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OPTIMISATION OF CULTURE MEDIUM TO OBTAIN MAXIMAL INCREASE OF THE EXTRACELLULAR STARCH DEGRADATION ACTIVITY BY THE ENZYMES AS PRODUCTS RELEASED OF THE *ACIDIPHILIUM* GENUS

Carmen Madalina CISMASIU

Institute of Biology Bucharest of Romanian Academy, 296 Splaiul Independentei, 060031, P.O. Box 56-53, Romania, Phone: +40212219202, Fax: +40212219071, E-mail: carmen.cismasiu@ibiol.ro, madalinabio@yahoo.com

Corresponding author email: carmen.cismasiu@ibiol.ro

Abstract

Acidophilic heterotrophic bacteria belonging to the Acidiphilium genus adapted frequently to the environmental concentration of toxic compounds developing protective mechanisms of resistance which are much diversified in those bacteria. The aim of the present paper was the extracellular enzymes production from the acidophilic heterotrophic bacteria belonging to Acidiphilium populations which hydrolysis the starch in the presence of $CaCl_2$ *or/and NaCl solutions. These bacterial populations were isolated from acid waters and mine sediments containing high concentrations of heavy metals collected from Baia (Tulcea County) and Roşia Poieni (Alba County). In the culture medium, the optimum of the substrate concentration represented by starch was 0.2 % in the presence of mixed* $CaCl_2$*-NaCl solutions. The most important physic-chemical parameter that influence extracellular enzymatic activities of the Acidiphilium genus are the contact time between bacterial populations and culture medium with metallic ions solutions. Also, an extended exposure of Acidiphilium populations in the GYE medium with different starch concentrations and 0.1%* $CaCl_2$ *induce intense starch degradation by extracellular enzymes in acidic conditions.*

Key words: *starch, acidophilic bacteria, extracellular enzymes, metallic ions*

INTRODUCTION

The research activities on the microbial ecology of acidic extreme environments provide a basis for the coal processing technologies have been developed. The pollution carbon compounds has determined an increasing of the knowledge body interest regarding the microbial resistance to carbon compounds; therefore the coal mining activities using acidophilic heterotrophic bacteria in biotechnology is important to environmental quality improvement [1, 4, 13, 26]. The capacity of the acidophilic heterotrophic bacteria capacity to convert a wide range of synthesis organic compounds into inorganic products plays an important role in the development of bioremediation technologies [20, 27, 32].
The oxidative degradation degree of organic contaminants and the obtained results within a bioremediation program contribute to a complex knowledge on the different processes which affect spreading organic substances in polluted environments [2, 15, 28].
The recent studies have shown that these bacteria can be used both for remediation of contaminated environments, by reducing the concentration of carbon compounds, as well as in biosorbtion of metallic ions from waste dumps.
The use of acidophilic heterotrophic bacteria to remove the metallic ions from polluting environments is an unconventional method, economic approach and perspective for their decontamination [19, 24, 31].
The acidophilic heterotrophic bacteria are important factors in the mineralization process of the organic substances from soil. They are involved in the solubilization of organic mineral resources from land, facilitating their use by other organisms.
It is essential to know the optimal physical-chemical conditions in which the acidophilic

bacteria are able to produce extracellular hydrolytic enzymes, in order to understand their distribution in the nature and their implication in the ecosystem [6, 22, 23].

The starch degradation by the extracellular enzymatic activity is influenced by the physiological characteristics of the acidophilic heterotrophic bacteria, the physical-chemical factors in the medium, the nutritive medium composition, all of them being important elements in the control activity of the microbiological removal process of carbon compounds from waste waters and coal sludge [6, 10, 22].

The study on metabolism of the acidophilic heterotrophic bacteria shows a great importance due to their biotechnological potential to the adaptation ways to increased values of acidity, and a theoretical importance for the knowledge and explanation of biodiversity in extreme environment [8, 16, 35].

In this context by their activities, acidophilic heterotrophic bacteria have a significant role both in the nature as well as the assessment of the anthropogenic impact on environment. These bacteria take part in the aggregation process of the soil through the ability to secrete viscous and sticky substances. In this way, they influence both the physical and the chemical structure of the soil [7, 16, 29].

It is considered that chemical and physiological reactions involved in the extracellular enzymatic activities depend on the physiological conditions of the bacterial cells and the chemical valence of the metallic ions that come in contact with them and act as cofactors. That is, the starch degrading enzymes are metalloenzymes, because they need a metallic ion for their catalytic activity as well for the settle of active conformation of the protein [5, 9, 25, 34].

