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I hope this letter finds you in good health and high spirits. It is my distinct pleasure to address you as the Editor-in-Chief of Integrative Omics and Applied Biotechnology (IIOAB) Journal, a multidisciplinary scientific journal that has always placed a profound emphasis on nurturing the involvement of young scientists and championing the significance of an interdisciplinary approach.

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I would like to extend my gratitude to our authors, reviewers, editorial board members, and readers for their unwavering support. Your dedication is what makes IIOAB Journal the thriving scientific community it is today. Together, we will continue to explore the frontiers of knowledge and pioneer new approaches to solving the world's most complex problems.

Thank you for being a part of our journey, and for your commitment to advancing science through the pages of IIOAB Journal.

Yours sincerely,

Vasco Azevedo

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RESEARCH ARTICLE OPEN ACCESS

ADSORPTION OF CR(VI) FROM TEXTILE WASTE WATER BY USING NATURAL BENTONITE

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ABSTRACT

*Cr(VI) chromium was removed from the artifical wastewater using natural bentonite in batch. The adsorption of the chromium on bentonite was investigated during a series of batch adsorption experiments carried out to determine the effect of three different temperatures and contact time. The amounts adsorbed at equilibrium were measured. The experimental results have been fitted both Langmuir and Freudlich isotherms. The maximum adsorption capacities of the bentonite were found to be 12.65, 10.99 and 9.50 mg/g bentonite at 25, 30 and 35^{0}C, respectively. Several thermodynamic parameters such as ΔH^o, ΔG^o and ΔS^o have been calculated. The results of thermodynamic parameters indicates, spontaneous and an exothermic process. The results showed that bentonite could be used as effective adsorbent for the removal of Cr(VI). In addition, the color changes in the artificial wastewater were measured using colorimetric method. The color difference CIE L *a *b* values between before and after adsorption were determined.*

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KEY WORDS

Chrom dyes; Waste water; Bentonite; Thermodynamics; Langmuir model; Freundlich model; CIE L a* b* colour space system*

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[I] INTRODUCTION

Chromium is found naturally in rocks, soil, plants, animals, volcanic dust and gases. It is present in aqueous solution mainly in Cr(III) an Cr (VI) oxidation states. Cr (Chromium) is widely used of modern industries such as leather tanning, electroplating, cement, steel, paint dyes and textiles. Also, Cr (VI) is considered to be potentially careinogenic to humans [1] and is reported to be bioaccumulated in to flora and fauna, creating ecological problems [2, 3] Besides in 1978, (USEPA) the United States Protection Agency Environmental prepared a list of inorganic and organic pollutants Which can be found in wastewaters. The following 13 metals found in list are Cr (Chromium) Cd (cadmium), Cu (copper) Pb (lead) Hg (Mercury), Ag (Silver), Ni (Nicel), Se (Selenium), As (Arsenic), At (Antimony) Be (Berylilum), Zn (Zinc) and Thallium [4] And EPA recommends that the amount of Cr (VI) in drinking water should be less than 100 µg/L .In order to enhance the most common metods for removal of Cr(VI) from industrial effluents, many sorbents have been benefited such as bentonite [5] diatomite [6], silica [7], activated carbon [8,9,10,11], sawdust [12], sludge [13] and others [14,15,16]

Resently, natural and activated bentonite for the adsorption of heavy metals at different temperatures have been reported such as Cr (III), Cr(VI), Cd (II), Pb(II) and Zn(II) in the literature.[17,18]. In previous papers have been make that bentonite can be effectively used to remove Cu, Pb and Ni from aqueous solution [19, 20]. Bentonites are highly influential for their sorptive properties, which stem from their surface area and their tendency to absorb water in interlayer sites [21, 22]

The aim of this study was to investigate the possibility of Cr(VI) removal from dye solutions by bentonite, the optimum sorption conditions were determined at kept pH 5.6 contact time, sorbent dosage. Besides, the Langmuir and freundlich isotherm models were applied to describe adsorption.

[II] MATERIALS AND METHODS

A stok solution of Cr (VI) (50 ppm) was prepared from $K_2Cr_2O_7$ in bidistilled water. Chemicals used were of analytical reagent grade. In all experiments used were 5.0 ppm $K_2Cr_2O_7$ solutions and it was kept at pH5.6. Bentonite was obtained from Çankırı Bentonite Co. Ltd. (Ankara/Turkey).

2.1. Batch adsorption experiments

Adsorption experiments were carried out using 50 ml 5.0 ppm of chromium solution at pH 5.6, adsorbent dosage 0.5-5g in 250ml Erlenmeyer flask at 25^{0}C, 200 rpm for 8h. Then the samples were separated by centrifugation at 20.000rpm for 20 min. Residual chromium concentration in the supernatant was determined by atomic absorption spectrophotometer (Solaar AA. Series spectrophotometer). It was also investigated the effect of temperature and time on adsorption at the recorded experimental conditions. In these experiments, the samples were treated for variable time 15, 30, 60 and 300 min. at 25, 30 and 35^{0}C. At the end of the each experiment, supernatants were analyzed by atomic absorption spectrophotometer and treatment time, temperature were determined. The percent removal of Cr(VI) was calculated as follows:

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% Removal of Cr (VI) = x100

2.2. Adsorption isotherms

The Langmuir and Freundlich adsorption isotherms using of the linear forms were also applied for the removal of Cr(VI) on bentonite as follows [12]

$$\frac{1}{q_e} = \frac{1}{Q_o} + \left(\frac{1}{K_L Q_o} x \frac{1}{C_e} \right)$$ Langmuir linear form

Where Ce is the equilibrium concentration in (mg/l), X/m is the amount adsorbed in (mg/g) at equilibrium, Qo is a measure of adsorption capacity of adsorbent (mg/g) and b is the Langmuir constant which is a measure of energy of adsorption (l/mg).

ln qe = ln KF + ln Ce Freundlich linear form

Where Ce is the equilibrium concentration in (mg/l), X/m is the amount adsorbed in (mg/g) at equilibrium, K and 1/n are Freundlich constants respectively.

2.3. Color measurements

Measurement was made according to a colorimetric method. The color changes in the artifical wastewater before and after adsorption were measured by using a Minolta CM-3600d spectrophotometer. CIE L* a* b* values of artificial waste water was determined

[III] RESULTS

3.1. Batch adsorption experiments

Figure–1 shows the effect of bentonite amount on percentage adsorption of chromium. As can be found from the **Figure–1**, the adsorption of chromium increased with increasing adsorbent amount. In initial conditions, the effect of increase in adsorbent amount was high in the removal of chromium but it has no impact after about 1 g bentonite amount. From this result it has been that chromium was held in 75 % according to 1.0 g of adsorbent amount. Maximum holding efficiency could be obtained in 5.0 g bentonite but it has no impact according to 1.0 g sorbent amount. For this reason it was gone on with about 1.0 g bentonite in after these experiments. As shown **Figure–2**, chromium holding efficiency was increasing sharply in 60 min time, but increase in holding efficiency has no important in higher time. After these experiments, it was investigated the effect of temperature in holding efficiency at constant 60 min time, 200 rpm and 1.0 g bentonite amount. There was no significant difference effect between 30 and 35^{0}C. However, it was obtained a slightly decrease in holding efficiency at 35^{0}C temperature. A decrease in holding efficiency may be explained because physical adsorption decreases with increasing temperature [6]

3.2. Adsorption isotherms

The linear plot of $\frac{1}{q_e}$ versus $\frac{1}{C_e}$ shows that the adsorption seems to follow the Langmuir model **[Figure–1]**. Using Langmuir isotherm of the equilibrium data yieldde the ultimate adsorption capacity were obtained at 30^{0}C value as 10.992 mg g^{-1} and 2.500 l/mg respectively. The equilibrium adsorption data at three different temperatures fitted well to the Langmuir adsorption model according to regression coefficient. Put another way, the values n between 2 and 10 shows good adsorption [6 -20] Similar types of observations have already been reported in the literature [5, 6] Freundlich adsorption model results fitted both the regression coefficient and n values too. The effect of temperature on the adsorption is shown in **[Figure– 3, –4]**. It is observed that at higher temperatures the adsorption is shower, and adsorption process was exothermic process.

The plot of ln qe against ln Ce shows in the **Figure–2**, the isotherm data is well fitted with the Freundlich model (R^2= 0.99). Slope and intercept give the values of $\frac{1}{n}$ and KF The constant n and KF were found to be 2.267 mgg-1 and 1.518 l/mg respectively.

As seen from **Table–1**, the Q^o of maximum adsorption capacity, corresponding to monolayer coverage is formed on the surface of the sorbent, was obtained at 25^{o}C.

Table: 1. The Langmuir and Freundlich adsorption isotherm constant for adsorption of Cr (VI) on bentonite

	Langmuir isotherm			Freundlich isotherm		
Ttemperature	*Q_o (mg/g)*	*K_L*	*R^2*	*K_F (mg/g)*	*n (l/mg)*	*R^2*
25^{o}C	12.658	2.706	0.999	4.815	2.345	0.987
30^{o}C	10.992	2.500	0.994	1.518	2.267	0.999
35^{o}C	9.500	2.010	0.920	1.511	2.200	0.999

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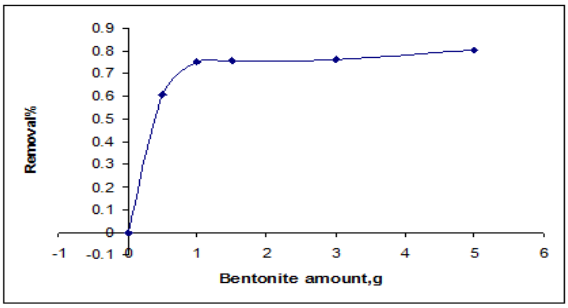

Fig: 1.

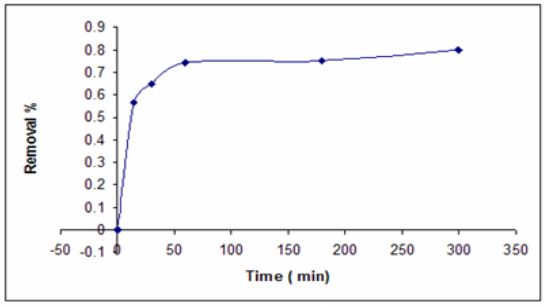

Fig: 2.

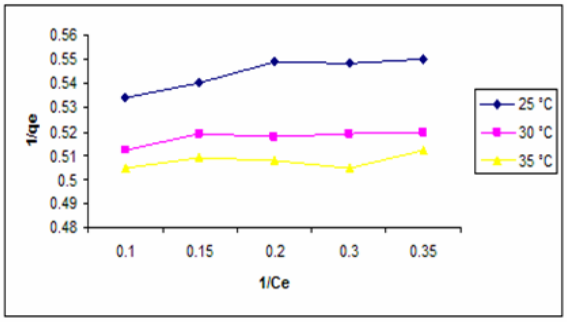

Fig: 3.

