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Original scientific paper

BIOCHEMICAL RESPONSES OF PURE AND MIXED FUNGAL CULTURES ISOLATED FROM WASTE WATER TO THE PRESENCE OF SODIUM TRIPOLIPHOSPHATES

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Summary

Sodium tripolyphosphate (STPP) has a wide application in different industrial processes but mainly as the builder in household cleaning products. The use of STPP has been associated with the environmental problem referred to as eutrophication. From the environmental point of view, it is important to identify microorganisms which have the ability to reduce STPP from environment. This paper investigated the chemical and biochemical responses of pure cultures *Trichoderma viride* and *Geotrichum candidum* and their mixed culture to the presence sodium tripolyphosphate (STPP) at 0.5% concentration during 8 days of cultivation in the growth liquid medium. For this purpose, the changes in the pH value, redox potential, proteolytic activity of liquid growth media and total dry weight biomass (DWB) of cultures were measured. The addition of STPP in growth medium affected the increase in initial pH values and decrease in redox potential values compared to the control. The STPP caused an inhibitory effect on protease activity of all fungal cultures in the following order: *T. viride* (3.60%), mixed culture (9.77%) and *G. candidum* (60.33%). The dry weight biomass (DWB) of *T. viride* was slightly inhibited (1.06%) whereas DWB of *G. candidum* (0.58%) and mixed culture (9.53%) were slightly and moderately stimulated by STPP. The results obtained in this study indicate a potential role of tested cultures in polyphosphates removal from industrial and waste water treatment plants and their potential application in the biotechnological processes.

Key words: sodium tripolyphosphate, pH, redox potential, proteolytic activity, *T. viride, G. candidum,* mixed cultures

INTRODUCTION

Sodium tripolyphosphate (STPP) is an inorganic compound with formula $Na_5P_3O_{10}$. STPP is applied in various industries (detergents, toothpastes, ceramics, textile, rubber, food, drug manufactures, etc). The high percentage of STPP incorporated in commercial detergents (20-45%) has led to the problems of environmental pollution, associated with eutrophication. As a component of detergents, STPP present in domestic waste waters is mainly discharged to the aquatic compartment via waste water treatment plants, infiltration or other autonomous waste water systems. In water and activated sludge, STPP has the ability to hydrolyze to

pyrophosphate and finally to orthophosphate, which can be assimilated by microorganisms (bacteria and fungi), algae or protozoa (Jiang *et. al.*, 2020). STPP thus ends up being assimilated into the natural phosphorus cycle. Microorganisms that are largely responsible for phosphorus removal are referred to as polyphosphate accumulating organisms (PAOs). These organisms have the ability to store phosphate as intracellular polyphosphate (polyp), leading to phosphate removal from the bulk liquid phase in the waste activated sludge (Ekama *et al.*, 1984; Jeon *et al.*, 2003; Oehmen *et al.*, 2007). *Acinetobacter* is the first microbial strain responsible for phosphate removal described by Fuhs and Chen (1975). Other bacteria that have been implicated in dominant phosphate removal include *Aeromonas*, *Vibrio*, *Pseudomonas* and the coliforms (Kavanaugh, 1991; Momba and Cloete, 1996; Seviour *et al.*, 2003; Snaidr *et al.*, 1997). The results of acute aquatic ecotoxicity test using *Daphnia*, fish, algae revealed no toxic effect of STPP to aquatic organisms: EC/LC_{50} value was above 100 mg/L. Considering both the mentioned results and the fact that STTP is rapidly hydrolyzed in the aquatic environment, no studies have been carried out to date concerning the chronic effects of STPP on mentioned aquatic organisms (HERA, 2003). However, in certain circumstances, nutrient enrichment can lead to negative effects, ranging from ecosystem modifications, through algal blooms, to oxygen depletion and collapse of the biocenosis in a surface water in extreme cases (through decomposition of plant biomass). Requirements to reduce phosphate and nutrient fluxes from sewage (of which, detergents where used are a minority proportion of phosphates) are directly addressed by the EU Urban Wastewater Treatment Directive 91/271/EEC.