The metallic ions act upon the physiological processes and they are essential for the bacterial growth. All of these are strongly influenced by the physical-chemical factors of the surrounding environment. Through their activities, bacteria belonging to the *Acidiphilium* genus lead to changes in the values of the pH and the oxidation-reduction potential [10, 18, 31].

An essential characteristic of these metalloenzymes responsible with the starch degradation, produced by the *Acidiphilium* genus, is their high thermodynamic stability when a deposit compound is formed between metallic ions and side protein [11, 17, 30].

Metallic ions can affect the catalytic function of the enzyme by acting through: (1) substrate binding and/or enzyme cofactors; (2) activation of enzyme-substrate complex; (3) induce certain changes in the conformation of the enzyme; (4) contribution to some oxidation-reduction reactions, as enzyme cofactors [3, 21, 33].

This study shows how the metallic ions, such as Ca^{2+} ($CaCl_2$) and Na^{+} ($NaCl$), acting as cofactors, affects the extracellular enzymatic activity of acidophilic heterotrophic bacteria belonging to the *Acidiphilium* genus in the starch degradation process.

MATERIALS AND METHODS

1. The growth of bacterial cultures

In order to obtain *Acidiphilium* populations have recolted waters and sediments mining from Baia (Tulcea county) and Rosia Poieni (Alba county), both containing high concentrations of metallic ions.

The bacterial strains belonging to the *Acidiphilium* genus have been isolated on selective GYE medium with pH 3.0, that have the glucose as source of carbon and energy.

The populations of heterotrophic bacteria tabulated in the *Acidiphilium* genus was observed after the incubation for 21 days at $28^{0}C$ on the liquid GYE medium by the modifying of medium composition correlated to the decreasing of the initial pH value of the organic medium [14].

Acidophilic heterotrophic bacteria have been represented by populations of *Acidiphilium* sp. isolated from the Rosia Poieni area noted P_4 (photo 1) and *Acidiphilium* sp. isolated from the Baia area noted P_7 (photo 2).

Regarding the obtaining populations of acidophilic heterotrophic bacteria were used for this study isolated colonies on agarized selective culture media, following the dynamics of the physiological activity in organic media specific to the *Acidiphilium* genus (photo 1-2).

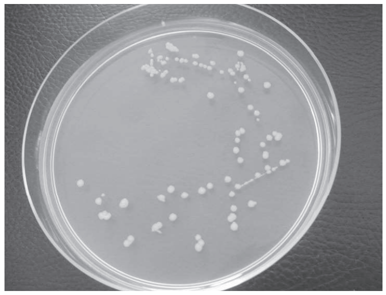

Photo 1. White colonies of *Acidiphilium* sp. isolated from mining effluents of the Rosia Poieni area

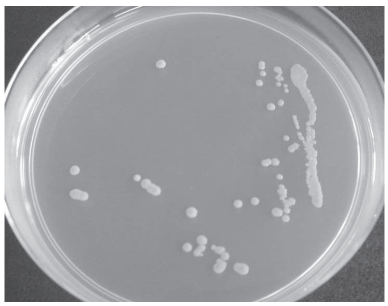

Photo 2. Red colonies of *Acidiphilium* sp. isolated from mining effluents of the Baia area

2. Shake flask experiments

The bacterial cultures have been grown in 100 ml Erlenmeyer glasses containing 50 ml growth medium and 5 ml inoculums (bacterial culture of 7 days old). These cultures were incubated up to 7, 14 and finally 21 days at 28^0C, under continuous agitation (150rpm). The bacterial density in the culture media was measured spectrophotometrically, by monitoring the optical density at 660nm for up to 21 days.

3. Extracellular starch degrading assays of bacterial cultures

The test of *Acidiphilium* populations for their capacity to produce extracellular starch degrading enzymes was made by using nine types of GYE medium: (1) medium with 0.1% NaCl; (2) medium with 0.1% $CaCl_2$; (3) medium with 0.1% NaCl and 0.1% $CaCl_2$; (4) medium with 0.1% starch and 0.1% NaCl; (5) medium with 0.1% starch and 0.1% $CaCl_2$; (6) medium with 0.1% starch, 0.1% NaCl and 0.1% $CaCl_2$; (7) medium with 0.2% starch and 0.1% NaCl; (8) medium with 0.2% starch and 0.1% $CaCl_2$; (9) medium with 0.2% starch, 0.1% NaCl and 0.1% $CaCl_2$.

