UNIVERSITY THOUGHT
Publication in Natural Sciences, Vol. 10, No. 1, 2020, pp. 13-19.
DOI: https://doi.org/10.5937/univtho10-21042
Original Scientific Paper

EFFECT OF DETERGENTS ON *ALKALINE INVERTASE* AND *ALKALINE PHOSPHATASE* ACTIVITY OF FUNGI *MUCOR PLUMBEUS* Bonord, 1864, *ASPERGILLUS NIGER* Tiegh, 1867 AND *TRICHODERMA HARZIANUM* Rifai, 1969

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ABSTRACT

Due to their diverse metabolic potential, many filamentous fungi have a great ability for degradation of different waste substances. The present research was aimed to investigate ability of fungi *Mucor plumbeus, Aspergillus niger* and *Trichoderma harzianum,* isolated from sewer and industrial waste water, to conduct degradation of high concentrations of commercial detergent (0.3%). Within enzyme extracts of samples taken during period of 3–16 days, activity of *alkaline invertase* and *alkaline phosphatase* was followed spectrophotometrically at room temperature. Obtained results proved that all examined fungi affected degradation of detergent. Quality (inhibition/stimulation) and quantity of action of detergent on activity of investigated enzymes depended on fungal species and incubation period. The highest inhibiting effect of detergent was recorded on 9th day of incubation in samples of *T. harzianum* and *A. niger,* while its most obvious stimulating effect was noticed on 3rd day of incubation in samples of *T. harzianum* and *M. plumbeus*. Investigated fungi can be used for purification of waste water containing high concentrations of detergent.

Keywords: *Aspergillus niger*, *Mucor plumbeus*, *Trichoderma harzianum*, Waste waters, Detergent, *Alkaline invertase*, *Alkaline phosphatase*.

INTRODUCTION

Increased use of detergents in households led to their accumulation in waste water and natural water ecosystems. It also caused numerous unfavorable consequences for microbiological community and hydro-bionics (Kumar et al., 2007; Chaturvedi & Kumar, 2010; Abu-Zreig et al., 2003). As pollutants, detergents and their products of degradation, reach environment through industrial and sewer waste water, usage of pesticides and disposal of waste active mud (Cavalli, 2004; Ying, 2006).

Literature includes very few data related to degradation of commercial detergents under effect of fungi (Ojo & Oso, 2008). It is known that products with high level of SASs (Surface Active Supstance) such as detergents, in water ecosystems can cause numerous harmful effects: they inhibite growth of one-cell seaweed, decelerate process of water purification by creation of foam, which delays dissolution of oxygen important for breathing and photosynthesis, and causes eutrophication. Many studies showed that tensides modify conformation of proteins and in such way enzyme activity, stability and specifity (Kamiya et al., 2000; Buvaneswari et al., 2013). Recent researches showed that filamentous fungi *Myceliophthora thermophila*, *Geomyces* sp., *Alternaria alternata*, *Verticillium alboatrum*, *Aspergillus flavus*, *Trichoderma* sp., *Aspergillus oryzae,* isolated from river mud are able to degrade different synthetic detergents of anion type (Ojo & Oso, 2008). These fungi have specific apical growth, which provides ingrowth of hyphae into various substrates and excretion of extracellular enzymes. Under effect of these enzymes, complex organic compounds of substrates degrade into simplified compounds which can be absorbed by fungi through hyphae and used for growth (Raimbault, 1998; Saucedo-Castañeda et al., 1992; Raimbault, 1981). Filamentous fungus *Mucor plumbeus* produces different enzymes such as *invertase*, *alkaline phosphatase*, *proteinase* and *lipase* which are variously applied in biotechnology. Genus *Aspergillus* includes more than 180 species of fungi which present main decomposers of plant polysaccharides (Brookman & Denning, 2000). Especially important species in biodegradation is *Aspergillus niger* which is commonly used due to its ability to produce different extracellular enzymes. *Trichoderma* spp. are saprophytic fungi which are located in soil and roots of plants but also highly metabolically valued (Saucedo-Castañeda et al., 1992). *Trichoderma harzianum* can decompose different compounds such as following: dichlorodipheniltrichloroethan (DDT), dieldrine, endosulphan, pentachloro-nitrobenzene (PCNB) and pentachlorophenol (PCP) (Brookman & Denning, 2000). Species of *Trichoderma* have an important role in bioremediation of soil which is contaminated by pesticides, herbicides and insecticides (Brookman & Denning, 2000).