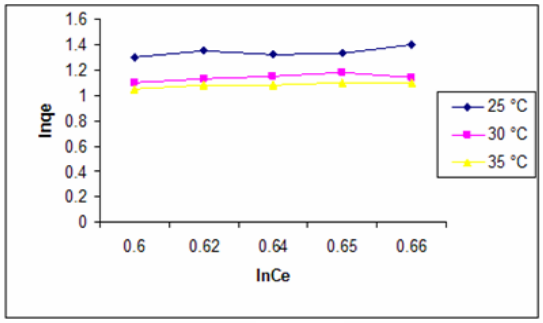

Fig: 4.

Fig: 1. Percentage removal of chromium by different amount of bentonite; 30°C, 8h, 200rpm. **Fig: 2.** Percentage removal of chromium for different times; 1.0 g bentonite, 30°C, 200rpm. **Fig: 3.** Langmuir plots for the adsorption of Cr(VI) onto bentonite. **Fig: 4.** Freundlich plots for the adsorption of Cr(VI) onto bentonite

3.3. Thermodynamics of adsorption

The values of ΔH^o, ΔG^o and ΔS^o were calculated by using the following equations [6]. The calculated values ΔH^o, ΔG^o and ΔS^o are given in **Table–2**.

Table: 2. Thermodynamic parameters for the adsorption of Cr (VI) on bentonite

T(K)	ΔH^0(kJ/mol)	ΔG^0(kJ/mol)	ΔS^0 (J/mol K)
298	-2.381	-2.466	0.285
303	-2.223	-2.308	0.280
308	-1.702	-1.787	0.275

$$\ln KL = \left(\frac{\Delta H}{RT}\right) + \text{- constant}$$

$$\Delta G^o = -RT \ln KL$$

$$\Delta S^o = \frac{\Delta H - \Delta G}{T}$$

Where b is Langmuir constant, T the temperature, and R is the gas constant equal to 8.3143 Jmol-1K-1. The calculated values are given in **Table–2**. Negative values of ΔH^o indicates an exothermic, adsorption. The negative values of ΔG^o confirm that the Cr(VI) adsorption on bentonite is a spontaneous process.

3.4 Colorimetric properties

As can be seen from **Table–3**, after adsorption of wastewater is more light than before adsorption. This fact can be explained by the state of colour in the solution, which means Colour of wastewater is removed by sorbent and the value of L* was increased after adsorption.

Table: 3. *CIE L* a* b values of artifical wastewater before and after adsorption**

	Time (min)	CIELab values			
		*L**	*a**	*b**	*d L**
Before adsorption	---	20.76	1.94	-3.17	---
After adsorption	15 30 60 180 300	29.85 30.32 31.77 34..68 40..81	-0.25 0.26 -0.35 -4.41 -0.92	-4.41 -4.86 -4.86 -5.29 -8.09	9.09 9.56 11.01 13..92 20..05

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[IV] CONCLUTION

Batch adsorption studies for the removal of Cr(VI) from aqueous solutions have been carried out using bentonite. The obtained results may be summarized as follows:

Increase in adsorbent dosage leads to increase in Cr(VI) adsorption, it was observed that 75% of Cr(VI) removal was achieved by using 1.0 g of bentonite amount. The % adsorption increases by increasing the dosage of adsorbent

The equilibrium experimental data showed good fit to two isotherms, Langmuir and Freundlich models. Different thermodynamic parameters ΔG^o, ΔH^o and ΔS_o have also been evaluated and it has been found the spontaneous nature of adsorption process and exothermic process.

As a result of colorimetric parameters calculations, the colour of solution was decrease which means lighter and brighter colors. In other words, the value of L* is increase after adsorption which means chromium in the solution was held by adsorbent.

CONFLICT OF INTEREST

Author declares no conflict of interest.

FINANCIAL DISCLOSURE

The work is not supported by any grant.

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IN SILICO GENETIC VARIATION PATHOGENICITY ANALYSIS OF HEMAGGLUTIN, MATRIX 1, AND NON STRUCTURAL 1 PROTEIN OF HUMAN H5N1 INDONESIAN STRAIN

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ABSTRACT

Avian influenza (H5N1) is a disease caused by type A influenza viruses. Although in general influenza type A is not lethal to humans, several cases did. In silico mutation analysis could be utilized using multiple alignment methods, AminoTrack server, and phylogenetic tree. Mutations were observed for the cleavage site of haemagglutin (HA) protein, while for the Non-Structural 1 (NS1) and Matrix1 (M1) mutations was observed on their entire region. It was followed by prediction of the pro-P (furin) HA specific cleavage, secondary structure, mutation (exposed/buried) prediction, epitope prediction, and 3D structures prediction. Based on the analysis of mutations in the HA cleavage site, the conserved pattern for Indonesia and Hong Kong H5N1 is RXK / RR, while the sequence of subtype H1N1, H1N2, and H3N2 did not have it. Furin prediction showed this pattern causes the HA of H5N1 could be cleaved. Specific mutations in the NS1 control sequences: (A/Indonesia/5/2005 (H5N1), A/Indonesia/CDC1032/2007(H5N1), A/HongKong/156/97(H5N1), A/BrevigMission/1/18(H1N1), A/Mexico/InDRE4487/2009(H1N1)) with the subtype H1N1, H1N2, and H3N2 were found in position 53. Identical specific control mutation on the M1 for the three subtypes was not found. 248 positions have changes in the H1N1 and H3N2. Epitope prediction explains that control sequences NS1 and M1 of H1N1, H1N2, and H3N2 subtypes have similar IC_{50} values below 50 nM. Specific mutations do not occur in epitope recognition. Occurred region-specific mutations for NS1 and M1 did not affect the secondary and tertiary structure of proteins significantly compared with the controls sequences.

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HA; influenza; in silico; M1; NS1

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[I] INTRODUCTION

Since 1997, a highly pathogenic avian influenza (HPAI), H5N1 influenza A virus, has caused massive deaths in poultry and humans [1]. This virus has spread in Asia including Japan and Indonesia. The first influenza virus causing the pandemic and many casualties are the subtype H1N1 in 1918 (Spanish Flu) [2]. Recent cases in 2009, the subtype H1N1 that occurred in Mexico (Swine Flu) at least causes hundreds of casualties. Until June 12, 2009, the H1N1 virus caused 145 casualties in various countries. The pandemic outbreak of threat of HPAI H5N1 could be fatal to the human population and poultry. Therefore, the estimation of pandemic occurrence still needs further evaluation because this pandemic occurrence can cause economic and investment losses. To overcome the threat, more insight on H5N1 molecular mechanism is necessary. Genome and Proteome of H5N1 need to be further studied for designing remedy of the disease [1].

Evolution and mutations that occurred in H5N1 viruses are strongly affected by their ability to perform antigenic drift/shift. During its development, H5N1 could be easily transmitted from human to human due to both antigenic processes, so it becomes a real pandemic threat [1].

There are three strains of influenza viruses: type A, B and C. Type A is responsible for the deadly influenza pandemics (worldwide epidemics) that occur every 10 to 40 years [3]. Influenza A virus belongs to the Orthomyxoviridae family. It consists of eight segments of single stranded RNA, that is HA, NA, PB2, PB1, PA, NS, NP, and M. Currently there are 16 known types of HA and 9 types of NA [4].

Viral attachment to sialic acid receptors in the host cell surface is the most decisive phase in the viral infection process. In the avian influenza virus, HA protein can only recognize the [Neu5Ac (α2-3) Gal] receptors that exist in birds. This is different with [Neu5Ac (α2-6) Gal] receptors in humans. Theoretically, the avian influenza virus could not infect humans because these receptors are different. However, subsequent developments indicate a possible change in the amino acid composition as a result of HA antigenic drift/shift. It culminated in inter-human transmission, which marks the beginning of the global pandemic threat of avian influenza [2].

The main aspects in determining the pathogenicity level of influenza virus are (i) the interaction at the mucus layer, (ii) the process of viral entry into host cells, (iii) the process of viral replication, (iv) the influence of viral infection on the immune

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system, (v) the spread of the virus replicates (vi) the level of cell damage by viral infection, and (vii) the specificity of the host cell [5].

Avian influenza can be found in two forms namely the acute form of HPAI and low pathogenic form of mild avian influenza (LPAI). In HPAI, morbidity and mortality can reach 100% [2].

Pathogenicity Level of H5N1 viruses is influenced partly by the termination site of HA (cleavage site) comprising amino polybasic region that allows the HA to be highly sensitive to endogenous proteases/cellular host. As a result, the virus can spread in a wide range of cells and not limited only in the respiratory tract. HA addition and mutation of other genes (i.e., NA, PB1, PB2, NS, and M) also affect the level of pathogenicity of the virus [2].

Our research group has conducted research on HA mutation of H1N1 [6]. The question is whether there is a common genetic variation of H5N1 Indonesia, which has a high level of pathogenicity of influenza cases or there are other genetic differences that determine the degree of pathogenicity of the virus.

The purpose of this study is to analyze the in silico genetic variation of HA protein (i.e., NS1) and the M1 protein of Indonesian H5N1; this variation may affect the level of human pathogenicity compared with other subtypes of influenza. The effect of mutation on the change in secondary and tertiary structures of protein as well as the introduction of the epitope will be expected.

[II] MATERIALS AND METHODS

The data for Influenza A protein were obtained from the GenBank database. It was issued by the National Center for Biotechnology Information (NCBI) through the Influenza Virus Resource.

2.1. Search sequence database of H5N1 proteins

H5N1 protein sequences were taken from the website http://ncbi.nlm.nih.gov/ which issued by the NCBI. The downloaded protein data were the HA, NS, and M1 Indonesian H5N1 virus isolates from humans, H5N1 from Hong Kong, H1N1 from 1918 outbreak, and H1N1 from outbreak at Mexico in 2009. They have high degree of malignancy characteristics. These data were also supported by other types of influenza, which had relatively low level of malignancy, such as H1N1, H1N2, and H3N2.

2.2. Performing Multiple Sequence Alignment

Having obtained the desired protein sequence, multiple alignments were then performed to determine protein sequences similarity in each strain of H5N1 influenza subtype with others that have relatively low levels of malignancy. Program used in this step was the CLC Main Workbench 5.0. The results of this alignment would be useful for the analysis of mutations of the H5N1 virus.

2.3. Generating Phylogenetic Tree

Making phylogenetic tree was intended to look for sequences that have a close kinship with virus HPAI type. Phylogenetic tree-making process was done using software CLC Main Workbench 5.0 with the input alignment of obtained sequences.

2.4. Mutation Analysis

This step was performed to look for areas where there were differences and similarities of protein sequences of Indonesian H5N1 influenza virus subtype to another. It is expected to find areas that determine the nature of viral pathogenicity by comparing with the LPAI cases. Literature study was necessary to look for possible areas that decisive for the pathogenicity of influenza A viruses, especially H5N1.
The program used to analyze of mutation is called the Amino Track on a server, which can be accessed through http://apps.sbri.org/AminoTrack/. This program would detect the amino acid mutation that occurred in the protein. The data input is from the result of previous sequence alignment, and the output is their specific mutations.

2.5. Site cleavage Prediction HA0 by furin

One of the pathogenicity determinants is the pattern of HA intracellular protein breakdown by furin. Patterns that occur by furin can be predicted using the furin online server, which can be accessed through the following Web site http://cbs.dtu.dk/services/ProP.