Until now, there has been a lack of data on the role of fungi (mycromicetes) in phosphate removal. Some preliminary studies indicated the ability of some fungi isolated from municipal wastewater, which originated from households, to grow and metabolize 0.5% of STPP. However, their respond to the presence of STPP has a taxonomic character. From all mentioned above, the current study investigates chemical and biochemical responds of *Trichoderma viride*, *Geotrichum candidum* and its mixed culture on the presence of STPP at 0.5% concentration in liquid growth medium and thus, their potential role in STPP removal.

MATERIAL AND METHODS

Isolation and identification of fungi from wastewater

Fungal cultures used in this study originated from municipal wastewater, collected at a location where municipal wastewater discharges into the Lepenica river basin (Kragujevac, Serbia). Potato-dextrose-agar (PDA) was used for fungi isolation. PDA was composed of (g/L): potato – 200, dextrose – 20, agar – 15. The PDA medium prepared according to aforementioned procedure was sterilized by autoclaving at 121 °C for 15 min. After sterilization, 15 mL of medium was aseptically dispensed into sterile Petri dishes and allowed to solidify. Afterwards, Petri dishes were inoculated by cultures and incubated at (28 ± 2) °C for 5 to 7 days. The isolates were identified by examining both microscopic and macroscopic characteristics. *Trichoderma viride* Pers., and *Geotrichum candidum* Link were identified by Systematic keys at the Faculty of Biology, University of Belgrade, Serbia. The cultures were maintained at 4 °C in nutrient agar. The fungi were sub-cultured periodically in aseptic conditions.

Preparation of spore inoculum

Spore suspensions were prepared according to the procedure described in our previous work. The concentration of spores in the suspension was evaluated by microscopic enumeration with a cell-counting hemocytometer, Neubauer chamber (Lonza Cologne AG, Germany) and adjusted to 5.0×10^6 spores/mL.

Cultures cultivation conditions

Shake flask cultivation was carried out in 250 mL Erlenmeyer flasks containing 200 mL of Czapek-Dox liquid medium (g/L): $NaNO_3$ – 3, K_2HPO_4 – 1, $MgSO_4\times7H_2O$ – 0.25, $FeSO_4\times7H_2O$ – 0.01, sucrose – 30 (control – C). The same medium was supplemented with 0.5% STPP (Henkel, "Merima" Kruševac, Serbia) (medium STPP). The pH value of C medium was about 6.8±0.2 (without adjustment) before sterilization. Thereafter, 1 mL of spore suspension (5.0×10^6 spores) was inoculated in liquid media. Inoculated flasks were incubated at 150 rpm on a shaker (Kinetor-m, Slovenia) and ambient temperature (28 °C±3 °C) for 8 days in triplicate. The mycelium was collected by filtration of fermentation broth using Whatman No.1 (Germany) filter paper at 8^{th} day.

Determination biomass dry weight (BDW)

The mycelia previously removed from fermentation broth were washed with sterile distilled deionized water several times. Both filter paper and mycelia were then dried in an oven at 80 °C to a constant weight. The dry weight of the mycelia was determined by subtracting the initial weight of the filter paper from the weight of mycelia and filter paper.

Measurement of pH and redox potential

PH-meter (type MA-5705, the product “Iskra”, Kranj) was used for measuring the value of pH and electrochemical potential. Redox potential was determined by Petersen’s method (Petersen, 1966).

Assay of proteolytic activity

Activity for the alkaline protease was determined spectrophotometrically by Anson’s method, with casein as substrate (Anson, 1938). Reaction mixture was incubated at 37 °C for 10 min and arrested by addition of 1 mL 5% trichloroacetic acid (TCA). The mixture was centrifuged at 4000 rpm and the supernatant 5 mL of 6% Na_2CO_3 and 1 ml diluted Folin–Ciocalteu phenol reagent were added. The resulting solution was incubated at room temperature for 30 min and absorbance of the blue color developed was read at 660 nm using tyrosine standard. One unite enzyme activity (IU) was defined as the amount of enzyme that liberated 1µg of tyrosine from casein per minute under assay condition.