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Invertases are enzymes which hydrolize disaharide sucrose to glucose and fructose. Invertases are isolated and defined in many filamentous fungi of species *Penicillium, Neurospora, Aspergillus*, etc. (Poonawalla et al., 1965; Ashok Kumar et al., 2001; Guimaraes et al., 2009). Most of fungi synthesize intracelullar and extracellular *invertases* (Gillespie et al., 1952; Crewther & Lennox, 1953), which differ in the position within a cell (cell wall, vacuoles or cytoplasm), solubility (soluble or insoluble in buffer of a small ionic strength), optimum pH (acid or neutral/alkaline) and isoelectric point (pI).

The aim of this research was to investigate ability of filamentous species *Mucor plumbeus, Aspergillus niger* and *Trichoderma harzianum* isolated from sewer and industrial waste water for degradation of high concentrations of commercial detergent (0.3%) and to estimate their potentials for waste water purification.

MATERIAL AND METHODS

Isolation of fungi from waste water

Samples of waste water were collected using sterile glass bottles from three different localities (Sport Center 'Mladost', Industrial zone in Čačak and village Stančići) on the territory of the municipality of Čačak, during the end of May in 2013. After transport at temperature of 4 °C, collected samples were sown in Petri dishes on *Maltose agar* (MA, Sigma Aldrich) which included streptomycin (0,5 mg/ml) for purpose of inhibition of bacteria growth. Sown samples were incubated at the room temperature during 5 days. After that, isolation of fungi into clean cultures was conducted on sterilized *Potato Dextrose Agar* (PDA, Sigma Aldrich) without streptomycin. Isolated individual species are identified on the base of macroscopic and microscopic morphology at Department for Biology and Ecology at the Faculty of Science University of Kragujevac, Serbia. All isolated fungi were cultivated until sporulation on tilted PDA at room temperature during 5–7 days. After that, isolated fungi were used for preparation of suspensions of spores in 10 ml of sterile distilled water. Spores were counted under the microscope using *Noebauer* chamber in three repetition cycles, and their final concentration in inoculum was set on 10^6 spores ml^{-1} (Stojanović et al., 2010).

Enzymes extracts preparation

Prepared suspension (1 ml) of each investigated fungus was sown into two sterile Erlenmeyer flasks each containing 200 ml of modified sterile *Czapek Dox* broth (CDB, pH value adjusted to 4.7 using 1 mol l^{-1} HCl; Stojanović et al., 2010). After that, detergent (D) was added into one of two Erlenmeyer flasks to reach the final concentration 0.3% (w/w). The second Erlenmeyer flask was used as a control (K). Both Erlenmeyer flasks with samples were incubated during 16 days at the room temperature on electric shaker (Kinetor, Ljubljana, Slovenia) on 250 rpm. For further analysis samples were taken after 3, 6, 9, 12 and 16 days of incubation. The liquid in amount of 10 ml was taken from each Erlenmeyer flask. This liquid was filtered through Whatman filter paper in order to remove traces of mycelia. After that, liquids were centrifuged for 10 min at 10,000 rpm, at the temperature of +4 °C (Stojanović et al., 2010). Obtained supernatants were used as a raw enzyme extracts for defining of activity of enzymes of *alkaline invertase* and *alkaline phosphatase*. The experiment was conducted in three repetitions.

Determination of alkaline invertase activity

Invertase enzyme activity was determined by Sumner & Howell method (1935). Firstly, reactive mixture was developed in two tubes. This mixture included following components: 0.5 ml of raw enzyme extract, 0.5 ml 0.02 mol l^{-1} of phosphate buffer (pH 8.0) and 1 ml 1% (w/w) of sucrose. One tube (trial one) was incubated in water bathroom at 37 °C for 15 min and the second one (control) was placed on ice during the same period. After that, 1 ml of mixture from each of tube was transferred into new tubes and 2 ml of dinitrosalicylic reagent was added into each of them. Both tubes were then incubated at 100 °C for 5 min. After cooling to the room temperature, the amount of reducing sugars was determined spectrophotometrically at absorbance of 540 nm. The standard line was constructed using six tubes which contained 0.05, 0.10, 0.20, 0.30, 0.40 and 0.50 ml of standard solution of glucose (1 mg ml^{-1} of solution of 5.56×10^{-3} mol^{-1} glucose standard – Sigma Aldrich) in distilled water which was used as a base line. All tubes were incubated at 55 °C for 20 min. The results were presented as a mean value of three repetitions.