2.6. Secondary Structure Prediction

The data of amino acid changes from the Amino Track analysis were utilized to predict whether there are changes in protein secondary structure. Secondary structure prediction was evaluated using Jpred server 3, which was accessed through http://compbio.dundee.ac.uk/ by entering amino acid sequences of the isolates in FASTA format.

2.7. Prediction of Protein Sequences Buried / Exposed Location

Prediction whether a specific mutation occurred in buried or exposed areas was done using the Conseq server. It could be accessed through the Web Site http://conseq.bioinfo.tau.ac.il

2.8. Prediction epitope HA and NA

Programs used for epitope prediction was Immuneepitope. This program was accessed through the website http://tools.immuneepitope.org/analyze/html/mhc-binding.html. From the server analysis, it is expected to know the changes that occur from the introduction of the epitope. The changes in amino acid composition could affect the epitopes.

2.9. Protein 3D structure Prediction.

The depiction of the proteins 3D structure could help to show the physiological structure similarity of the existing protein sequence. Online courses were accessed via http://ps2.life.nctu.edu.tw.

[III] RESULTS

3.1. Protein Sequence Search

Utilized sequences of influenza A viruses were H1N1 subtype,

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H1N2, H3N2, and H5N1 from human host. The selected influenza protein sequences in this study were the HA, NS1, and M1. The search towards entire sequence of the desired protein was done by accessing the web site of NCBI with the following address: http://ncbi.nlm.nih.gov/genomes/FLU/Database.html.

This study utilized the influenza A H1N1, H1N2, H3N2, and H5N1 subtypes from human hosts. H1N1 and H3N2 sequences were isolated from Asia, while the H1N2 sequences were taken from the entire region in the database. This was due to the scarce availability of the Asian subtype. The H5N1 subtype sequences came from Indonesia. The derived protein sequences were HA, NS1, and M1 as well as selected full-length sequences in order to facilitate the subsequent analysis **[Supplementary Table– 1]**.

3.2. Multiple Sequence Alignment

This study was aimed to determine the differences in Indonesian H5N1 human host protein sequences from the other cases of influenza virus subtypes. To facilitate the causal factors determination of the Indonesian H5N1 human high pathogenicity, two sequences of Indonesian H5N1 human were retrieved as the representative for the HA protein, NS1, and M1. Selected sequences were based on the first case of Indonesian H5N1 human infection (A/Indonesia/5/2005) and the last case reported to the NCBI database (A/Indonesia/CDC1032/2007).

Sequences that have a high level of human malignancy were also retrieved for comparison. They were subtype H1N1 in 1918 (A / Brevig Mission/1/18) and 2009 (A/Mexico/InDRE4487/2009) and the Hong Kong H5N1 strain of the human host (A / Hong Kong / 156/97). Sequences that have a high level of pathogenicity were subsequently used as a control to see the occurred protein sequences differences when compared with other relatively low pathogenicity human influenza subtypes cases.
The sequences with high level of pathogenicity were then carried out by sequence alignment with H1N1, H1N2, and H3N2.

3.3. Generating Phylogenetic Tree

The results of the created phylogenetic tree were interesting. It was found that the control strains with high pathogenicity are on adjacent branches. It could be concluded that they have a close kinship. This trend is found on each sequence for low pathogenicity H1N1, H1N2, and H3N2; they have relatively pretty close kinship with the controls **[Supplementary Table– 2]**. Sequences of these proteins can be used as a comparison and can be searched for specific mutations that affect the pathogenicity level of a virus.

3.4. Mutation Analysis

Amino Track is a server that could identify the occurred mutations such as deletion, substitution, or insertion an amino acid to the resulted multiple sequence alignment in the form of comparisons between one sequence as a reference with others based on statistical programming [7].

The result of multiple sequence alignment must be converted into FASTA format. It required Jalview software that could convert the *.aln format alignment into FASTA. The output of Amino Track was in zip format that included the Microsoft Excel format. This server makes it easy to see changes in position in the protein sequences of each isolated virus.

3.4.1. Mutation HA

The results of mutation analysis using Amino Track for HA protein sequences on the HA0 cleavage site are shown in **[Supplementary Table– 3]**. In addition to its role in receptor binding, HA also plays a role in viral fusion process to release its genetic material into host cells. This process requires activation by the HA precursor molecule that cuts into HA1 and HA2. It was done by host cell proteases [8].

It can be seen that the Indonesian human H5N1 sequences and Hong Kong have a polybasic amino acid insertion region RXK / RR while the sequences of H1N1, H1N2, and H3N2 have only one arginine in the cleavage area **[Supplementary Table– 3]**. The pattern of the Indonesian H5N1 sequences is in accordance with the A/HongKong/156/97 avian influenza cases. They are classified as HPAI (H5N1).

3.4.2. Mutations in NS1

NS1-specific mutation sequence is shown in **Supplementary Table– 4**. The Table shows that specific mutations with the H1N1 control sequences occurred in position 3, 47, 51, 53, 137, 145, 164, 173, 176, and 216. Control with H1N2 specific mutations occurred at positions 53, 56, 57, 66, 73, 180,183, and 211. While specific mutations in the H3N2 control occurred at position 53, 56, 66, 73, 98, 180, 183, and 185. The interesting thing is found in the deletion occurrence at positions 80, 81, 82, and 83 for Indonesian H5N1 isolates sequences. Mutations occurred at position 53 to either the control sequences with the H1N1, H1N2, and H3N2. D53N mutation happened in the H1N1 and H1N2 D53E / H3N2.

Pathogenicity Level of influenza viruses is strongly influenced by the work of the NS1 protein, which is involved in innate immune response inhibition process mediated by type 1 interferons (IFN). The presence of glutamic acid at position 92 of NS1 causes the virus to be resistant to IFN. If the aspartic acid residue is at position 92, the influenza virus becomes sensitive to IFN [9].

Glutamic acid was found at position 92 in the sequence

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A/HongKong/156/97 (H5N1), whereas the Indonesian H5N1 as well as H1N1, H1N2, and H3N2 isolates have the aspartic acid residue at position 92. The difference is that the origin of Indonesian H5N1 has a deletion area; this is different with other viral sequences. This deletion region could possibly exert influence on the pathogenicity level of influenza viruses, especially H5N1 [8].

The sequences of A/Brevig Mission/1/18 (H1N1) and A/Mexico/InDRE4487/2009 (H1N1) also have aspartic acid at position 92. However, both of these sequences have high level of pathogenicity toward humans. This may illustrate that the D92E mutation on the NS1 is not the sole determinant of influenza virus A pathogenicity.
The results of the specific mutations analysis were observed on the changes in secondary structure and epitope prediction.

3.4.3. Mutation in M1

Amino Track servers obtained several mutations in M1 sequence. From these results then sought a position in which specific mutations occurred with the selected H1N1, H1N2, and H3N2 control sequences **[Supplementary Table– 5]**.

Specific mutations that occurred between the H1N1 and H3N2 control occurs at only one position. They are positions 248 and 193 for H1N1 and H3N2. While specific mutations among control with H1N2 occurred in position 9, 10, 11, 12, 13, 14, 201, 208, and 248. The mutation position was not identical for all three subtypes. Only position 248 is experiencing changes in the H1N1 and H3N2.

The specific mutations analysis would be observed for its influence on changes in secondary structure and epitope prediction for protein NS1.

[IV] DISCUSSION

4.1. Prediction Site Cutting HA0 by furin

LPAI usually has a single arginine residue in the cleavage region. HPAI viruses have the polybasic amino acids that could be cleaved by many intracellular proteases such as furin. This would cause a systemic infection on the host [8]. The results of multiple alignment and mutation analysis using the Amino tracks on the utilized sequences show that Indonesian H5N1 human isolates already have an RXK/RR pattern similar to that of A/Hongkong/156/97. It was seen that the entire sequence of the Indonesian H5N1 human host virus has the pattern, and it belongs to the HPAI class.

Moreover, for proving that this pattern affects the ease of cleavage by using intracellular proteases such as furin, the online server furin was employed to determine the cleavage site(s) on HA. Scores are given to indicate the most likely position of the HA cleavage process. Here, the results of pro-P prediction server for HA sequences are shown in **Supplementary Table– 6**. The Table shows that the addition of H5N1 polybasic amino acid sequences could be easily broken down by furin. While the subtype H1N1, H1N2, and H3N2 did not fit with furin pattern.

1N1 in 1918 (A/SouthCarolina/1/18) and H1N1 in 2009 (A/Mexico/InDRE4487/2009) are strains that cause substantial mortality for humans as well as H5N1 case. However, the two sequences do not have a pattern such as those owned by H5N1. This illustrates that the virulence factors or pathogenicity of human influenza viruses are not merely influenced by the cleavage sites of HA alone. Moreover, there are other genetic factors influencing the level of malignancy of influenza virus. Although the H1N1 influenza virus of 1918 has no polybasic amino acids at the HA-cutting areas, it has trait for ease of HA cleavage. This proved that the virus could also be cleaved by the absence of trypsin. Until now, the mechanism of ease of cleavage for HA in the 1918 H1N1 has not been clearly identified [8].

4.2. Secondary Structure Prediction

Determination of secondary structure of influenza virus was predicted. The results were obtained in the form of secondary structure prediction for each protein.

The result for NS1 protein was that the amino acid change at a specific position of mutation did not significantly affect the coherence of secondary structure. It was found that there is only one position (position of 51) that acts as a specific mutation site in the H1N1 versus control. It changes the secondary structure of the coil into an extended strand.

The secondary structure prediction on a specific mutation on M1 found that changes in secondary structure occurred only in H1N2 sequences. However, in position 11 and 12 there were changes from a helix and extended strand, and at position 14 changes were found from the extended strand to coil. Mutations in the H1N1 and H3N2 did not change the secondary structure of M1 protein. To this end, it can be inferred that the structure of M1 sequences of Indonesian H5N1, H5N1 Hong Kong, 1918 H1N1, and H1N1 Mexico 2009 are more homologous to the sequences of H1N1 and H3N2. They have not had a high level of pathogenicity.

4.3. Mutation Prediction Burried / Exposed

Specific mutation prediction that occurs in areas that are buried or exposed can be evaluated using the Conseq server. FASTA-formatted protein sequences were used as input, and the protein

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sequence A/Indonesia/5/2005 (H5N1) was employed as a comparison.

Specific mutations among controls were carried on H1N1 in the exposed region (positions 3, 53, 164, 173, 176, 216) and the buried region (positions 47, 51, 137, 145). Specific mutations among controls were carried out to H1N2 in the exposed region (positions 53, 56, 66, 211) and in the buried regions (positions 57, 73, 180, 183). There are specific mutation among controls which exposed to H3N2 in positions 53, 56, and 66; whereas in buried regions occurred in positions 73, 98, 180, 183, and 185. Mutations region that occur in the NS1 protein could detect both the exposed and buried area. Position 53 is the point of specific mutations among the controls within H1N1, H1N2, and H3N2. They are located in exposed areas, and it could influence the specific activity of the protein.