Starch was selected as substrate, carbon and energy source.

The starch hydrolytic activity was assessed through spectrophotometer determination at 580 nm of the compounds formed between starch and iodine.

Extracellular starch degrading enzyme activity was evaluated by Wohlgemuth method [12].

RESULTS AND DISCUSSIONS

The results referring to the influence of mixed $CaCl_2$-NaCl solutions on the growth of the *Acidiphilium* populations, cultivated in GYE medium with starch as source of carbon and energy at 28^0C in continuous agitation conditions, are represented in figures 1-3.

Comparative studies made on the *Acidiphilium* populations show that those isolated from mining effluents of the Rosia Poieni area has a growth and a extracellular starch degrading enzyme activity which is more intense than the bacterial population isolated from mining effluents of the Baia area in same cultivation conditions (fig. 1-3).

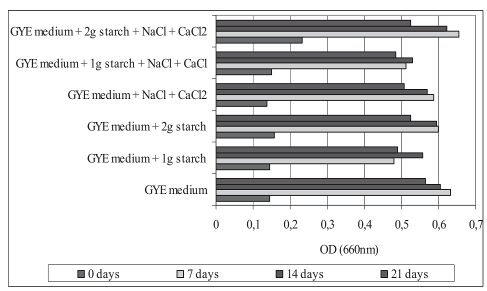

Fig.1. The bacterial density of the P_4 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and different concentrations of starch

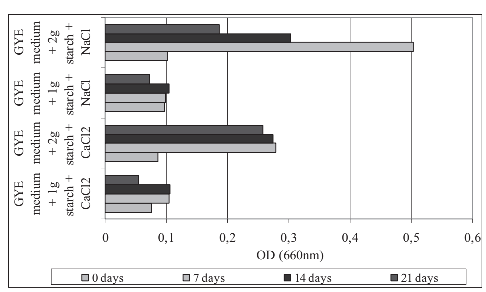

Fig.2. The bacterial density of the P_4 population in GYE medium with different concentrations of starch and 0.1% $CaCl_2$, respectively 0.1% NaCl

On the other hand, the *Acidiphilium* population P_4 is more sensitive at substrate variations because at the starch concentration between 0.1% - 0.2% the bacterial density is lower than in the case of the bacterial population isolated from Baia (fig. 2-3).

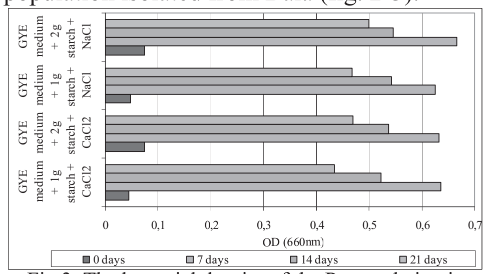

Fig.3. The bacterial density of the P_7 population in GYE medium with different concentrations of starch and 0.1% $CaCl_2$, respectively 0.1% NaCl

The analyses about the influence of mixed $CaCl_2$-NaCl solutions on the extracellular starch degradation enzymatic activity of bacterial populations, cultivated in GYE medium with different substrate concentrations at a temperature of 28^0C, are illustrated in figures 4 - 9. The compared data within this study pointed out that the two bacterial populations present the same optimum growth conditions, but the *Acidiphilium* P_4 population is more efficiently at decreasing the starch concentration of the culture medium than the *Acidiphilium* P_7 population.

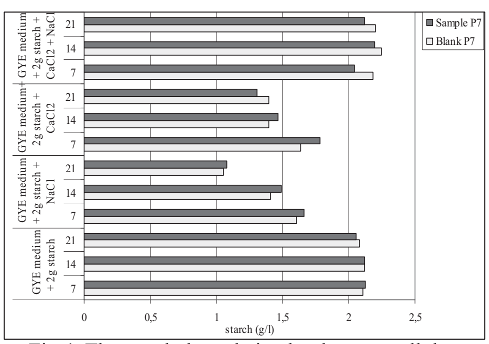

Fig.4. The starch degradation by the extracellular enzymatic activity of the P_7 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and 2g/l starch

The comparative studies regarding the influence of mixed $CaCl_2$-NaCl solutions on the growth and the extracellular enzymatic activity is developed with a maximum intensity up to 7 and 21 days of incubation periods at the same substrate concentration.