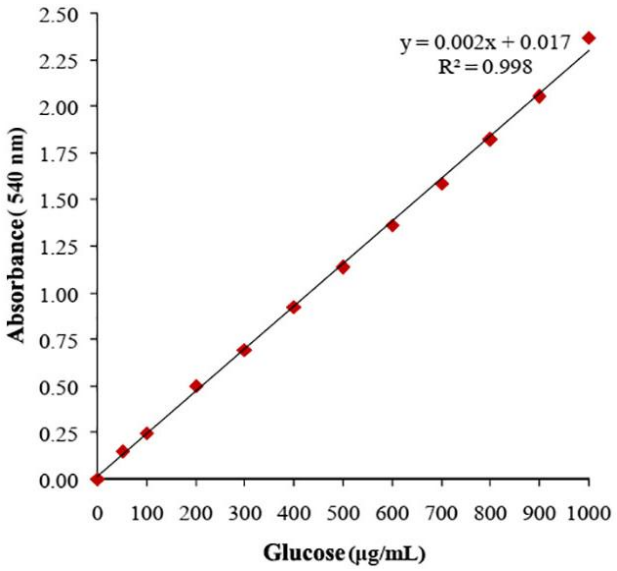

Figure 1. Standard line for glucose.

Determination of alkaline phosphatase activity

Enzyme activity of *alkaline phosphatase* was determined using the certain amount of inorganic phosphorus by Allen method according to Stojanović (2007). Enzyme activity was

measured in reaction mixture prepared of 1 ml of enzyme extract, 1 ml of glycol buffer (pH 9.0) with Mg^{2+} and 1 ml of *β*-glycerophosphate (substrate). Tubes with reactive mixture were incubated at 37 ºC for 30 min, after which the enzyme reaction is stopped by adding 3 ml of 10% trihydroxyuric acid (TCA). After that, tubes were held on ice for 15 min, after which their content was filtrated and the filtrate was collected. Control tubes were prepared in the same way except the incubation process. The phosphorus concentration in samples was determined through Allen reaction. The amount of 1 ml of filtrate for trial and 1 ml of filtrate for control were measured in two tubes each. After that, 0.4 ml of amido (Sigma Aldrich), 0.4 ml of 60% TCA (Sigma Aldrich), 0.2 ml of ammonium molybdate and 3 ml of distilled water were added to each of tubes. Tubes were incubated at the room temperature for 11 min. In order to construct standard line for phosphorus, the series of phosphorus standard solutions with lowered concentration were prepared and the described procedure was repeated. The measurement of absorbance was conducted at 720 nm. The results were expressed as a mean value of three repetitions (Heinonen & Lahti, 1981).

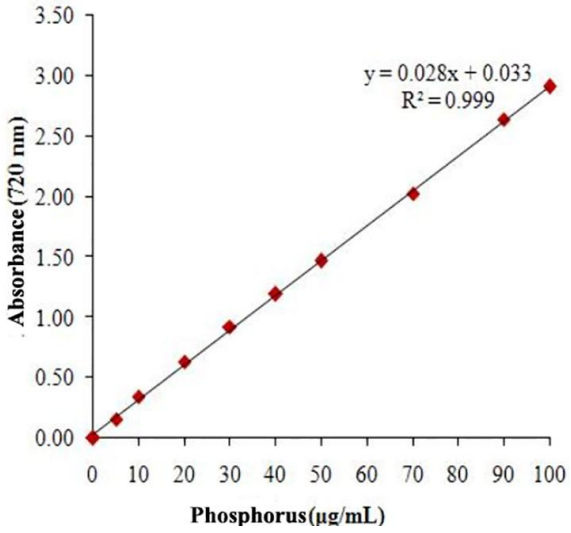

Figure 2. Standard line for phosphorus.