Controls with H1N1 specific mutations occurred in the buried area (position 248). Specific mutation between controls with H1N2 occurred in exposed regions (position 9, 201, 208), while in buried regions, they are some different positions. They are position 10, 11, 12, 13, 14, and 248). Specific mutation between controls with H3N2 occurred in exposed regions (position 93).

4.4. Predicted epitope

The immuoepitope server predicts the epitope recognition using the FASTA-formatted protein sequences input [10]. In this server, MHC class selection (Major Histocompatibility Complex) will be used in epitope prediction. This study is using MHC class I-related peptide fragment that is activated by intracellular signaling and CD8 + [11].

The output prediction is given in immune-epitope units (IC_{50} nM). From these predictions if there are peptides that have the IC_{50} value (inhibitory concentration) of less than 50 nM, then the peptide is having high affinity. IC_{50} value of less than 500 nM has intermediate affinity and the IC_{50} value of less than 5000 nM has a low affinity (http://tools.immuneepitope.org/analyze /html/MHC-binding.html, accessed May 18, 2009, 10:45 GMT).

By comparing the NS1 and M1 epitope between controls with subtype H1N1, H1N2, and H3N2, we can see if there are changes in the predicted epitope recognition and changes the level of virus pathogenicity.

NS1 epitope predictions detected LKANFSVVF peptide sequence that has the lowest IC_{50} value of 0.98 nM. This protein sequence presents in isolates of A/Thailand/271/2005 (H1N1) at position 130-138 with recognition by HLA B * 1503. This recognized position also occurs in isolates of A/HongKong/156/97 (H5N1), A / BrevigMission / 1 / 18 (H1N1), A/Mexico/InDRE4487/2009 (H1N1), A/Philippines/344/2004 (H1N2), A/HongKong/1774/99 (H3N2) with peptide sequence LKANFSVIF and IC_{50} of 1.2 nM. The predicted epitope of Indonesian sequence that has IC_{50} of 1.0 nM at position 196-204, which has ETIQRFAWR sequence with the recognition of HLA A * 6801. IC_{50} value is less than 50 nM, suggesting that that all isolates of the virus are easily inhibited by the immune system. It can be concluded that specific mutations that occur have less effect on this epitope prediction [12].

Epitope predictions for M1 obtained RKLKREITF sequence that has the lowest IC_{50} value of 1.1 nM. This sequence was found in isolates of A/ Brevig_Mission/1/8 (H1N1) at position 101-109 with recognition by HLA B * 1503. This position also occurs in isolates of A/Mexico/InDRE4487/2009 (H1N1), A/Philippines/344/2004 (H1N2), A/HongKong/1774/99 (H3N2) with KKLKREITF sequence and has IC_{50} of 1.4 nM. The A/HongKong/156/97 isolates (H5N1) also have the epitope region with this position and have KKLKREMTF sequence with IC_{50} of 1.3 nM. For Indonesian isolates, the RSHRQMATI predicted epitope has IC_{50} of 1.3 nM at position 160-168 and it is recognize by HLA B * 1517. Just as predicted epitope for the NS1, all isolates had IC_{50} values below 50 nM so they can be recognized by HLA and easy to inhibit. Specific mutations occurred did not affect the change of epitope prediction and the obtained IC_{50} values.

4.5. Structure Prediction

Predicted 3D structures of NS1 and M1 proteins were determined and the extent of changes occurred due to mutation was evaluated. This prediction method is known as the homology modeling. This process uses an online server that can be accessed through http://ps2.life.nctu.edu.tw. Input of the predicted protein sequences was in FASTA format. Then, Blast was performed to search for template that has the highest homology level.

The search results of NS1 protein template have similarity for all viral isolates. Template to the RNA Binding Domain is 2ZoaA and to the effectors domain is 2gx9A. M1 template for the whole virus isolates is 1aa7A. **Figures– 1 and –2** show the 3D structures of protein NS1 of the selected sequences and superimposed with the protein from A/Indonesia/5/2005. **Figure– 3** shows the 3D structure of the selected M1 protein sequences and superimposed with protein from A/Indonesia/5/2005.

Value of RMSD shows a very close resemblance to each structure prediction results. So it can be concluded that the specific mutations that occurred did not affect the tertiary structure of proteins significantly. The **Supplementary Table– 7 and– 8** show the RSMD data for M1 and NS1 protein. These results could be scaled up with molecular docking and modeling, with the example of our previous research [13]

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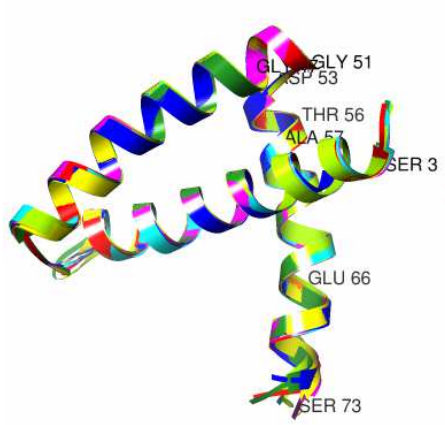

Fig: 1.

Fig: 2.

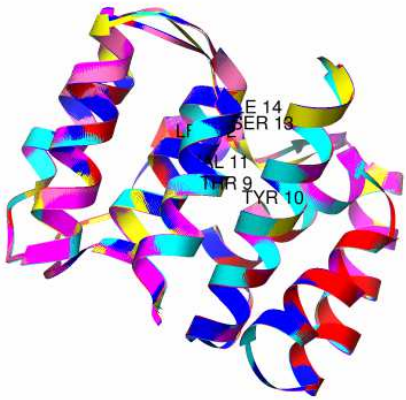

Fig: 3.

Fig: 1. Superimposed RNA Binding Domain NS1. **Fig: 2.** Superimposed NS1 effector domain. **Fig: 3.** Superimposed M1

[V] CONCLUSION

Mutation analysis found specific mutations in the HA sequences of Indonesian and Hong Kong H5N1 isolates in the site which cleaved HA0 into HA1 and HA2. The results concluded that avian influenza H5N1 virus could cause a systemic infection on the host compared with other isolates. Epitope prediction shows that the sequences NS1 and M1 of H1N1, H1N2, and H3N2 subtypes have similar IC_{50}. Henceforth, it still could be recognized by the host immune system. The results of these specific mutations that occurred for both NS1 and M1 did not affect any significant changes in secondary structure and tertiary structure. The next step will be conducting molecular docking and modeling of our designs.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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SUPPLEMENTARY TABLES (As supplied by author)

Supplementary Table: 1. Number of Obtained Influenza A Protein Sequences

Subtype	Protein		
	HA	NS1	M1
H1N1	79 sequence	34 sequence	69 sequence
H1N2	30 sequence	32 sequence	34 sequence
H3N2	430 sequence	130 sequence	510 sequence
H5N1 Indonesia	97 sequence	85 sequence	81 sequence

Supplementary Table: 2. Sequences of Influenza A Virus H1N1, H1N2, and H3N2 that Has Kinship Close to Control

Subtype	Protein		
	HA	NS1	M1
H1N1	A/Thailand/271/2005	A/Thailand/271/2005	A/Thailand/271/2005
H1N2	A/Philipine/344/2004	A/Philipine/344/2004	A/Philipine/344/2004
H3N2	A/HongKong/1774/99	A/HongKong/1774/99	A/HongKong/1774/99

Supplementary Table: 3. Mutations that occurred in the Regional HA Cleavage Site

Sample	320	321	322	323	324	325	326	327	328	329	330	331	332
A/Indonesia/5/2005 (H5N1)	N	S	P	Q	R	E	S	R	R	K	K	R	G
A/Indonesia/CDC1032 /2007(H5N1)	N	S	P	Q	R	E	S	R	R	K	K	R	G
A/HongKong/156/97 (H5N1)	N	T	P	Q	R	E	R	R	R	K	K	R	G
A/SouthCarolina/1/18 (H1N1)	N	I	P	S	I	Q	S	R	-	-	-	-	G
A/Mexico/InDRE4487/ 2009 (H1N1)	N	V	P	S	I	Q	S	R	-	-	-	-	G
A/Thailand/271/2005 (H1N1)	N	I	P	S	I	Q	S	R	-	-	-	-	G
A/Philippines/344/ 2004(H1N2)	N	I	P	S	I	Q	S	R	-	-	-	-	G
A/HongKong/1774/99 (H3N2)	N	I	P	E	K	Q	T	R	-	-	-	-	G

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Supplementary Table: 4. Specific mutations NS1

Sample	3	47	51	53	80	81	82	83	84	137	145	164	173	176	216
A/Indonesia/5/2005 (H5N1)	S	G	G	D	-	-	-	-	-	I	I	P	D	N	P
A/Indonesia/CDC1032/ 2007 (H5N1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A/Brevig_Mission/1/ 18(H1N1)	-	-	-	-	T	I	A	S	V	-	-	-	-	-	-
A/HongKong/156/97 (H5N1)	-	-	-	-	T	I	A	S	V	-	-	-	-	-	-
A/Mexico/InDRE4487/ 2009(H1N1)	-	-	-	-	T	I	A	S	V	-	-	-	-	-	-
A/Thailand/271/2005 (H1N1)	P	S	S	N	T	I	A	S	V	V	T	L	N	D	S

a. Control vs H1N1

Sample	53	56	57	66	73	80	81	82	83	84	180	183	211
A/Indonesia/5/2005 (H5N1)	D	T	A	E	S	-	-	-	-	-	V	G	R
A/Indonesia/CDC1032/2007 (H5N1)	-	-	-	-	-	-	-	-	-	-	-	-	-
A/Brevig_Mission/1/18 (H1N1)	-	-	-	-	-	T	I	A	S	V	-	-	-
A/HongKong/156/97 (H5N1)	-	-	-	-	-	T	I	A	S	V	-	-	-
A/Mexico/InDRE4487/2009 (H1N1)	-	-	-	-	-	T	I	A	S	V	-	-	-
A/Philipine/344/2004 (H1N2	E	P	S	K	Y	T	I	A	S	V	T	K	G

b. Control vs H1N2

Sample	53	56	66	73	80	81	82	83	84	98	180	183	185
A/Indonesia/5/2005 (H5N1)	D	T	E	S	-	-	-	-	-	M	V	G	L
A/Indonesia/CDC1032/ 2007 (H5N1)	-	-	-	-	-	-	-	-	-	-	-	-	-
A/Brevig_Mission/1/18 (H1N1)	-	-	-	-	T	I	A	S	V	-	-	-	-
A/HongKong/156/97 (H5N1)	-	-	-	-	T	I	A	S	V	-	-	-	-
A/Mexico/InDRE4487/2009 (H1N1)	-	-	-	-	T	I	A	S	V	-	-	-	-
A/HongKong/1774/99 (H3N2)	E	P	K	Y	T	I	A	S	V	I	I	K	F

c. Control vs H3N2

Supplementary Table: 5. M1 Specific mutations

Sample	248
A/Indonesia/5/2005 (H5N1)	M
A/Indonesia/CDC1032/2007 (H5N1)	-
A/HongKong/156/97 (H5N1)	-
A/Brevig_Mission/1/18 (H1N1)	-
A/Mexico/InDRE4487/2009(H1N1	-
A/Thailand/271/2005(H1N1)	I

