Invertase Production by Fungi, Characterization of Enzyme Activity and Kinetic Parameters

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The present paper presents the results of research carried out on 14 fungal isolates of various origins aiming to select new efficient sources for invertase production for further biotechnological application. Aspergillus flavus presented the highest protein content and Aspergillus niger, the most intense invertase activity. The relationship between enzyme concentration and enzymatic activity at 0.25 mM mL^{-1} sucrose as substrate assayed for successive decimal dilutions of Aspergillus niger enzyme ranging from 0.1 to 1mLrevealed a linear correspondence between 0.1 and 0.5mL. The kinetic parameters Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) for invertase activity of Aspergillus niger, Penicillium aurantiogriseum, Aspergillus wentii and Rhizopus stolonifer were calculated. The determination coefficients R^2 calculated from Lineweaver-Burk plots presented values very close or equal to 1.

Keywords: invertase, filamentous fungi, sucrose, kinetic parameters, proteins.

Plants and microorganisms have complex enzymatic equipment and scientists selected many enzymes by biotechnological methods for various practical applications.

One of the first enzymes described in 1828 in yeasts was known as invertase or β-D-fructofuranosid-fructohydrolase (E.C.3.2.1.26) [1]. The enzyme hydrolyses the β 1-2 linkage from disaccharide sucrose releasing a mixture of glucose and fructose called *inverted syrup* [2]. The invertases produced by fungi and yeasts are of particular interest because not only hydrolyse the sucrose but they also catalyse the synthesis of fructo-oligosaccharides (FOS) with short chains [3] of high biotechnological importance as functional food ingredients due to their prebiotic properties [4, 5]. Other studies [6] identified and characterized the genes responsible for FOS synthesis in fungi from species *Aspergillus niger* or described new efficient methods of invertase purification in fungi [7-10] and yeasts [11, 12].

Research on invertase immobilization for application in food industry revealed advantages such as: improvement of enzyme properties, including the stability at variable pH and temperature conditions, easy recovery of products and enzyme reutilization. Various methods and media for invertase immobilization have been cited in the literature, including CM-Sephadex, chitosane pearls [13] and nanogels [14] that conferred kinetic parameters similar to those of free invertase.

The objective of the present research was to evaluate the capacity to produce the enzyme invertase by 14 fungal isolates of various origins, to select the most active strains and to characterize the kinetics of enzyme for further practical application.

Experimental part

Fungal isolates and culture media

Pure cultures of fungi representing 14 new isolates of various origins from the collection of National Research-Development Institute for Soil Science, Agrochemistry and Environment-ICPA, Bucharest (table 1) it have been assayed for the capacity to produce the enzyme invertase. Microbial strains were cultivated on solid media PDA (purchased

No.	Fungal isolate	Sources
1	*Aspergillus niger*	Mixed brined vegetables
2	*Aspergillus flavus*	Pickled cucumbers
3	*Aspergillus flavus*	Contaminated bread
4	*Aspergillus wentii*	Lettuce leaf
5	*Aspergillus tamarii*	Air
6	*Aspergillus ochraceus*	Tomato rhizosphere (Buzau greenhouse)
7	*Aspergillus ochraceus*	Growth substrate in greenhouse (Sanjai farm)
8	*Trichotecium roseum*	Egg plant
9	*Trichoderma viride*	Lettuce roots (cultivated on perlite)
10	*Penicillium aurantiogriseum*	Air
11	*Penicillium echinulatum*	Contaminated garlic
12	*Rhizopus stolonifer*	Air
13	*Cladosporium sphaerospermum*	Tomato fruits
14	*Verticillium tenerum*	Soil polluted with heavy metals (NEFERAL factory)

Table 1
FUNGAL ISOLATES UTILIZED FOR EVALUATION OF INVERTASE PRODUCTION AND THEIR ORIGIN

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from Merck KGaA Germany) in Petri dishes at 28±2^{0}C for 144 h.