The starch concentration of 2.0 g/l is optimum for the growth and the extracellular starch degradation enzymatic activity of the two bacterial populations from the *Acidiphilium* genus (fig. 4-6).

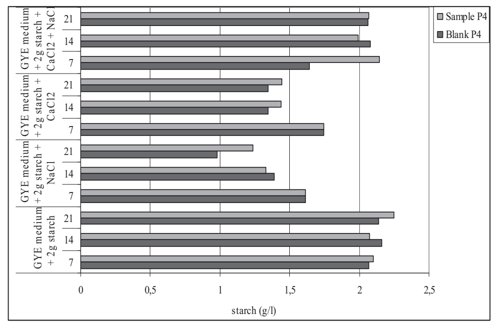

Fig.5. The starch degradation of the extracellular enzymatic activity of the P_4 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and 2g/l starch

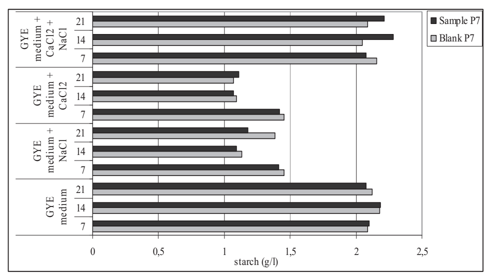

Fig.6. The starch degradation of the extracellular enzymatic activity of the P_7 population in GYE medium with 0.1% NaCl and 0.1% $CaCl_2$

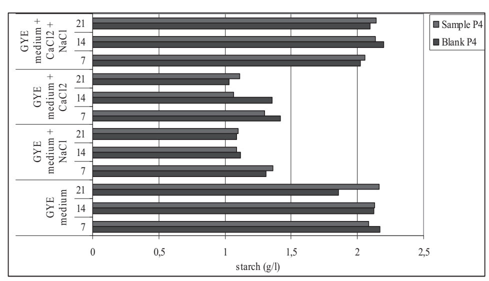

Fig.7. The starch degradation of the extracellular enzymatic activity of the P_4 population in GYE medium with 0.1% NaCl and 0.1% $CaCl_2$

The continuous agitation conditions determine a higher extracellular starch degrading enzyme activity of *Acidiphilium* populations correlated to the increasing of bacterial density in the culture medium with different substrate concentrations of starch. Also, lowering the starch concentration to 1.0 g/l determined a reduction of the bacterial density and the hydrolytic activity at 70-80% of the values obtained at an optimum substrate concentration (fig. 1-9).

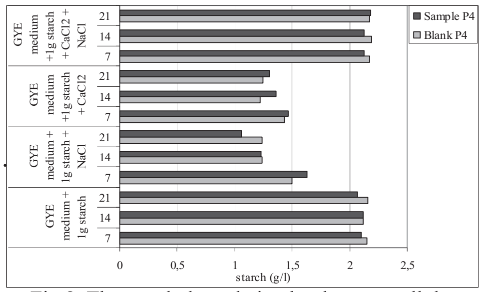

Fig.8. The starch degradation by the extracellular enzymatic activity of the P_4 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and 1g/l starch

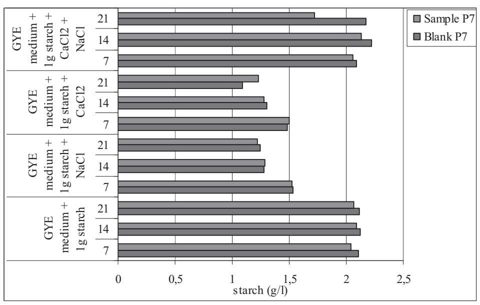

Fig.9. The starch degradation by the extracellular enzymatic activity of the P_7 population in GYE medium with 0.1% NaCl, 0.1% $CaCl_2$ and 1g/l starch

The results obtained lead to the increase of the knowledge body of the previous studies about the growth and the extracellular enzymatic activity of the heterotrophic bacterial populations from the *Acidiphilium* genus in which the extreme limits of temperature were established between 20^0C and 37^0C.

Regarding the optimum values of physical-chemical parameters is necessary in laboratory experiments that test the extracellular enzymatic activity of the bacterial cultures tabulated in the *Acidiphilium* genus to take place in the presence of raised concentrations of metallic ions in the polluted environment conditions [3, 14, 23, 34].