RESULTS AND DISCUSSION

The importance of inorganic polyP for living beings is manifold: it is a rich source of energy, reservoir for phosphate, channel for DNA entry, buffer against alkali ions, chelator of metal ions, regulator of stress and survival and helpful component in gene regulation

(Achbergerová and Nahálka, 2011). In microbial cells, the main physiological processes (mobility, development of biofilm, quorum sensing and virulence) are directly linked to polyp (Rashid and Kornberg, 2000; Rashid *et al.*, 2000). The effect of polyP on microbial cells depends on numerous factors including the polyP concentration, the composition of growth medium, pH and redox potential of medium, microorganism strain, etc. Exogenous PolyP has an antimicrobial effect on the most microbial strains such as *Sarcinalutea*, *Staphylococcus aureus* (Post *et al.*, 1963), *Listeria monocytogenes* (Zaika and Kim, 1993), and *Bacillus cereus* (Maier *et al.*, 1999), but also fungi, such as *Aspergillus flavus* (Knabel *et al.*, 1991). However, a great body of works revealed that *Acinetobacter johnsonii* had ability to accumulate up to 30% polyP. The identification of microorganisms' strains which have the ability to accumulate polyP is very important for environment point of view. For this reason, we investigated chemical and biochemical responds of pure fungal cultures isolated from waste water and their mixed culture to the presence of STPP at concentration 0.5%.

Chemical and biochemical properties of *T.viride*, *G.candidum* and their mixed culture grown in control (C) and medium with addition of 0.5% STPP were observed by measurement of pH, redox potential, proteolytic activity (from 4th to 8th day) and total DWB (at 8th day).

Changes in the pH value of growth media of pure and mixed cultures are presented on Figure 1. During the growth of pure cultures *T. viride* and *G. candidum* from 4th to 6th day the pH value of media decreased, whereas it was slightly increased until 8th day. The pH value of mixed culture varied, with tendency to increase between 4th and 5th day and 6th and 7th day. The addition of STPP in growth medium of *T. viride* influenced significant changes in pH value. In the mentioned medium, the pH value of *T. viride* notably decreased from 4th to 6th day. The change of pH value of *G. candidum* was stable during cultivation time with a decrease observed between 4th and 5th day. In STPP medium cultivated by mixed culture, the pH value slightly decreased between 4th and 6th day, afterwards it slightly increased until 8th day. The changes in the pH values of growth media are the result of the utilization of nutrients from the media and the release of metabolites; primarily organic acids (Jakovljević, 2020).