Preparation of total protein extracts and protein assay

In order to evaluate the presence of enzyme, the first step was to collect the fungal biomass developed on the solid culture media and to triturate it with quart sand in 10 mL distilled water. The extraction was ready after 24 h at 4^{o}C. After 15 min centrifugation at 5000 rpm, the supernatant was collected and kept at 4^{o}C for maximum 30 days. The quantity of protein for each fungal isolate was estimated by method of Lowry [15] were the intensity of colour spectrophotometrically read at 660 nm, permits the esatblishing of protein content in the sample.

Standard curve was performed with albumin solution.

Invertase assay

Invertase activity was assayed by Sumner & Howells method [16]. 1 mL sucrose as substrate in 0.02 M acetate buffer (*p*H-4.5) was incubated at 37^{o}C for 15 min. By addition of 2 mL of dinitrosalicylic acid (DNS) reagent, the reaction was terminated and tubes were kept at boiling water bath for 5 min. The tubes were cooled at room temperature and 6 mL of distilled water was added each tube to 10 mL final volume. The intensity of the colour was read spectrophotometrically after 30 min at 540/ 546 nm. Standard curve was performed with inverted sugar solution. One unit of enzyme activity was defined as the amount of enzyme required for release 1μmol inverted sugar/ mL/ min/37^{o}C.

Enzyme activity and determination of kinetic parameters

Enzyme activity (EA) versus enzyme concentration was assayed for concentrations ranging from non-diluted enzyme to 1/10 dilutions in the acetate buffer mentioned above. The assay was performed for *Aspergillus niger* enzyme and the substrate concentration was 0.25μM/mL.

The effect of substrate concentration on enzyme activity at fungal isolates of *Aspergillus niger*, *Penicillium aurantiogriseum*, *Aspergillus wentii* and *Rhizopus stolonifer* was assayed for sucrose concentrations from 25 μM to 250 μM.

The kinetic parameters Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) of invertase activity of these fungi were calculated by the Lineweaver-Burk linearization using the Michaelis-Menten kinetic model [2]. All experiments were conducted in triplicate and the results represent the mean values of three replicates. The derived data were evaluated using one-way ANOVA analysis of variance for $P<0.05$, according to Student test.

Results and discussions

Protein content of fungal extracts

The results concerning the protein concentration in fungal extracts are presented in figure 1. Data analysis revealed clear differences in the capability to produce protein between the 14 fungal strains.

The highest protein was estimated for *Aspergillus flavus* isolated from bread, followed by groups of fungi with similar protein contents.

The first group was represented by *Rhizopus stolonifer*, *Trichoderma viride, Cladosporium sphaerospermum* and *Verticillium tenerum*.

The second group showing intermediate values was represented by *Aspergillus wentii*, two strains of *Aspergillus ochraceus* and *Penicillium aurantiogriseum*.

The lowest values of protein content were registered for the group including *Aspergillus niger*, *Aspergillus flavus* from pickled cucumbers, *Aspergillus tamarii, Trichotecium roseum* and *Penicillium echinulatum.*

The source of isolation for microorganisms in each group was different, so it could not be established a clear influence of the provenance conditions on the induction of protein synthesis.

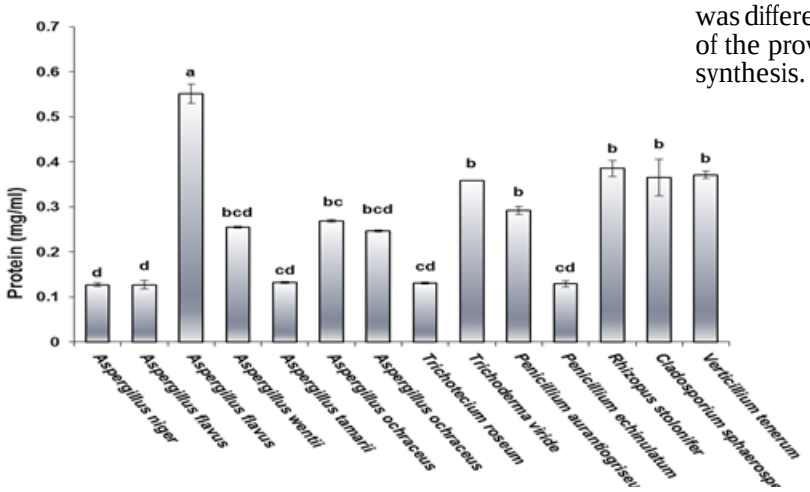

Fig. 1. The total protein content of extracts from14 fungal isolates. Different letters above the bars indicate statistically significant differences at $P<0.05$ (Student test)