CONCLUSIONS

The experimental results in which were compared the effects of the $CaCl_2$ and NaCl solutions on the acidophilic heterotrophic bacteria, isolated from the mining effluents at Rosia Poieni and Baia areas, revealed a significant increase of the bacterial density and extracellular hydrolytic activity in GYE medium with 0.1% $CaCl_2$ and 0.1% NaCl at a concentration value of the substrate is established at 2g/l starch.

The comparative results regarding the influence of mixed NaCl-$CaCl_2$ solutions on the growth and the extracellular starch degradation enzyme activity in the same experimental conditions revealed the fact that they present a maximum intensity of the two populations cultivated at optimum physic-chemical parameters, such as pH=3.0 and temperature of 28^0C.

Future studies will be focused on identifying a range of the metallic ions concentrations where *Acidiphilium* populations present their highest extracellular enzymatic activities, as well as on the implementation of these results in the reduction of pollution with carbon compounds produced from industrial activity.

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REFERENCES

[1] Ahluwalia, S.S., Goyal, D., 2007, *Microbial and plant derived biomass for removal of heavy metals from wastewater. Bioreources Technology*, Biological Wastes. Energy in Agriculture and Biomass. 98: 2243-2257.

[2] Aqeel, B.M., Umar, D.M., 2010, *Effect of Alternative Carbon and Nitrogen Sources on Production of Alpha-amylase by Bacillus megaterium.* World Applied Sciences Journal, 8: 85-90.

[3] Austin, C.B., Dopson, M., 2007, *Life in acid: pH homeostasis in acidophiles - a Review.* TRENDS in Microbiology, 15(4): 165-171.

[4] Baker, B.J., Banfield, J.F., 2003, *Microbial communities in acid mine drainage.* FEMS Microbiology Ecology, 44:139–152.

[5] Bouwer, E.J., Zehner, A.J.B., 1993, *Bioremediation of organic compounds-putting microbial metabolism to work.* Trends Biotechnology, 11(8): 360-367.

[6] Cismasiu Carmen Madalina, 2011, *The adaptation of gram-negative bacteria to acidic environmental conditions with implication in heavy metals removal*