Statistical analysis

All experiments were performed in triplicate and results were expressed as means ± standard error of mean. For statistical analysis, the following tests were used: Mann-Whitney and Kruskal-Wallis Tests. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Results of many researches confirmed that activity of *invertases* depends on kind of surface and phase of development of fungi. Vainstein & Peberdy (1991) proven that *Aspergillus nidulans* produces extracellular invertases when grown on medium containing sucrose or raffinose, and their enzyme maximum can be achieved after 15 h of incubation at 28 °C. Poonawalla et al. (1965) concluded that *Penicillium chrysogenum* and *Sascharomyces cerevisiae* grown on medium with 30 g l^{-1} sucrose in submerged fermentation produce intra- and extracellular invertase with maximum activity of enzymes achieved between 72 and 96 h.

In the present study we investigated activity of *alkaline invertase* and *alkaline phosphatase* in control medium (which included sucrose in the first case) and in the medium containing detergent. Summary results of the experiment are presented in the Table 1.

With the exception of *alkaline phosphatase* activity in *T. harzianum*, Mann-Whitney U Test indicated that the activity of both enzymes was significantly higher ($p < 0.05$) in detergent containing media than in control media (Table 2). Also, Kruskal-Wallis Test showed statistically significant difference between enzymes activity in different fungi both on control media and detergent containing media (Table 3).

Activity of *alkaline invertase* of *M. plumbeus* (Table 1) in control medium was negligible on the 3rd day of the experiment while it was almost totally inhibited on the 6th day (0.0082 IU ml^{-1}). Sudden increasing of enzyme activity was recorded in a period from the 9th to 16th day, when it reached maximum value of 0.0883 IU ml^{-1}. In case of medium containing 0.3% of detergent, the significantly higher activity of *alkaline invertase* was recorded. It did not change much during investigated time period and its enzyme maximum was reached on the 16th day of the experiment (0.1607 IU ml^{-1}) when the mycelium of fungus was totally developed.

Activity of *alkaline invertase* of *A. niger* (Table 1) in control medium was changeable. The maximum activity was recorded on the 3rd day of incubation, after which its slope was recorded. It reached its minimum value on the 9th day of the experiment (0.0680 IU ml^{-1}). Activity of *alkaline invertase* in medium containing detergent was expressed during the entire period of mycelium growth, from the 3rd to 9th day, while maximum activity was measured at the 3rd day of the experiment (3.7673 IU ml^{-1}).

Activity of *alkaline invertase* of *T. harzianum* (Table 1) in control medium was negligible till 9th day of experiment, when it reached sudden increase of enzymes activity to their maximum value (0.8097 IU ml^{-1}). In surface with detergent, the enzymes activity was the smallest on the 3rd day of the experiment (0.1620 IU ml^{-1}), while on the 6th day it had sudden increase to its maximum value (3.4257 IU ml^{-1}), which was measure on the 16th day.

Phosphatases hydrolize esters and anhydrides of phosphoric acid. These enzymes are involved in various biological processes such as cell cycle, differentiation of cells and other cell processes. Optimum value of pH beeing 9 is needed for effect of alkaline phosphatase, while numerous researches showed that enzyme is active in wide range of pH being 3–9. Characteristics of acid and alkaline phosphatase in nutritive medium (extracellular enzymes) and mycelium extracts (citoplasmatic

and cellular bonded enzymes) were investigated in different fungi grown in stationary conditions on liquid mineral medium (Raper & Fennell, 1965). Many *alkaline phosphatases* have speciffic effects on substrate. Production of phosphatase of *A. awamor*i var. *kawachii* was investigated in media with small or large concentration of phosphate. This fungus has maximum of phosphatase activity in medium with small concentration of phosphate, such as bake yeast, *Escherichia coli* and *Neurospora crassa* (Schmidt et al., 1956). On the other hand, in medium with high concentration of phosphate, phosphatase activity was weak but still higher with β-glycerosphate as substrate than with glucose-6-phosphate. Researche conducted by Koffi et al. (2010) showed that SDS detergent displays strong inhibitory effect (cca 98%) on phosphatase activity.