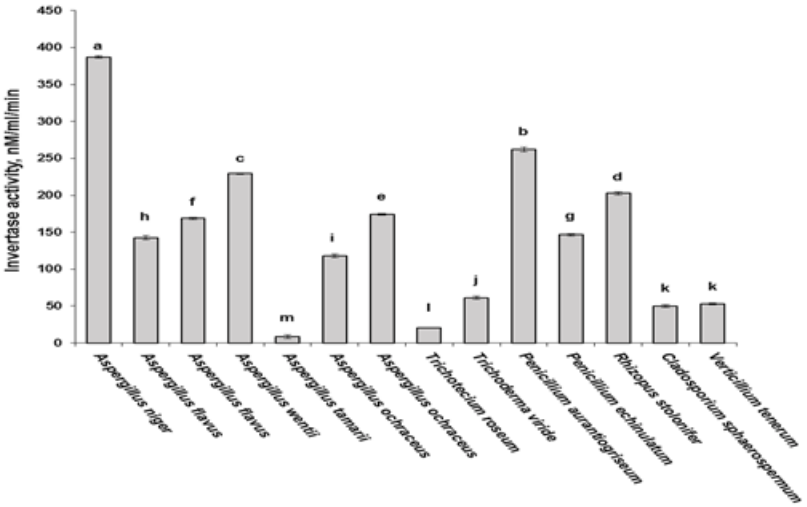

Fig. 2. Invertase activity of 14 fungal isolates. Different letters above the bars indicate statistically significant differences at $P<0.05$ (Student test)

Invertase activity

The invertase activity of the 14 fungal isolates is presented in the figure 2.

When compared the protein content with enzyme activity, it was evident that the highest invertase activity it was not detected for the strain with the highest protein production. Thus, *Aspergillus niger* with low protein contents presented the highest invertase activity (387.33 nM inverted sugar/ml enzyme/minute/37°C). Important enzyme activity values were also registered for *Penicillium aurantiogriseum, Aspergillus wentii* and *Rhizopus stolonifer*, significantly higher than for the rest of the fungal strains.

Cladosporium sphaerospermum and *Verticillium tenerum* presented similar invertase activity.The lowest values of invertase activity were registered for fungal isolates of *Trichotecium roseum* and *Aspergillus tamarii.*

Data from the present research are confirmed by literature results that showed that invertase synthesis in *Aspergillus niger* is governed by mechanisms different from those characteristic for other filamentous fungi [17-19] or yeasts [20].

Enzyme activity and kinetic parameters

The velocity of enzymatic reaction is direct proportional with the concentration of enzyme in the medium. When study the influence of enzyme concentration on reaction velocity, usually obtain a curve for many enzymes. This phenomenon shows a limited domain of enzyme concentration where this proportionality is linear.

In the present work, estimation of enzyme activity at different dilutions of the total protein extract (TPE}from the most active strain of *Aspergillus niger* have been done in order to find out in which domain of enzyme concentration can be obtained a linear response.

Aspergillus niger presented a low protein content, probably represented mainly by the enzyme invertase. A linear correspondence of enzyme concentration and enzyme activity was evident between 0.1 and 0.5 mL total protein extract (TPE) concentration (fig.3). At higher concentrations of enzyme, from 0.6 to 1.0 mL, the invertase activity rested practically the same (plateau values).

As a consequence, the best values were obtained at the dilution of ½ of the total protein extract at the substrate concentration of 0.25 mM/mL.

For concentrations ranging from 25 μM to 250 μM of sucrose as substrate the kinetic parameters (K_m and V_{max}) of invertase activity were determined at 37°C and *p*H-4.5.