processes. Romanian Biotechnological Letters. Supplements. 16(6): 10-18.
[7] Costa, M.C., Duarte, J.C., 2005, *Bioremediation of acid mine drainage using acidic soil and organic wastes for promoting sulphate-reducing bacteria activity on a column reactor.* Water Air and Soil Pollution, 165: 325-345.
[8] Djukie, D., Mandic, L., 2006, *Microorganisms as Indicators of Soil Pollution with Heavy Metals*. Acta Agriculturae Serbica, 22: 45-55.
[9] Dopson, M., Austin, C.B., Koppineedi, P.R., Bond, P.L., 2003*, Growth in sulfidic mineral environments. Metal resistance mechanisms in acidophilic micro-organisms.* Microbiology, 149**:** 1959–1970.
[10] Gadd, G.M., 2010*, Metals, minerals and microbes: geomicrobiology and bioremediation.* A journal of the Society for General Microbiology, 156: 609-643.
[11] Gianfreda Liliana, Rao Maria, 2004, *Potential of extracellular enzymes in remediation of polluted soils: a review, Enzyme and Microbial* Technology, 35(4):339-354.
[12] Gupta, R., Gigras, P., Mohapatra, H., Goswami, K. V., Chauhan, B., 2003, *Microbial α-amylases - a biotechnological perspective*. Process Biochemistry, 00: 1-18.
[13] He, M.C., 2009, *Application of HEMS cooling technology in deep mine heat hazard control. Mining* Science and Technology, 19: 269-275.
[14] Hiraishi, A., Imhoff, J.F., 2005, *Genus Acidiphilium.* In: Bergey's Manual of Systematic Bacteriology: The Alpha-, Beta-, Delta-, and Epsilonproteobacteria.; C Brenner, D.J., Krieg, N.R. and Staley, J.T. (eds); Springer Science + Business Media, Inc., New York, p. 54-62.
[15] Irwin Jane A., Baird, A.W., 2004, *Extremophiles and their application to veterinary medicine.* Irish Veterinary Journal, 57(6): 348-354.
[16] Johnson, B.D., Hallberg, K.B., 2005, *Acid mine drainage remediation options: a review*. Science of the Total Environment, 338: 3– 14.
[17] Johnson, D.B., 2012, *Geomicrobiology of extremely acidic subsurface environments*. FEMS Microbiology Ecology, 81(1): 2-12.
[18] Johnson, D.B., Hallberg, K.B., 2008, *Carbon, iron and sulfur metabolism in acidophilic micro-organisms*. Advances in Microbial Physiology, 54: 201-255.
[19] Kozlov, M.V., Zvereva, E.L., 2007*, Industrial barrens: extreme habitats created by non-ferrous metallurgy.* Review in Environmental Science and Biotechnology, 6(1-3): 231-259.
[20] Kumar, A., Bisht, B.S., Joshi, V.D., Dhewa, T., 2011, *Review on Bioremediation of Polluted Environment: A Management Tool*. International Journal of Environmental Sciences. 1(6): 1079-1093.
[21] Küsel, K., Dorsch Tanja, Acker, G., Stackebrandt, E., 1999, *Microbial Reduction of Fe(III) in Acidic Sediments: Isolation of Acidiphilium cryptum JF-5 Capable of Coupling the Reduction of Fe(III) to the Oxidation of Glucose.* Applied Environmental Microbiology, 65(8): 3633-3640.
[22] Lu, S., Gischkat, S., Reiche, M., Akob, D.M., Hallberg, K.B., Küsel, K., 2010, *Ecophysiology of Fe-Cycling Bacteria in Acidic Sediments*. Applied and Environmental Microbiology, 76(24): 8174-8183.
[23] Morozkina, E.V., Slutskaya, E.S., Fedorova, T.V., Tugay, T.I., Golubeva, L.I., Koroleva, O.V., 2010, *Extremophilic microorganisms: Biochemical Adaptation and Biotechnological Application*. Applied Biochemistry and Microbiology, 46(1): 1-14.
[24] Nancucheo, I., Johnson, D.B., 2012*, Selective removal of transition metals from acidic mine waters by novel consortia of acidophilic sulfidogenic bacteria.* Microbial Biotechnology, 5(1): 34-44.
[25] Pandey, A., Nigam, P., Soccol C.R., Soccol V.T., Singh D., Mohan R., 2000, *Advances in microbial amylases*. Biotechnology Applied Biochemistry, 31: 135-152.
[26] Pandey, B., Fulekar, M.H., 2012*, Bioremediation technology: A new horizon for environmental clean-up.* Biology and Medicine, 4(1): 51-59.
[27] Rawlings, D.E., Johnson, D.B., 2007, *The microbiology of biomining: development and optimization of mineral-oxidizing microbial consortia*. Microbiology, 153(2): 315-324.
[28] Riewer, P.W.F., Ormond, W.G., 1995, *International perspectives and the results of carbon dioxide capture disposal and utilisation studies.* Energy Conversion and Management, 36: 813-818.
[29] Rodriguez, R., Diaz, M., 2009, *Analysis of the utilization of mine galleries as geothermal heat exchangers by means of a semi-empirical prediction method.* Renewable energy, 34: 1716 – 1725.
[30] Shafaat, S., Akram, M., Rehman, A., 2011, *Isolation and characterization of a thermostable α-amylase from Bacillus subtilis.* African Journal of Microbiology Research, 5(20): 3334-3338.
[31] Sharma, A., Kawarabavasi, Y., Satyanarayana, T., 2012, *Acidophilic bacteria and archaea: acid stable biocatalysts and their potential applications.* Extremophiles: life under extreme conditions, 16(1):1-19.
[32] Singh, S., Sharma, V., Soni, L.M., 2011, *Biotechnological applications of industrially important amylase enzymes*. International Journal of Pharma and Bio Sciences. India. 2(1): 486-496.
[33] Sivaramakrishnan Swetha, Gangadharon Dhanya, Nampoothiri K.M., Soccol, C.R., Pandey, A., 2006, *α-Amylases from Microbial Sources – An Overview on Recent Developments.* Food Technology Biotechnology, 44(1): 173-184.
[34] Wang, J., Chen, C., 2009, *Biosorbents for heavy metals removal and their future.* Biotechnology Advances, 27: 195-226.
[35] Zeyaullah, M., Atif, M., Islam, B., Abdelkafe, A.S., Sultan P., Elsaady, M.A., Ali, A., 2009. *Bioremediation: A tool for environmental cleaning.* African Journal of Microbiology Research. Biological Sciences. South Africa. 3(6): 310-314.