Sample	193
A/Indonesia/5/2005 (H5N1)	A
A/Indonesia/CDC1032/2007 (H5N1)	-
A/HongKong/156/97 (H5N1)	-
A/Brevig_Mission/1/18 (H1N1)	-
A/Mexico/InDRE4487/2009(H1N1	-
A/HongKong/1774/99(H3N2)	V

a. Control vs H1N1
c. Control vs H3N2

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Sample	9	10	11	12	13	14	201	208	248
A/Indonesia/5/2005 (H5N1)	T	Y	V	L	S	I	E	Q	M
A/Indonesia/CDC1032/2007 (H5N1)	-	-	-	-	-	-	-	-	-
A/HongKong/156/97 (H5N1)	-	-	-	-	-	-	-	-	-
A/Brevig_Mission/1/18 (H1N1)	-	-	-	-	-	-	-	-	-
A/Mexico/InDRE4487/2009(H1N1)	-	-	-	-	-	-	-	-	-
A/Philippines/344/2004(H1N2)	N	V	C	S	L	Y	D	K	I

b. Control vs H1N2

Supplementary Table: 6. Prediction results of pro-P

Sample	*Furin-type Cleavage Site Prediction*
A/Indonesia/5/2005 (H5N1)	ESRRKKRIGL
A/Indonesia/CDC1032 /2007(H5N1)	ESRRKKRIGL
A/HongKong/156/97 (H5N1)	ERRRKKRIGL
A/SouthCarolina/1/18 (H1N1)	*none*
A/Mexico/InDRE4487/2009 (H1N1)	*none*
A/Thailand/271/2005(H1N1)	*none*
A/Philippines/344/2004(H1N2)	*none*
A/HongKong/1774/99(H3N2)	*none*

Supplementary Table: 7. NS1 RMSD data

Sequence	RMSD (Å)	
	RNA Binding domain	Effector Domain
A/HongKong/156/97 (H5N1)	0,361	0.222
A/SouthCarolina/1/18 (H1N1)	0,27	0,135
A/Mexico/InDRE4487/2009 (H1N1)	0,356	0,152
A/Thailand/271/2005(H1N1)	0,427	0,17
A/Philippines/344/2004(H1N2)	0,379	0,235
A/HongKong/1774/99(H3N2)	0,433	0,117

Supplementary Table: 8. RMSD Data M1

Sequence	RMSD (Å)
A/HongKong/156/97 (H5N1)	0,139
A/SouthCarolina/1/18 (H1N1)	0,191
A/Mexico/InDRE4487/2009 (H1N1)	0,201
A/Thailand/271/2005(H1N1)	0,134
A/Philippines/344/2004(H1N2)	0,148
A/HongKong/1774/99(H3N2)	0,134

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RESEARCH ARTICLE OPEN ACCESS

STUDIES ON A MALTOHEXAOSE (G6) PRODUCING ALKALINE AMYLASE FROM A NOVEL ALKALOPHILIC STREPTOMYCES SPECIES

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ABSTRACT

The present paper describes the production and characterization of alkaline amylases from a novel obligate alkalophilic Streptomyces sp. This strain was isolated from the alkaline soil of the meteorite Lonar lake, situated in Maharashtra, India. This is the first report of a maltohexaose producing alkaline amylase from an alkalophilic Streptomyces sp and the fourth report of an alkalophilic Streptomyces sp producing an alkaline amylase. The optimum growth of the culture occurred in the range of pH 8 to 10 at 28° C. No visible growth occurred at neutral or acidic pH. Optimum enzyme production occurred only at alkaline pH, the optimum being pH 10.5. Soluble starch as well as raw starch induced the enzyme, induction being higher with raw starch. Substantial quantities of the enzyme was also produced in a non-starch medium containing malt extract, yeast extract and glucose. The enzyme was active in the range of pH 7-10, the optimum pH being 9.0 at 45°C. Starch electrophoresis of the culture filtrate revealed the presence of two amylolytic bands. The molecular weights of the two bands as determined by native electrophoresis were 36,920 and 36,300 daltons respectively. The enzyme was found to be endo in action, the predominant products being maltohexaose (48%) followed by maltotetrose, maltotriose and maltose, at the initial stages of hydrolysis as well as after 24 hours and hence would have a potential application in the food and pharmaceutical industry. The enzyme preparation was also effective in removing starch based stains. Hence, the alkaline amylases from the Streptomyces sp would have a potential application in the food and detergent industries.

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KEY WORDS

Alkalophilic Streptomyces sp; alkaline amylase; maltohexaose; application in food and detergent industries;

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[I] INTRODUCTION

Amylases are ubiquitous and have been isolated, purified and characterized from a number of sources. Most of these amylases are active at acidic or neutral pH. In contrast, alkaline amylases have been reported mainly from the genus Bacillus [1], with a few reports from other genera i.e. Natronococcus [2], Myxococcus [3], and Micrococcus [4]. The alkalophilic *Streptomyces sp*. as well as its amylases is the fourth report, besides the three available reports of alkalophilic *Streptomyces* strains producing alkaline alpha-amylases [5-7].

An important characteristic of the amylase is that it hydrolyses starch to produce maltohexaose (G6) as the major product along with maltotriose (G3), maltotetraose (G4) and maltose (G2) and hence would have a potential application in the food and pharmaceutical industry. To date, in the genus *Streptomyces* there are two reports of maltotriose producing amylases [7,8] and a single report each of maltotetraose [9] and maltohexaose [10] producing strains. However, this is the first report of a maltohexaose producing alkaline amylase from an alkalophilic *Streptomyces* strain. In recent years, there has been an increasing interest in the use of malto-oligosaccharides as biopreservatives [11, 12]. Since these oligosaccharides are natural components of foods such as fruit, vegetables, milk and honey, they are increasingly being favoured in lieu of chemical additives which are becoming less welcome by the consumers [13-15]. Amongst the malto-oligosaccharides, G3 ie maltotriose, G4 ie maltotetraose, G5 i.e. maltopentaose and G6 ie maltohexaose have attracted attention because they not only provide useful modifications to flavour and physiocochemical characteristics of the food, but also have properties that are beneficial to human health [11,12,14, 16,17]. Major uses of maltooligosaccharides are in beverages, infant milk powders, confectionery, bakery products, yoghurts and dairy desserts. The price of pure maltooligosaccharides is extremely high because the chemical manufacture of malto-oligosaccharides larger than maltotriose has been very difficult. However, the discovery of microbial enzymes that specifically act on starch substrate to produce specific malto-oligosaccharides has made it possible to produce syrups containing various malto-oligosaccharides [16]. As compared to the vast number of amylases from different sources

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that have been isolated, purified and characterized, there are very few amylases which act on starch to specifically produce malto-oligosaccharides with DP of 3, 4, 5 and 6.

Our experiments have also shown that the alkaline amylases from the *Streptomyces sp*. could effectively remove starchy stains. This paper thus describes the production and characterisation of the alkaline amylases which could have potential application not only in the food and pharmaceutical industry but also in the detergent industry.

[II] MATERIALS AND METHODS

2.1 Materials

Malt extract, yeast extract, peptone, tryptone, casaminoacids, glucose, potato starch, corn starch were from Hi- media chemicals, India. DNSA, Maltotetraose, maltose, Brilliant Blue G 250, bis acrylamide and TEMED were from Sigma Chemicals, USA. Pre-coated TLC silica gel 60 sheets were from Merck KgaA, Germany. Acrylamide was from Sisco Research Laboratories, India. All other chemicals used were of analytical grade. Tapioca starch, rice flour, wheat flour and soyabean flour were purchased from the local market.

2.2. Isolation of the organism

The soil sample used for isolation was collected from the vicinity of the Lonar Lake, an alkaline salt lake situated in Buldhana district of Maharashtra, India. The protocol and media for isolation of alkalophilic actinomycetes was as described by Mikami et al [18]. The pH of the medium was adjusted to 10.5 by addition of sterile Na_2CO_3, prior to inoculation. Purification of the isolated cultures was carried out by the dilution technique repeatedly to overcome bacterial contamination. No antibiotics were used. The cultures were screened using the plate assay method as described below.

2.3. Plate Assay

Screening of cultures for amylase activity was carried out on agar media in petri plates containing 1% soluble starch, 0.5% peptone, 0.5% yeast extract, 0.05% K_2HPO_4, 0.1% $MgSO_4$ and agar 2.4% (PSA). The pH of the medium was adjusted to 10 by the addition of sterile Na_2CO_3 to a final concentration of 1% prior to pouring the plates. The culture was inoculated at the center of the plates and incubated for 96 h at 28^{0}C. The plates were then flooded with 0.1% iodine in 2% KI. Amylolytic cultures were identified by a zone of clearance against a dark blue background. The amylolytic strain used in the present study was identified as a *Streptomyces sp*. based on morphological characteristics and rDNA analysis (Unpublished results).

2.4. Medium composition and culture conditions

Amylase production was carried out in three steps as follows:

Step1: The organisim was subcultured on an alkaline potato starch-agar plate (PSA) as described above. A five day old agar plate was used to prepare the inoculum. The plates were inoculated and incubated at 28^{0}C.

Step 2: The medium had the following composition: 0.5% yeast extract, 0.5% peptone, 0.5% raw starch, 0.05% K_2HPO_4. 0.01% of $MgSO_4$. The starch was dry heated at 110^{0}C for 20 min and dry sterilized separately for 20 min at 15 lbs. The pH of the medium was adjusted to 10.5 by the addition of sterile 10% Na_2CO_3 to a final concentration of 1%. The raw starch and Na_2CO_3 were added to the medium prior to inoculation. Growth equivalent to one cm was inoculated after maceration.. The flasks were incubated on a rotary shaker at 28^{0}C for 48 h.

Step 3: Production of the enzyme was carried out in 250 ml Erlenmeyer flasks containing one-fifth volume of medium. The fermentation medium consisted of 0.5% yeast extract, 0.5% peptone, 0.1% K_2HPO_4, 0.02% $MgSO_4$ and 3% potato raw starch. Raw starch was sterilized and added as described for the inoculum. The pH of the medium was adjusted to 10 by Na_2CO_3 as described above. Vegetative inoculum 10% (v/v) was transferred to the experimental flasks which were then incubated at 28^{0}C. with shaking at 200 rpm. The cell-free supernatant liquid obtained by centrifugation at 8000 rpm for 30 min at 4^{0}C, was used for determining alkaline amylase activity.

