***, which fit into two general classes of methods for determining the antioxidant activity: ***DPPH method and β-carotene bleaching test***
- ❖ Biocatalyst identification and the biocatalytic optimal conditions through mathematical modeling and statistical analysis methods
- ✓ It was carried out by a design of experiments model Plackett-Burman, two levels of the variation of each of the seven independent variables





Methods

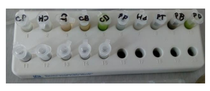
Extraction of biocatalyst

Preparation of crude biocatalysts for cycloaddition reactions

50 ml of YEPD liquid medium was inoculated with 5% (v/v) of Y.lipolytica inoculum (106 CFU ml-1). Cultivation took place in submerged conditions on an orbital shaker at 180 rpm and 28°C, during 144 h.

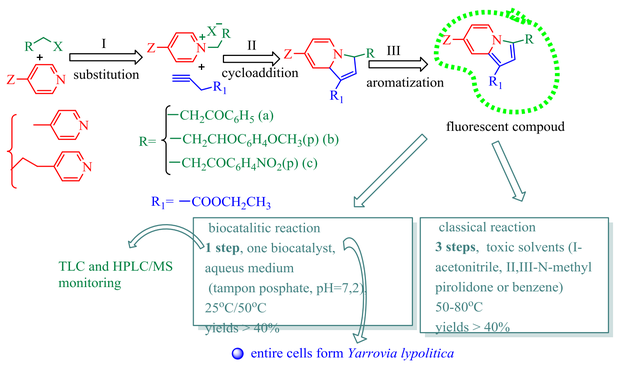
The whole culture was centrifuged for 20 min at 6000 rpm and 4°C. After centrifugation, the cell free supernatant was collected in a sterile tube, filtered and was used as such as crude enzyme extract.

The biomass thus obtained was washed twice with sterile saline (0.9% NaCl) and used as a whole cell biocatalyst after resuspension in 5 ml 250 mmol l-1 phosphate buffer, pH 7.0.



Crude enzyme extract

Biosynthesis of indolizines



• 4,4'- bipyridil or 4-[2-(pyridin-4-yl)ethyl], (0.05 mmols) + 1 mL phosphate buffer pH 7.2 + 29.85 mg 2-bromacetophenone (0.15 mmols) + 500 µL biocatalyst + 15.3 µL ethyl propiolate (0.15 mmols) ➡ room temperature orbital shaker ➡ extraction (chloroform) ➡ HPLC-MS

•indolizine structure and purity were characterized by NMR, IR and mass spectroscopy

Antioxidant activity

Characterization of antioxidant profile by investigating scavenging activity of compounds on DPPH radicals in 96-well plates

The absorbance of different concentrations of indolizines (20 - 600 µg/mL.) was measured with Microplate Spectrophotometer (NanoQuant, Tecan) at 515 nm. The radical scavenging activities of each sample were expressed as scavenging rate (SR), which was calculated using following formula::

Ac - absorbance of control

As - absorbance of sample

$$SR\% = \frac{(A_c - A_s)}{A_c} \times 100$$

Antioxidant activity (b–carotene bleaching test)

The absorbance of indolizines (20 - 1200 µg/mL) was measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The measurement was carried out at initial time (0) and successively at 30, 60, 90, 120, 150 and 180 min. The antioxidant activity (AA) was measured in terms of successful bleaching b-carotene by using the following equation:

$$AA = [1 - (A_0 - A_t)/(A_0^o - A_t^o) \times 100]$$

A_0 and A^o_0 -the absorbance values measured at the initial incubation time for samples/standard and control

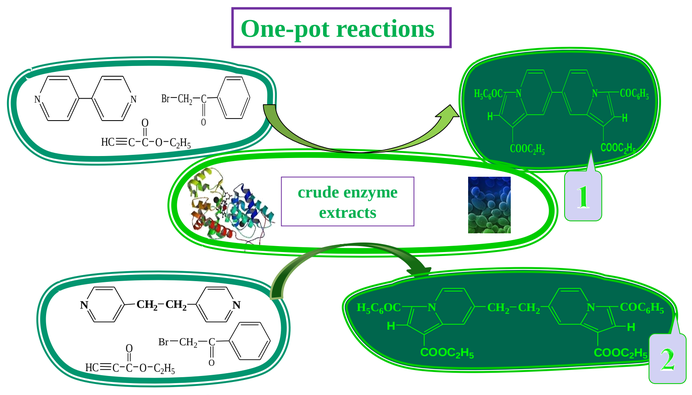
A_t and A^o_t -the absorbance values measure in the samples/ standard and control respectively at t =30 min and t = 60, 90, 120,150, 180 min.