The values of kinetic parameters calculated from Lineweaver-Burk plots for *Aspergillus niger*, *Penicillium aurantiogriseum*, *Aspergillus wentii* and *Rhizopus stolonifer* (fig.4) are summarized in table 2.

The coefficients of determination R^2 represented on Lineweaver-Burk plots as a statistic evaluation of the real data presented high values, very close to 1 (for *Aspergillus niger*, *Penicillium aurantiogriseum* and *Rhizopus stolonifer*) or equal to 1 (for *Aspergillus wentii).* The results from the present study are in concordance with other data from literature that reported the production of invertase enzyme by fungal isolates from genus *Aspergillus.*

A fructosyltransferase from *Aspergillus aculeatus* showed a high transferase/hydrolase ratio at elevated sucrose concentrations that conferred it a great potential for industrial application in obtaining prebiotic fructooligosaccharides [5].

Aspergillus japonicus strain 3.3556 producing b-fructofuranosidase with kinetic parameters K_m and V_{max} of

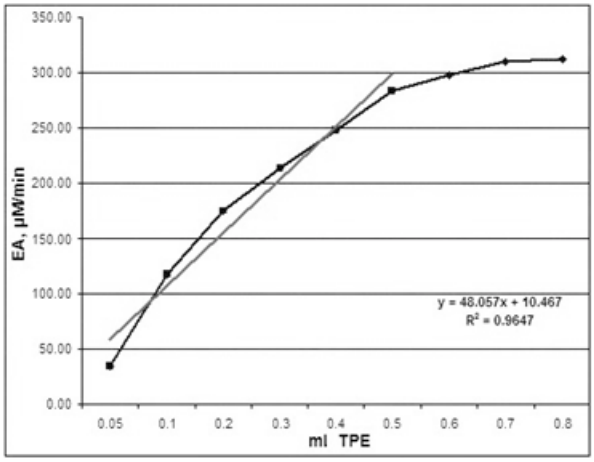

Fig. 3. The influence of enzyme concentration on enzyme activity (for *Aspergillus niger*)

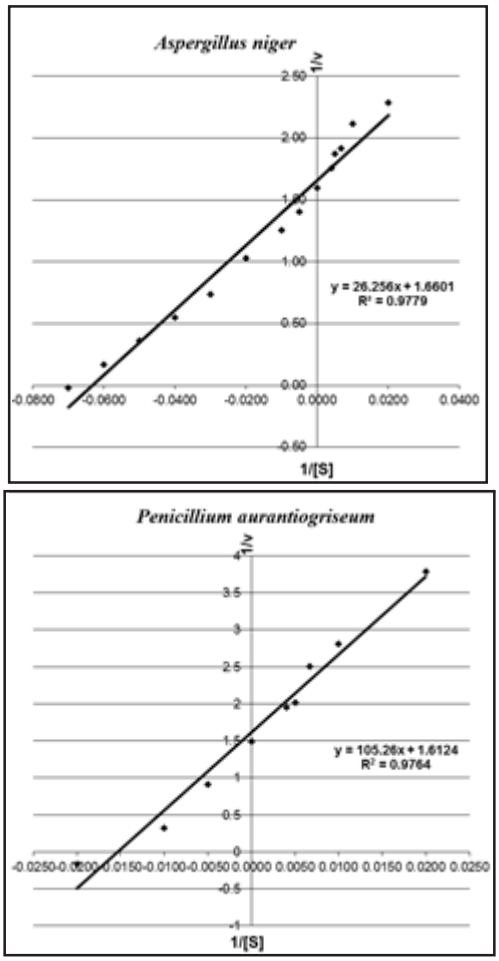

Fig. 4. *Lineweaver-Burk plots for A. niger, P. aurantiogriseum*

Fungal isolate	V_{max} (nM inverted sugar/ ml enzyme/minute/37°C)	K_m (μM)
Aspergillus niger	0.600	15.82
Penicillium aurantiogriseum	0.620	65.28
Aspergillus. wentii	0.015	8.29
Rhizopus stolonifer	0.130	119.30