2.5. Enzyme Assay

a) Saccharifying activity

The amylase activity of the culture was estimated using soluble starch as the substrate by the dinitrosalicylic method as described by Bernfeld [19]. The reaction mixture of one ml contained 0.5 ml of suitably diluted enzyme in 50 mM glycine NaoH buffer pH 9 and 0.5 ml of 1% starch solution. The reaction mixture was incubated at 45^{0}C for 30 min and the reaction was terminated by the addition of one ml of DNSA. The tubes were heated in a boiling water bath and the color intensity was read at 540 nm after dilution with 10 ml of distilled water. In the case of assays carried out at other pH values, the corresponding amount of soluble starch prepared in water was used and the final concentration of buffer was 50 mM. A standard maltose curve was used for calculating enzyme activities. One International unit (IU) of amylase activity was defined as the amount of enzyme required to produce one micromole of maltose or equivalent in one min under the assay conditions.

b) Dextrinizing activity

This was estimated as described by Krishnan and Chandra [20]. The reaction mixture contained 0.5 ml of 1% soluble starch, 0.4 ml of 0.125 M glycine NaOH buffer pH 9 and 0.1 ml of suitably diluted enzyme. In the control tube, the enzyme was replaced by an equivalent amount of buffer. The reaction mixture was incubated at 45^{0}C for 5 min and was terminated by the addition of 0.5 ml of 1N HCl. A volume of 0.1 ml of this reaction mixture was diluted with 14.3 ml of distilled water. To this 0.5 ml of 1N HCl was added, followed by the addition of 0.1 ml of 0.3% Iodine in 3% KI. The blue color was read at 600 nm. One dextrinizing unit is defined as the amount of enzyme which hydrolysed 1 mg of starch in 5 min.

2.6. Protein estimation

Amount of protein was determined according to Bradford et al [21] using bovine serum albumin as the standard.

2.7. Electrophoresis

Native PAGE in 10% slab gels was carried out as described by Davis [22]. The protein bands were visualized by staining the gels by silver staining [23].After the run, the gels were sliced into two halves and one half of the gel was stained with silver stain to visualize the bands while the second half was processed for detecting amylase activity. For activity staining, 0.1 IU of enzyme was used. The gel was sandwiched between two 10% PAGE gels containing 0.3 % starch and incubated at 37^{0}C for 2 h. The sandwiched gel was then flooded with 0.1% Iodine in 2% KI. The amylase bands appeared as white bands on a dark blue background

2.8. Molecular weight determination

Molecular weight of the amylases was determined by the method of Hedrick and Smith [24] using the native gel system containing starch. The standard proteins used were bovine serum albumin (66,000 daltons),

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carbonic anhydrase (29,000) daltons), soybean trypsin inhibitor (20,000 daltons) and myoglobin (18,800 daltons). The proteins were detected by Brilliant Blue G-250 [23]. Each of the standard proteins and the partially purified sample was loaded on native PAGE gels of different acrylamide concentration (5%, 7.5%, 10%, 12% and 16%). The slopes of the standard proteins and the sample were determined by plotting log Rm versus gel concentration. A plot of the slopes versus the molecular weight of standard proteins was drawn and the molecular weights of the amylases were determined from this graph by co-relating the slopes.

2.9. Determination of isoelectric pH

This was determined in polyacrylamide tube gels as described by Vesterberg [25].

2.10. HPLC

To determine the mode of action of the enzyme, the degree of polymerization (DP) of the products was determined by HPLC as described by Nirmala [26].Hydrolysis of starch was carried out as follows: 2% soluble starch was hydrolysed in a total volume of 2 ml containing 2 IU of enzyme in 50 mM sodium phosphate buffer (pH 8.0), for different intervals of time ranging from 1 h to 24 h. At the end of the reaction time, the mixture was neutralized by the addition of 1 N HCL followed by boiling for 5 min. 3 vol. of absolute ethanol was added to the hydrolysate and the mixture was kept at 4^0C for 6 h for precipitation after which it was centrifuged at 10,000 rpm. The supernatant was concentrated in a rotavap and the product was dissolved in ultra pure water and filtered through a 0.22 µM membrane and analyzed by HPLC. 20 µL sample was injected and analyzed by HPLC. The amounts of oligosaccharides (DP1-DP7) were quantified by peak integration, with standards detected using a refractive index detector. HPLC was carried out in a Shimadzu system equipped with a RI detector with the following specification: Column: µBondapak NH2 (3.9 x 300 mm, 10 µm)

Solvent system used was acetonitrile: water (70 : 30); Temperature : 25^0C; Flow rate : 1 ml/ min, isocratic; Injected volume : 20 µL. Standards used:–maltose to heptaose

2.11. Raw starch adsorption

Amylase adsorption on raw starch was measured as described by Gashaw et al with slight modifications [27]. The reaction mixture (1 ml) contained 1.2 IU of enzyme in 50 mM phosphate buffer (pH 7.0) and 0.5 g of corn / potato starch. The reaction mixture was gently stirred for 15 min. at 30^0C. The suspension was then microfuged for 5 min. at 10,000 rpm and the amylase activity in the supernatant was determined. The percentage adsorption of the enzyme was calculated with respect to the original activity.

2.12. Test for checking efficacy of the enzyme preparation in removing starchy stains

The application of the amylase preparation as a detergent additive was evaluated as described by Kamal Kumar et al [28]. The application of amylase as a detergent additive was evaluated on pieces of white cloth (2x2 cm) stained with 0.3 % starch solution. The stained cloth pieces were dried at 50 ^{0}C for 30 min. The soiled cloth pieces were washed with 10 ml of water containing 2 mg and 7 mg/ml of surf Excel and 50 U/ml of crude amylase enzyme respectively. The flasks were incubated for 30 min, 1 h and 4 h respectively. The controls consisted of soiled cloth pieces without enzyme treatment. The treated and untreated samples were compared visually, to evaluate the efficacy of the enzyme treatment.

[III] RESULTS

3.1. Isolation and characterisation of the organism

The obligate alkalophilic *Streptomyces sp* was isolated from the alkaline soil of the meteorite Lonar Lake, situated in the state of Maharashtra, India. The colony showed the typical dry powdery characteristic of an actinomycete colony. The aerial mycelial formation was extensive. This actinomycete strain was found to grow only at alkaline pH indicating the strain to be alkalophilic in nature. The optimum growth of the culture occurred in the range of pH 8 to 10 at 28^oC. No visible growth occurred at neutral or acidic pH **[Figure–1a and 1b]**. The strain was identified to be a *Streptomyces* species based on its morophological characteristics and 16s rDNA (unpublished results).

3.2. Plate assay

The *Streptomyces sp* gave a distinct zone of clearance on starch agar medium when flooded with iodine solution in KI indicating the strain to be amylolytic.

3.3. Alkaline Amylase production inoculum

Enzyme production was studied in media containing soluble starch and raw starch as an inducer. The optimum concentration of raw starch in the inoculum was found to be 0.5%. Equivalent amounts of soluble starch gave 56% of the activity as that obtained with raw starch (data not shown). Hence, raw starch was used in all further studies, unless otherwise mentioned.

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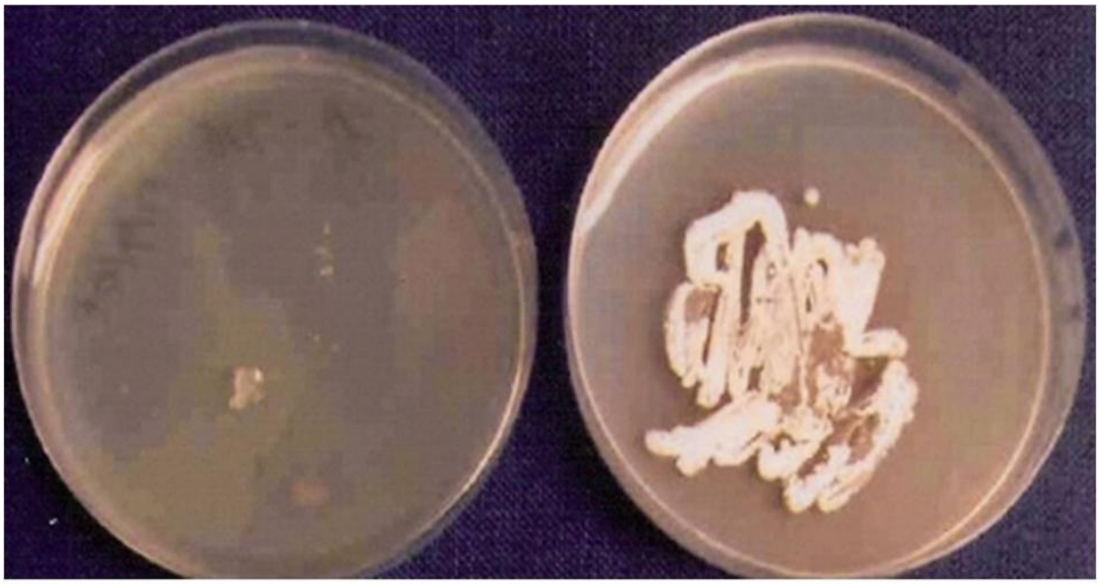

Fig. 1a

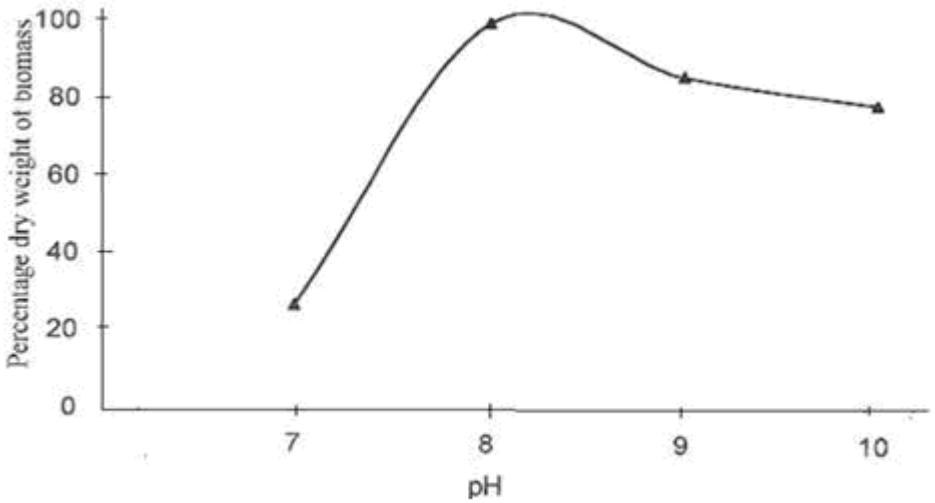

Fig. 1b

Fig: 1. (a) Growth characteristics of the alkalophilic *Streptomyces sp* at neutral and alkaline pH **(b)** Effect of pH of the medium on growth of the culture.

3.4. Requirement of Na^+ and K^+

A number of alkalophilic bacteria are known to have a stringent requirement for Na+ and which cannot be replaced by K+ [1, 29]. The growth of the *Streptomyces sp* was not affected when Na+ ions was replaced with K+ ions in alkaline medium **[Figure– 2a]**. However, amylase production was only 50% when Na_2CO_3 was replaced by K_2CO_3 **[Figure– 2b]** The organism was not halotolerant as only 20% growth was observed in 5% NaCl as compared to the control.