Results

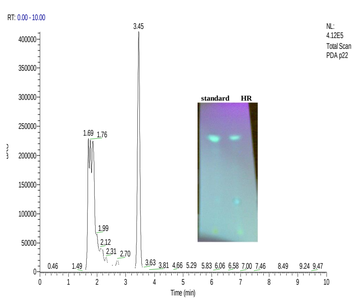
The reaction mechanism

➢One–pot cycloaddition reaction of dipolarophyle compound (ethyl propiolate) with a stable ylide prepared in situ from pyridinium compounds (reactions 1,2,3) and w-bromo-acetophenone in the presence of biocatalysts, crude enzyme extracts

One-pot reactions



HPLC chromatogram for reaction 2 using Yarrowia lipolytica biomass



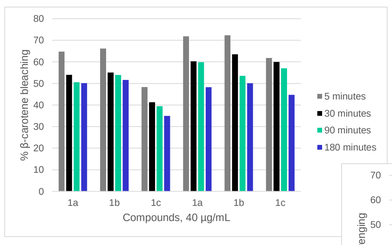
Yarrovia lypolitica biomass	Concentration of indolizine 1a 1(µg/mg DW)
120 h of cultivation, 25 C	43,61
168 h of cultivation 25 C	**233,14**
120 h of cultivation, 45 C	94,83
168 h of cultivation 45 C	**245,6**

Antioxidant activity of indolizines obtained by biocatalitic process

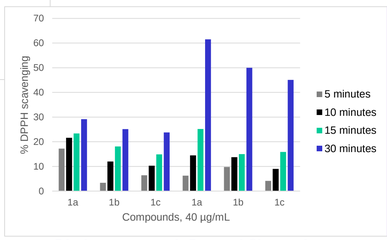
➢A higher purity degree for indolizine compounds was obtained with the HR biocatalysed reaction.

➢The yield obtained for biocatalysed reactions is heavily influenced by crude enzyme extract biocatalyst ; a more efficient extraction technique is currently under investigation.

Scavenging activity of compounds on DPPH radicals

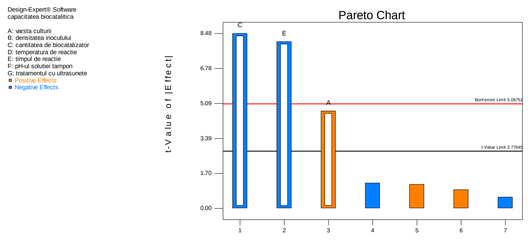


β-carotene bleaching test

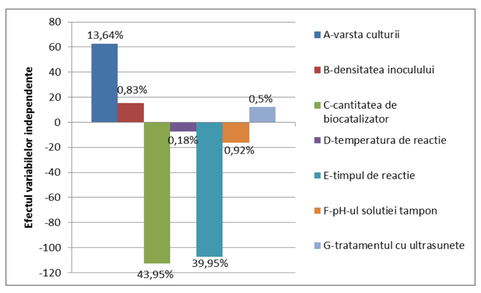


Biocatalytic optimal conditions identifications through mathematical modeling

Table 2. Comparison of metrics for enzymatic and classical reaction



Pareto chart that describes the influence of independent variables on biocatalytic process biomass produced by Yarrowia lipolytica strain RD14



The influence of the independent variables on the process of biomass catalyst produced by Yarrowia lipolytica strain RD14