Table 1. Activity of *alkaline invertase* and *alkaline phosphatase* (IU ml^{-1}) in fungi *M. plumbeus*, *A. niger* and *T. harzianum* followed period of 3–16 days*.

Sample	*Alkaline invertase* (IU ml^{-1})			*Alkaline phosphatase* (IU ml^{-1})		
	M. plumbeus	*A. niger*	*T. harzianum*	*M. plumbeus*	*A. niger*	*T. harzianum*
K-3	0.0273±0.0198	3.5337±0.0024	0.0410±0.0295	0.1373±0.0033	0.1043±0.0024	0.4673±0.0050
K-6	0.0082±0.0057	0.1967±0.0038	0.0623±0.0023	0.3640±0.0026	0.0317±0.0023	0.0023±0.0009
K-9	0.0220±0.0026	0.0680±0.0029	0.8097±0.0052	0.5130±0.0087	0.0857±0.0035	1.8213±0.0064
K-12	0.0430±0.0023	0.3363±0.0033	0.2870±0.0023	0.0051±0.0004	0.1343±0.0041	0.4557±0.0091
K-16	0.0883±0.0043	0.9167±0.0035	0.3340±0.0017	0.6760±0.0061	0.1733±0.0050	0.7120±0.0040
D-3	0.1543±0.0024	3.7673±0.0055	0.1620±0.0032	2.1987±0.0034	0.2507±0.0018	1.4467±0.2467
D-6	0.1487±0.0043	3.6140±0.0032	0.9563±0.0041	1.0943±0.0029	0.3577±0.0029	0.7607±0.0015
D-9	0.1527±0.0026	3.6777±0.0032	0.5767±0.0043	1.2490±0.0032	0.0710±0.0017	0.0061±0.0005
D-12	0.1570±0.0021	2.3010±0.0032	0.8937±0.0041	0.3510±0.0038	0.7627±0.0020	0.3170±0.0026
D-16	0.1607±0.0035	2.7010±0.0044	3.4257±0.0041	1.5840±0.0021	0.4683±0.0033	0.2613±0.0032

*K – control samples without detergent (K3–K16); D – samples with addition of 0.3% (w/w) detergent (D3–D16); results were expressed as means ± standard error of mean

Table 2. Mann-Whitney U Test for comparison of *alkaline invertase* and *alkaline phosphatase* activity between control samples and samples with addition of detergent.

	Ranks				Test Statistics		
	Group	N	Means (IU ml^{-1})	Sum of Ranks	Mann-Whitney U	Z	p-level*
Alkaline invertase activity	*M. plumbeus*						
	Control	15	0.0378	120.00	0.00	-4.66628	0.000003
	Detergent	15	0.1547	345.00			
	A. niger						
	Control	15	1.0103	138.00	18.00	-3.91968	0.000089
	Detergent	15	3.2122	327.00			
	T. harzianum						
	Control	15	0.3068	156.00	36.00	-3.17307	0.001508
	Detergent	15	1.2029	309.00			
Alkaline phosphatase activity	*M. plumbeus*						
	Control	15	0.3391	147.00	27.00	-3.54637	0.000391
	Detergent	15	1.2954	318.00			
	A. niger						
	Control	15	0.1059	156.00	36.00	-3.17307	0.001508
	Detergent	15	0.3821	309.00			
	T. harzianum						
	Control	15	0.6917	243.00	102.00	0.435520	0.663186
	Detergent	15	0.5584	222.00			

*Values of $p < 0.05$ were considered statistically significant

Activity of *alkaline phosphatase* of investigated fungi in control medium and in medium containing detergent is also presented in Table 1. In control medium, activity of alkaline phosphatase of *M. plumbeus* (Table 1) was significantly increased in period of mycelium growth (3rd to 9th day) after which its sudden decrease appeared to negligible value (0.0051 IU ml^{-1}) measured on the 12th day. In medium with detergent enzyme activity varied from maximum value on the 3rd day of the experiment (2.1987 IU ml^{-1}) to minimum value on the 12th day of experiment (0.3510 IU ml^{-1}) after which it increased till 16th day of the experiment (1.5840 IU ml^{-1}).