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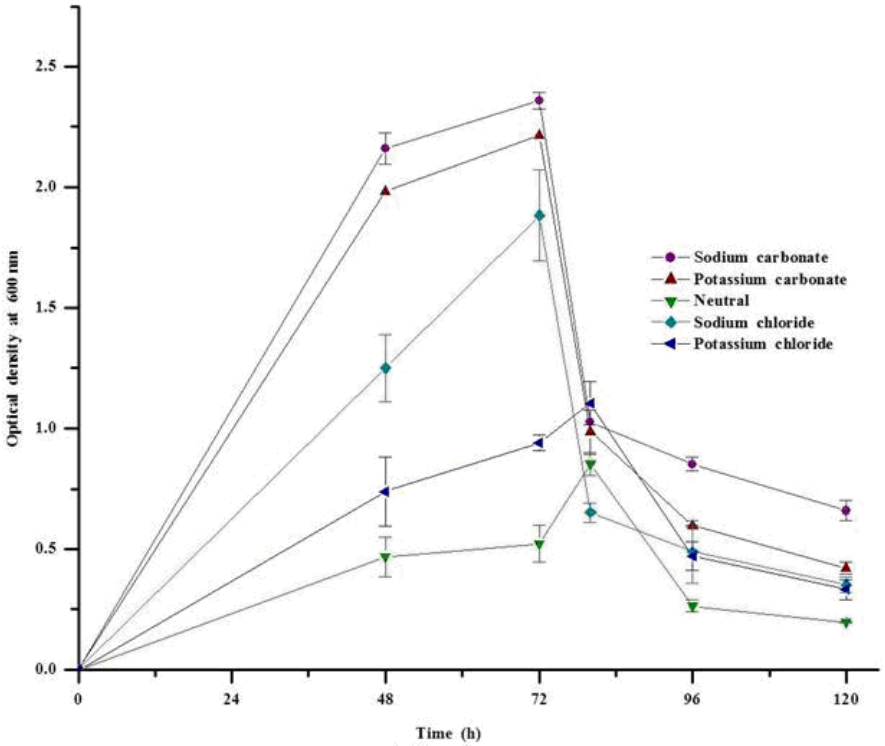

Fig. 2a

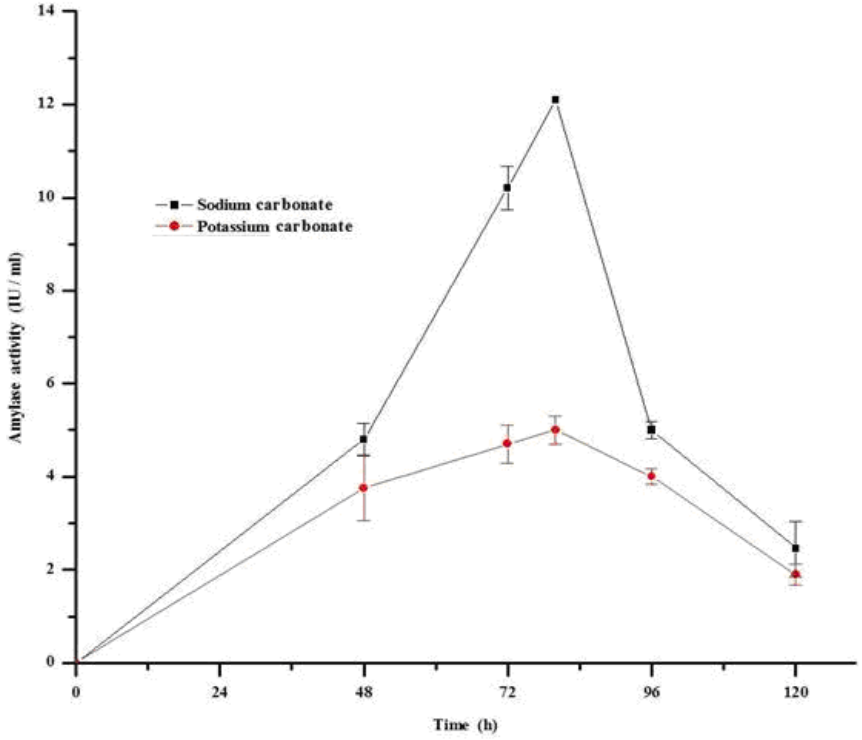

Fig. 2b

Fig: 2. Effect of Na^+ and K^+ on **(a)** growth **(b)** alkaline amylase production

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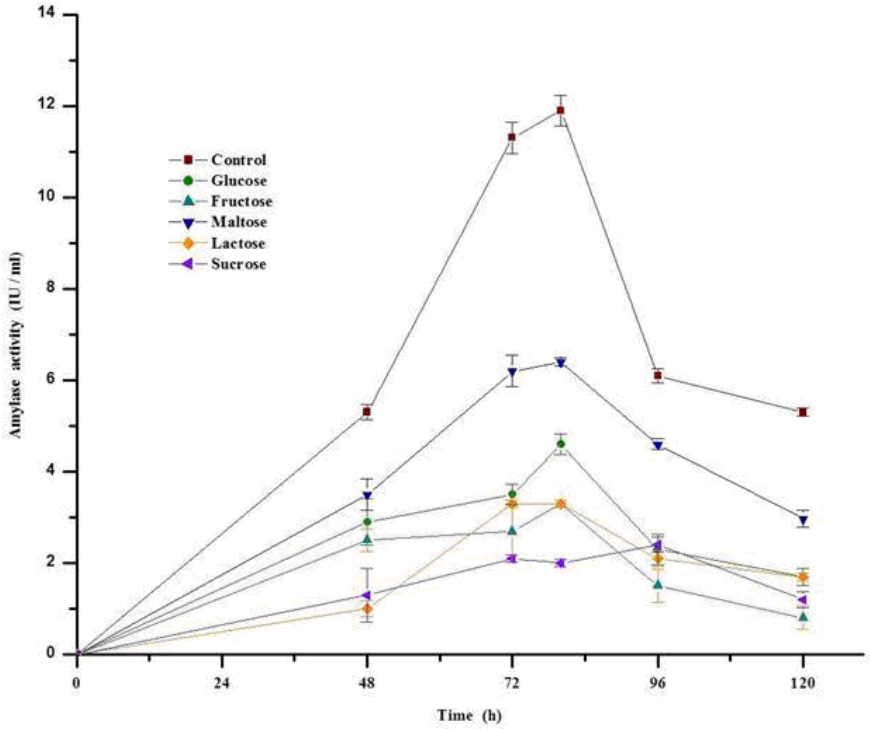

Fig. 3a

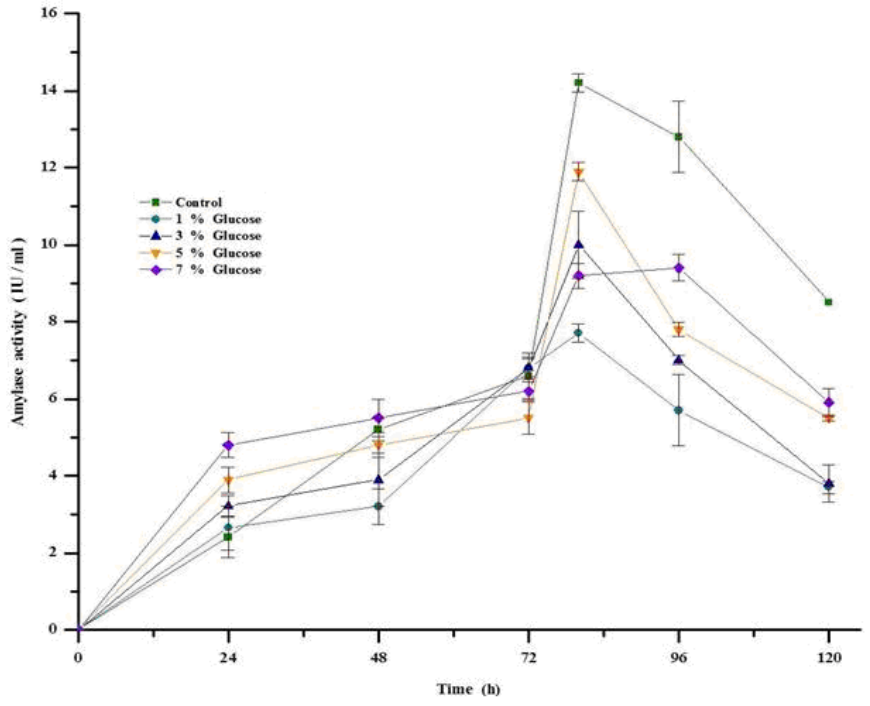

Fig. 3b

Fig: 3. Effect of carbon sources on alkaline amylase production. **(a)** different inducers (sugar inducers 3 %) yeast extract 0.5% ,peptone 0.5 %,$MgS0_4$ – 0.02 % K_2HPO_4 - 0.1 %, sodium carbonate 1 %.) n = 4, significance 1% (ANOVA). **(b)** Alkaline amylase production in MGYP medium, n= 4, significance 1% (ANOVA)

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3.5. Optimization of constituents in the fermentation medium

3.5.1. Starch concentration

The concentration of starch was varied between 1-7% in the fermentation medium, keeping the other constituents constant. It was observed that a maximum activity of 13.2 IU/ml was obtained in presence of 3% raw starch at 80 h. **[Supplementary Table– 1]**. Soluble starch when added at the same concentration gave only 70% of the maximal activity as compared to raw starch. Higher concentrations of soluble starch or incremental addition of soluble starch gave lower activities. Hence, in all further experiments raw starch was added to a final concentration of 3%.

3.5.2. Effect of various starch sources on alkaline amylase production

Starch from various natural sources was studied for their effect on amylase production. It was observed that starch from corn and potato gave maximum and equivalent activities followed by wheat flour **[Supplementary Table – 2]**. Enzyme production was only 36 to 40% of maximal activity with rice and refined wheat flour.

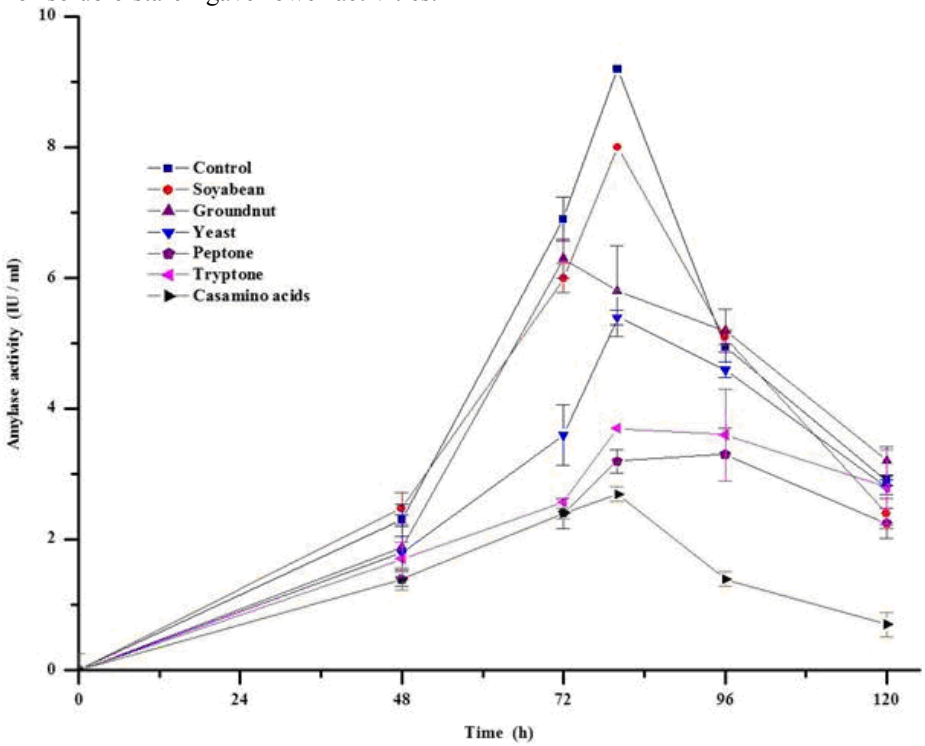

Fig. 4

Fig 4: Effect of organic nitrogen sources on amylase production. (n= 4, significance 1% (ANOVA))