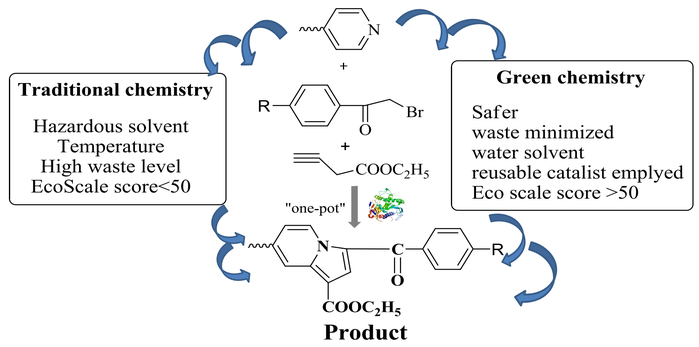
➢ parameters with the greatest influence on the biomass biocatalytic ability, produced by Yarrowia lipolytica strain RD14 are: the amount of biocatalyst reaction time, culture and age

✓ other variables influence the answer in a subunitary percentage. Thus the pH of the buffer solution and the reaction temperature have a negative influence, and the concentration of the inoculum and sonication have positive effects

Conclusions

- ❖ A higher purity degree for indolizine compounds was obtained by biocatalysed reaction with crude enzyme extracts
- ❖ All the tested extracts (crude fermentation products , biomass and cell free supernatant) have good biocatalytic activity.

Starting materials



❖Indolizines from ***4, 4'-bipyridine*** and ***4-[2-(pyridin-4-yl)ethyl]pyridine*** were DPPH radical-scavengers with inhibition being higher than 40% after 30 minutes.

❖In the linoleic emulsion system, oxidation of β-carotene was effectively inhibited by all indolizinic compounds.

❖The inhibition of β-carotene oxidation was higher than 50% for all the compounds.

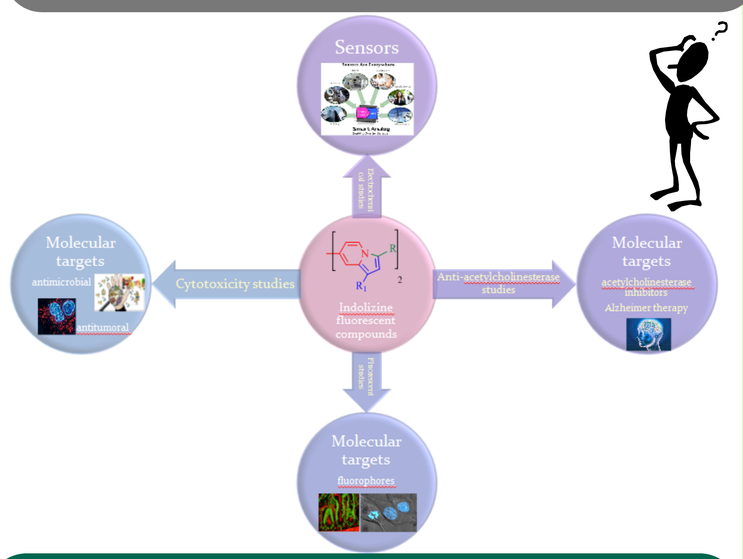
❖Antioxidant activity, which reflected the ability of the samples to inhibit the stable free radical DPPH, or the bleaching of b-carotene–linoleic acid emulsion systems, was measured and compared with that of compounds known for their antioxidant activity like vitamin C

❖These results suggest that the evaluated compounds exhibit good antioxidant activities

❖Analysis of the data revealed that from an economical and efficiency point of view, the best performances were furnished by the enzymatic reaction which confirms that the "green" reaction (in the presence of enzymes) is better than the traditional reaction

❖Our preliminary studies (unpublished results) show that these compounds are not cytotoxic and some of them could even stimulate biomass multiplication.

Future directions



References

•Bora, U.; Saikia, A.; Boruah, R.C. A novel microwave-mediated one-pot synthesis of indolizines via a three-component reaction. Org. Lett. 2003, 5, 435–438.

•Rodica Mihaela Dinica, Bianca Furdui, Ioana Otilia Ghinea, Gabriela Bahrim, Simon Bonte and Martine Demeunynck, Novel One-Pot Green Synthesis of Indolizines Biocatalysed by Candida antarctica Lipases, Mar. Drugs, 11, 431-439, 2013

Acknowledgments

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