Activity of *alkaline phosphatase* of *A. niger* in control medium decreased from the 3rd to 6th day. It was totally inhibited by 6th day. Enzyme maximum of *A. niger* in control medium was reached on the last day of the experiment (0.1733 IU ml^{-1}). In medium with detergent, the enzyme activity has been increased during development of fungi mycelium. Significant decreasing of enzyme activity was recorded on 9th day (0.0710 IU ml^{-1}), while the highest increase in activity was recorded on the 12th day (0.7627 IU ml^{-1}). Presence of detergent in medium stimulated activity of *alkaline phosphatase* compared to control nutritive medium.

Table 3. Kruskal-Wallis ANOVA by Ranks for comparison of *alkaline invertase* and *alkaline phosphatase* activity among fungi *M. plumbeus, A. niger* and *T. Harzianum.*

	Ranks				Test Statistics		
	Group	N	Means (IU ml^{-1})	Sum of Ranks	H(2)	Total N	p-level*
Alkaline invertase activity	Control samples						
	M. plumbeus	15	0.0378	164.00	21.24720	45	0.0000
	A. niger	15	1.0103	489.50			
	T. harzianum	15	0.3068	381.50			
	Samples with addition of detergent						
	M. plumbeus	15	0.1547	126.50	35.07609	45	0.0000
	A. niger	15	3.2122	552.00			
	T. harzianum	15	1.2029	356.50			
Alkaline phosphatase activity	Control samples						
	M. plumbeus	15	0.3391	369.00	9.857391	45	0.0072
	A. niger	15	0.1059	222.00			
	T. harzianum	15	0.6917	444.00			
	Samples with addition of detergent						
	M. plumbeus	15	1.2954	511.50	16.16232	45	0.0003
	A. niger	15	0.3821	251.00			
	T. harzianum	15	0.5584	272.50			

*Values of $p < 0.05$ were considered statistically significant

Activity of *alkaline phosphatase* of *T. harzianum* in control medium was totally inhibited on the 6th day, after which an increase was recorded. Maximum of enzyme activity was measured on the 9th day (1.8213 IU ml^{-1}). However, in the stage of autolysis (12th day), the activity was near to the value recorded on the 3rd day (0.4673 IU ml^{-1}). In medium with detergent, the enzyme activity was changeable. Enzyme maximum was recorded on the 3rd day (1.4461 IU ml^{-1}), after which a decrease of enzyme activity was recorded. It was inhibited on the 9th day and reached its minimum value (0.0061 IU ml^{-1}).

CONCLUSION

Effect of detergent on *alkaline invertase* and *alkaline phosphatase* activity of *Mucor plumbeus*, *Aspergillus niger* and *Trichoderma harzianum* was significantly different during experimental period of 16 days. In control medium, the lowest activity of *alkaline invertase* was recorded in *M. plumbeus* during the entire experimental period while maximum of enzyme activity was reached in *A. niger* (3rd day) and *T. harzianum* (16th day). In the medium containing detergent, activity of *alkaline invertase* was stimulated or inhibited depending on fungal species. The highest stimulating effect of detergent was related to activity of *alkaline invertase* of *A. niger* (3rd day) and *T. harzianum* (16th day). Inhibitory effect of detergent was recorded for enzyme activity of *M. plumbeus* (during the entire experiment period) and *T. harzianum* (3rd day). In control medium, the activity of *alkaline phosphatase* was the lowest in *T. harzianum* (6th day), but the same fungus reached the highest enzyme activity on 9th day. Detergent had stimulatory or inhibitory effect on this enzyme, mostly depending on fungal species. The strongest inhibitory effect of detergent was recorded in enzyme activity of *T. harzianum* (9th day) and *A. niger* (9th day). Stimulatory effect of detergent was recorded for enzyme activity of *M. plumbeus* (3rd day) and *T. harzianum* (3rd day).

Obtained results are important for possible practical application of mentioned fungus species in processes of waste

water purification and production of enzymes for use in chemical industry. Based on comparison of enzyme activity of different fungi in detergent containing media we can recommend *M. plumbeus* for waste water treatment due to high *alkaline phosphatase* activity as well as *A. niger* due to high *alkaline invertase* activity.

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