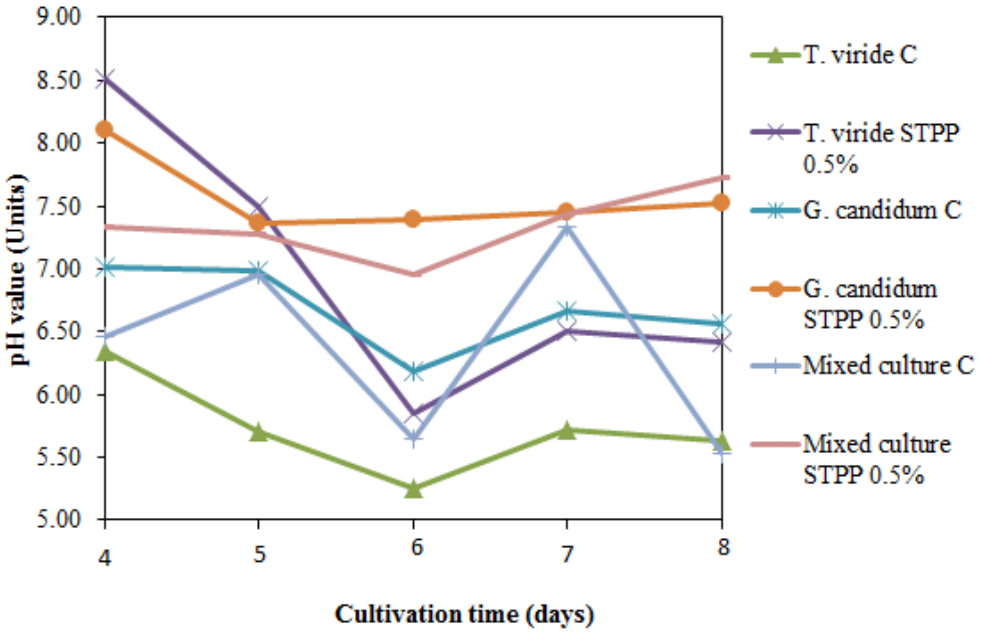

Figure 1. pH value of pure and mixed cultures growth media

The most intensive changes in pH values indicate intensive growth of cultures. The obtained results are in line with results of other researches who found metabolic changes related to pH of the culture medium, depending from phosphorus content and polyP chain size in different growth phases (stationary and logarithmic) of different microorganisms, including fungi (Barak and Rijn, 2000; Weitzman *et al.*, 1995). Hamilton *et al.* (2018) revealed that tripolyphosphate hydrolysis was significantly faster in acidic conditions whereas alkaline nature of soils reduced the abiotic hydrolysis rates.

Redox potential of all cultures grown in C and STPP nutrient media had a positive value throughout the whole experimental period, with exception of *T. viride* and *G. candidum* in STPP medium in early phase of growth (4th day), when redox potential had a negative value (Figure 2). The highest value of redox potential was noted in C medium with *T. viride*, followed by the mixed culture and *G. candidum* in same medium. In STPP medium, *T. viride* had the most significant changes in redox potential value between 4th and 6th day, when the maximal value was observed. The redox potential value of mixed culture slightly increased from 4th to 6thday; afterward it decreased until 8th day. In STPP medium with *G. candidum*, the value of redox potential increased from 4th to 5th day, and then decreased until 7th day. The negative values of redox potential in STPP media of *T. viride* and *G. candidum* indicate an ability of both cultures to use reduced P as source of P, in early growth phase. The same ability was observed in experiment with *Microcystis aeruginosa* by Shi *et al.* (2003). Glucose was fermented via EMP pathway, producing 2 ATP per molecular glucose, and PolyP only needs one ATP for extending P-P bond. Therefore, only about half of the energy was used for the synthesizes of PolyP, and the other half was available for the growth of *M. aeruginosa* as well as other requests.

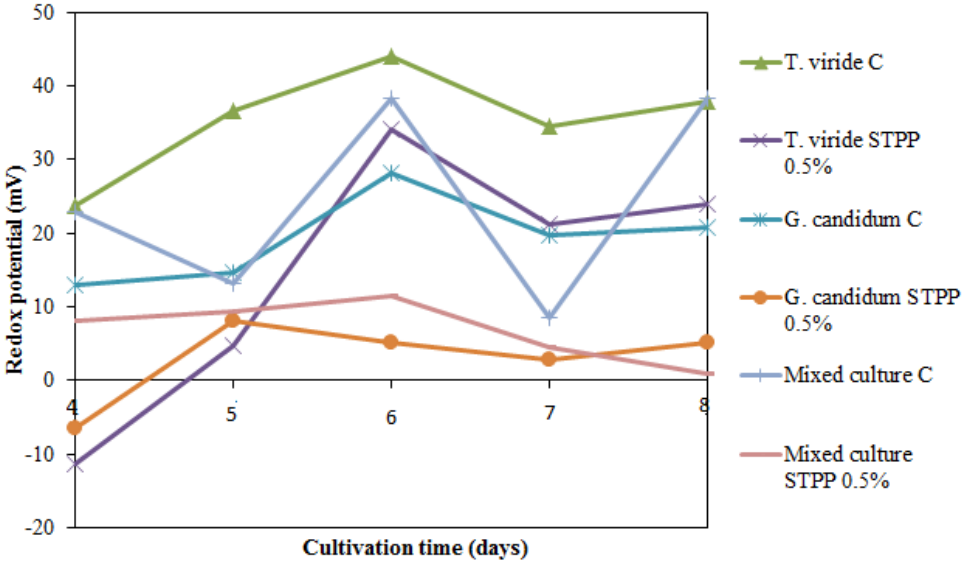

Figure 2. Changes in redox potential of cultures in growth media

The proteolytic activities of cultures in both liquid growth media (C and STPP) were particularly expressed on 5th and 8th day (Figure 3). In C medium, the highest proteolytic activity was measured in medium inoculated by *T. viride* (0.972 IU/mL) on 8th day, followed

by in medium inoculated by mixed culture (0.901 IU/mL) and *G. candidum* (0.668 IU/mL) on 5th day. The addition of STPP in the growth medium caused an inhibitory effect on protease activity of all fungal cultures. Slight inhibition of protease activity was noted in STPP medium of *T. viride* (3.60%), whereas a middle and very strong inhibitory effect was observed in STPP medium of mixed culture (9.77%) and *G. candidum* (60.33%). The inhibitory effect of STPP on the protease secretion of *Aeromona shydrophila* was confirmed by Venugopal *et al.* (1984). Also, inhibition of protease activity of *Pseudomonas fragi* with STPP at 0.8% and 1% was revealed by Marsh (1992).