Table 2
KINETIC PARAMETERS AT 37°C, FOR INVERTASE FROM FOUR FUNGAL ISOLATES

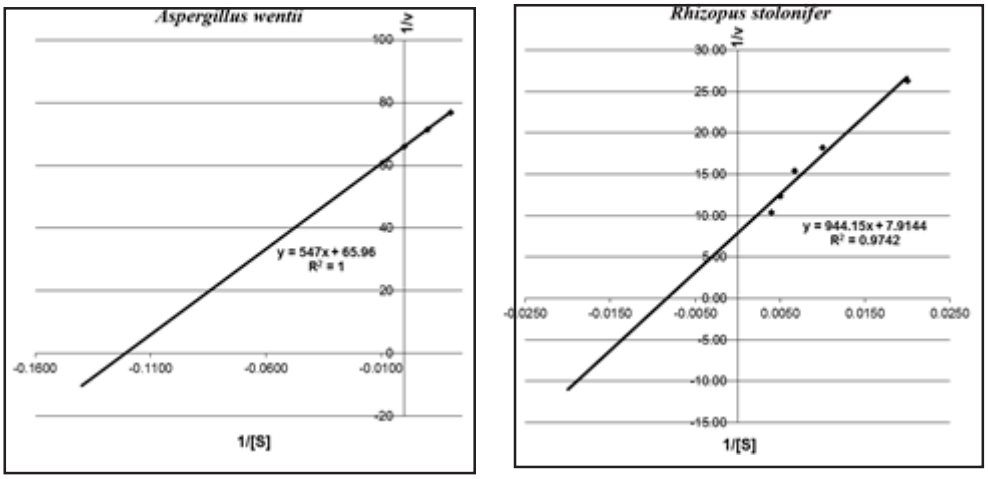

Fig. 4. Lineweaver-Burk plots for A. wentii and Rh. stolonifer

59.88 nM/L and 0.9421 μM/mL/min was selected for industrial production of 1-kestose and nystose from sucrose. Enzyme activity increased with the increase of sucrose concentration [21].

Invertase production by *Aspergillus flavus* presented the K_m = 0.23 mg/mL and V_{max} =15.8 U/mg [8].

Kinetic parameters reported for yeast *Saccharomyces cerevisiae* MK invertase for concentrations ranging from 0.5 to 5mg/mL of sucrose as substrate were K_m = 0.3410 mg/mL and V_{max} = 0.5953 μM/mg/min [2].

Other study [22] reported an increased invertase production by filamentous fungi from genera *Penicillium*, *Alternaria*, *Aspergillus* and yeasts by large-scale fermentation of sucrose.

Conclusions

Fourteen fungal isolates of various origins presented different protein contents and invertase production capabilities.

The highest protein content was synthesized by *Aspergillus flavus* isolated from bread, followed by the other strains grouped according to closed values of this parameter.

In each group, the origin of fungal isolates was different and it cannot be established if the provenance environment presented similar factors for induction of protein synthesis.

Fungal isolates with the highest protein production did not present the highest invertase activity. The most intense enzyme activity was determined for *Aspergillus niger*, with low protein content, where a linear correspondence between enzyme concentration and enzyme activity at 0.25 mM/ mL substrate concentration was registered from 0.1 to 0.5mL total protein extract, beyond this concentration the activity being practically the same.

At 37°C the kinetic parameters K_m and V_{max} of invertase activity for the most active fungi were:

Aspergillus niger (K_m =15.82 μM and V_{max} = 0.60 nM inverted sugar/ mL/min),

Penicillium aurantiogriseum (K_m =65.28 μM and V_{max} = 0.62 nM inverted sugar/ mL/min),

Aspergillus wentii (K_m =8.29μM and V_{max} = 0.015 nM inverted sugar/ mL/min) and

Rhizopus stolonifer (K_m =119.30 μM and V_{max} = 0.13 nM inverted sugar/ mL/min) and values for coefficients of determination R^2 very close or equal to 1.

The most efficient fungi can be a source for invertase production and selected for various practical applications as a cost-effective alternative to yeasts.

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