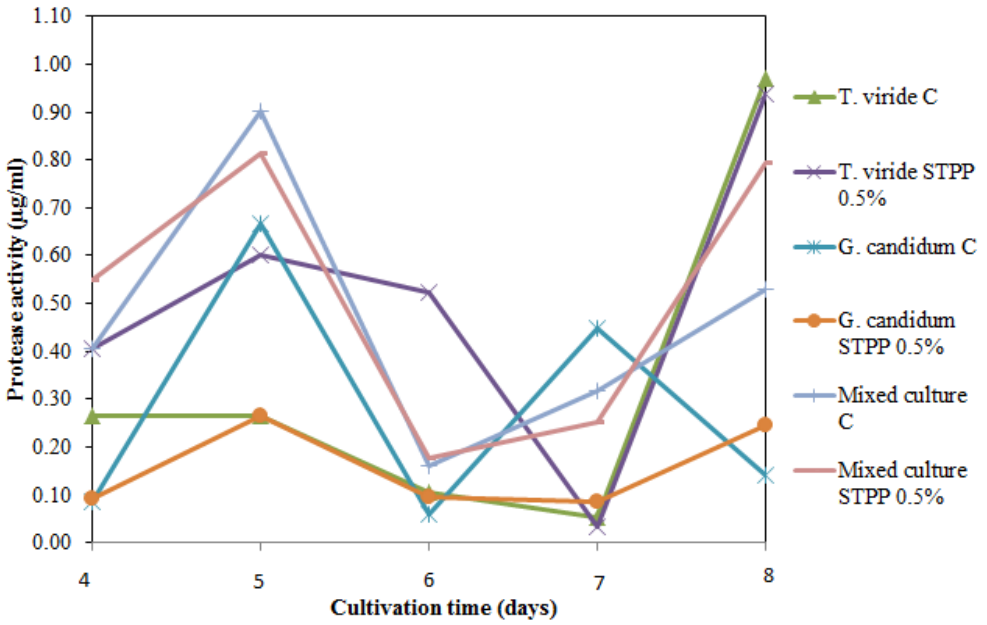

Figure 3. Proteolytic activity of cultures in growth media

The quantity of total DWB of pure and mixed cultures grown in C and STPP liquid media is showed in Figure 4. In Czapek-Dox liquid medium (C) the highest DWB was produced by *T. viride*, whereas significantly lower DWB was produced by *G. candidum* and mixed culture. In same medium with addition of STPP 0.5%, the highest DWB was also produced by *T.viride* and mixed culture whereas significantly lower DWB was produced by *G.candidum*. The addition of STPP 0.5% in growth medium of cultures caused a very slight inhibitory effect (1.06%) on the biomass production of *T. viride*. However, the same phosphates compound caused a very slight and middle stimulatory effect on production of DWB by *G.candidum* (0.58%) and mixed culture (9.53%).

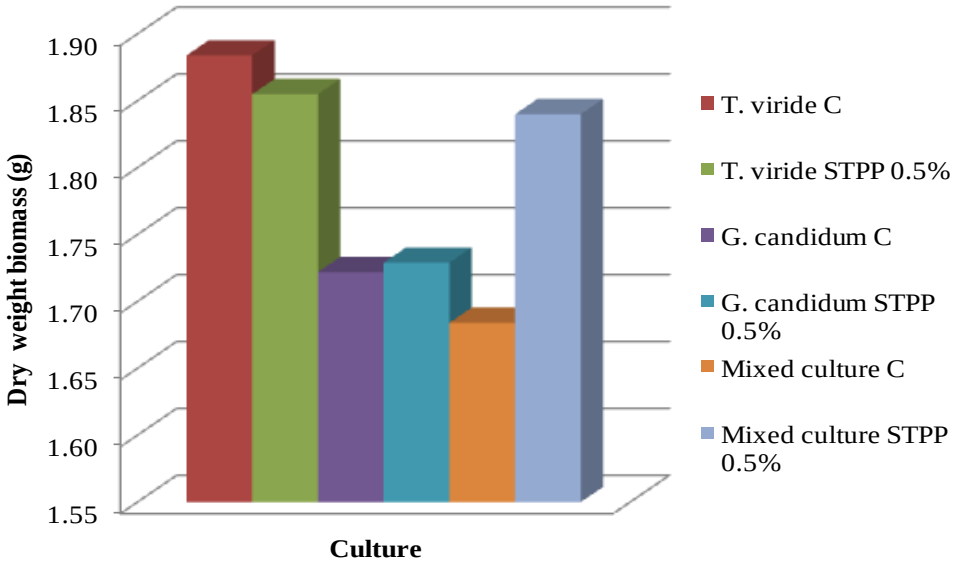

Figure 4. The total DWB of cultures after 8th day cultivation in growth media

These results confirmed that during growth the tested cultures accumulated different amounts of orthophosphate (Pi) from the medium depending on fungi species or type of cultivation. As a result of this phenomenon, DWB of *G. candidum* and mixed culture was stimulated compared to control. Lima *et. al.* (2003) found that *Cunninghamella elegans* produced different amounts of biomass depending on concentration Pi (0.5 and 2.5 g/L) in medium, thus the maximal biomass amount was obtained at concentration 2.5 g/L. In addition, Marsh (1992) revealed that growth of *P. fragi* was inhibited at 0.8% and 1% solution of STPP.

CONCLUSION

Based on the obtained results, we may infer that both pure and mixed cultures of fungi had a different response to STPP at concentration 0.5%. The addition of STPP in growth medium of cultures had influenced changes in pH and redox potential values, and inhibited their protease activities. The applied polyP stimulated the growth and production of DWB of *G. candidum* and mixed culture but only slightly inhibited DWB of *T. viride*. This study indicates a potential of *G. candidum* and mixed culture in the polyP accumulation, and suggests a future application of mentioned cultures in biotechnological processes.

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Sažetak

Natrijum tripolifosfat (*Sodium tripolyphosphate* STPP) ima široku primjenu u različitim industrijskim procesima, ali uglavnom kao bilder u proizvodima za čišćenje u domaćinstvima. Upotreba STPP povezana je sa ekološkim problemom poznatim kao eutrofikacija. Sa ekološke tačke gledišta, važno je identifikovati mikroorganizme koji imaju sposobnost redukcije STPP iz okoline. U ovom radu je ispitivan hemijski i biohemijski odgovor čistih kultura *T. viride* i *G. candidum* i njihove mješovite kulture na prisustvo STPP u koncentraciji 0,5% tokom 8 dana kultivacije u tečnoj hranljivoj podlozi. Sa tim u vezi su ispitivane promjene vrijednosti pH, redoks potencijala, proteolitičke aktivnosti tečnih podloga i ukupne suve mase biomase (*dry weight biomass* DWB) kultura. Dodatak STPP hranljivoj podlozi uticao je na povećanje početnih vrijednosti pH i smanjenje vrijednosti redoks potencijala u poređenju sa kontrolom. STPP je imao inhibitorno dejstvo na aktivnost proteaza svih kultura: *T. viride* (3,60%), mješovita kultura (9,77%) i *G. candidum* (60,33%). Suva biomasa (DWB) *T. viride* bila je blago inhibirana (1,06%), dok je DWB *G. candidum* (0,58%) i mješovite kulture (9,53%) blago i srednje stimulisana STPP. Rezultati dobijeni u ovoj studiji ukazuju na potencijalnu ulogu ispitivanih kultura u uklanjanju polifosfata iz postrojenja za prečišćavanje industrijskih i otpadnih voda i njihovu potencijalnu primjenu u biotehnološkim procesima.

Ključne riječi: natrijum tripolifosfat, pH, redoks potencijal, proteolitička aktivnost, *T. viride, G. candidum*, mješovita kultura

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