3.5.3. Effect of carbon sources

The effect of different carbon sources was evaluated on amylase production **[Figure– 3a]** Amylase synthesis is known to be induced by starch and maltose. The only report of a constitutive production of an amylase has been from *Pseudomonas sp* IMD 353 where a higher amylase activity was observed in a non-starch medium [30]. In our studies it was observed that 3% maltose gave 54% of the control activity (potato starch), while glucose gave 30% of the control activity indicating that there is a substantial basal level of constitutive production of the enzyme. In a MGYP medium (1% glucose, 0.3% malt extract, 0.5% yeast extract, 0.5% peptone) where the effective concentration of maltose from malt extract was approximately one tenth of that added in the 3% maltose experiment, 65% of the control activity was obtained indicating that a factor in addition to maltose from malt extract is inducing the amylase **[Figure–3b]** When the concentration of glucose was increased from 1% to 5% there was a gradual increase in activity and at 5% glucose concentration, 83% of the control activity was obtained. Further increase in glucose concentration resulted in a decrease in amylase activity. These results indicate that the repressive effect of glucose could be overcome partially by the addition of malt extract. Similar observations were made by Diaz et al [31] in the case of ß amylase production from *Xanthophyllomyces dendrorhous*. Eighty eight percent of ß amylase activity was obtained in a MGYP medium when compared to a control medium containing starch and glucose. It

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has been shown that the repressive effect of glucose was reduced in solid state fermentation [32]. A number of amino acids are known to increase the production of various enzymes such as amylases [33] and xylanases [34]. Chandra et al [35] showed that in the presence of a mixture of amino acids the repressive effect of monosaccharides, especially glucose at low concentrations did not effect amylase synthesis. It is therefore likely that amino acids present in the malt extract could have played a role in enhancing the activity besides induction by maltose.

3.5.4. Effect of nitrogen sources on amylase production

Various nitrogen sources individually or in combination was evaluated using an optimum starch concentration of 3%. **[Supplementary Table– 3, Figure– 4]** Maximum activity was obtained in control flasks containing a combination of yeast extract and peptone followed by soybean. Yeast extract and peptone when used singly gave lower activities. Tryptone gave poor yields. A combination of tryptone and yeast extract gave only 57% of the control activity. Similar results were obtained by Hayashi et al [36] in the case of alkaline amylase production in *Bacillus sp H-167*. A review of the literature showed that the requirement of an optimal nitrogen source varied with the organism. Tigue et al [37] have shown that yeast extract alone gave maximum yields as against a combination of yeast extract and peptone. Narang and Satyanarayana [38] have shown that tryptone or yeast extract individually or in combination gave optimal levels of amylase production in *Bacillus thermooleovorans*. In a few cases inorganic nitrogen sources such as ammonium hydrogen phosphate, ammonium sulphate and ammonium nitrate in combination with an organic nitrogen source has given enhanced amylase production [39]. In the case of most organisms yeast extract was found to be essential for optimal production of amylases.

The quality of the protein and the carbon –nitrogen balance appears to play an important role in optimal production of the enzyme.

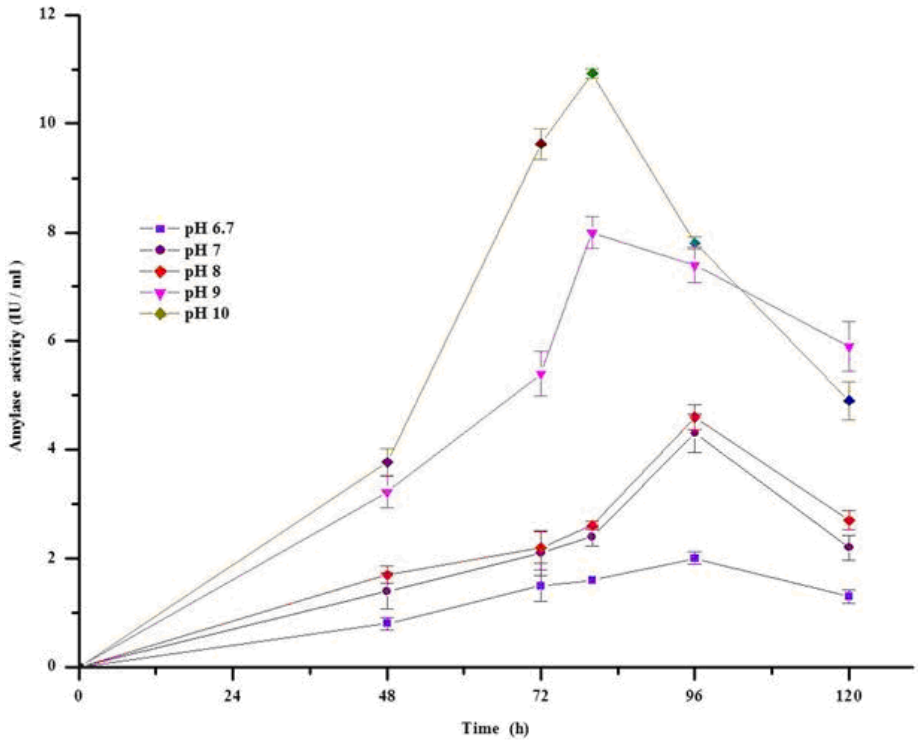

Fig. 5

Fig: 5. Effect of initial pH of the medium on alkaline amylase production n= 4, significance 1% (ANOVA)

3.5.5. Effect of metal ions on amylase production

Various metal ions were evaluated for enzyme production, keeping the concentration of the nitrogen and carbon source constant. The metal ions K_2HPO_4, $MgSO_4$, and $CaCl_2$ when added individually gave approximately 30 to 50 % of the maximal activity. Maximum amylase production occurred with a

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combination of K_2HPO_4 and $MgSO_4$. A combination of K_2HPO_4 and $CaCl_2$ gave only 65 % of the control activity indicating that calcium ions were not essential for amylase production **[Supplementary Table– 4]**. Malhotra et al [40] too showed that amylase production was calcium independent and Mg ions enhanced enzyme production. Similar observations were made by Chandra et al [35].

3.6. Effect of initial pH on amylase production

Since the growth of the *Streptomyces* culture occurred at pH 8 and above, fermentation was carried out at different pH in order to determine the optimum pH for amylase production. The pH of the medium was varied by the addition of 10% sodium carbonate which was autoclaved separately and added prior to inoculation. Other constituents of the fermentation medium were kept constant. To determine the extent of amylase production at various pH values, equal amounts of inoculum (10% v/v) grown at alkaline pH was added to all the flasks at different pH values. The flasks were incubated at 28^{0}C at 180-200 rpm. It was earlier seen that the growth of the culture at pH 7.0 in a MGYP medium was 20 % when compared with the growth at pH 10 indicating that the culture was truly alkalophilic in nature **[Figure– 1b]**. Concomitantly at neutral pH only 20 % of amylase production was observed even though the pH of the medium rose to a pH of 8. **[Figure– 5])** At 96 h the pH of the medium rose further to a pH of 9.45, but the corresponding amylase activity was only 35 % of the control activity. Thereafter the activity declined.

These results indicated that the initial pH of the medium was critical for maximum amylase production which was preferably above a pH of 9.0.

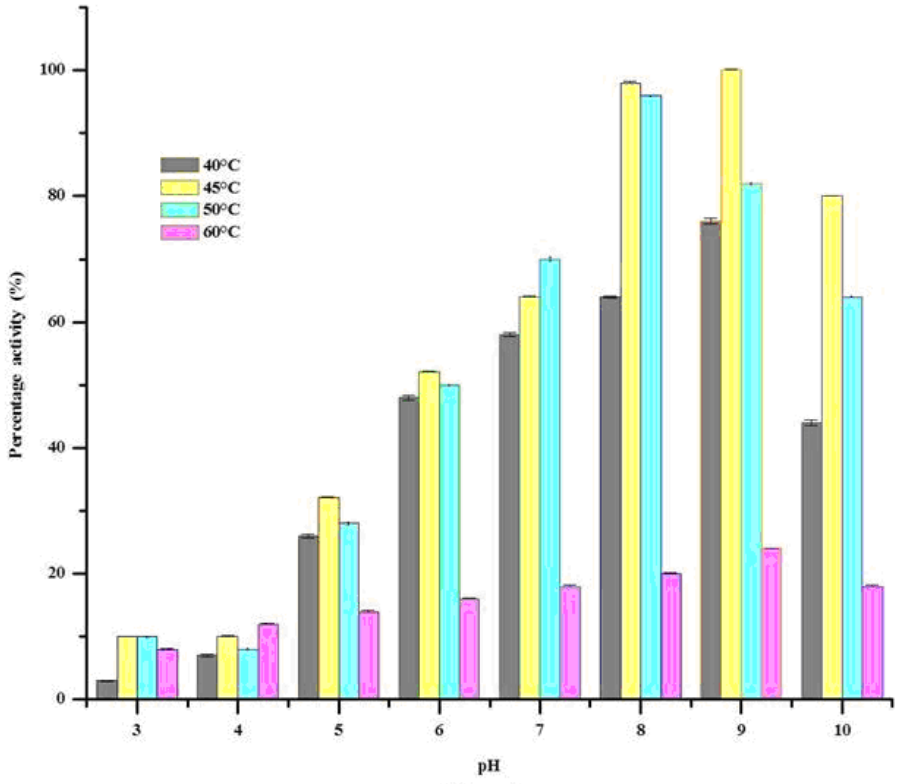

Fig. 6

Fig: 6. Profile of optimum pH and temperature of alkaline amylases from alkalophilic *Streptomyces sp*. Buffer system: sodium acetate buffer 50 mM (pH4.0-5.0); Potassium phosphate buffer, 50 mM (pH 6.0- 8.0); Glycine- NaOH buffer, 50 mM (pH 9.0 – 10.0). Temperature 45^{0}C, n= 3, significance 1% (ANOVA)

3.7. Characterisation of the amylases from the Streptomyces sp

3.7.1. Optimum pH and temperature

The optimum pH of amylase activity was determined at various pH values ranging from pH 4 to 10 as a function of temperature. From **Figure– 6** it can be seen that the amylase is active in the alkaline range. Maximum activity was obtained at pH 9 and at 45^{o}C. The enzyme was found to be active over a temperature range of 40^{o}C to 50^{o}C at pH 9 and 10. The enzyme was stable over a pH range of 7-10, maximum being at pH 7. The above pH

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and temperature parameters make the enzyme suitable for detergent, leather and textile applications where enzymes active at alkaline pH are desirable.

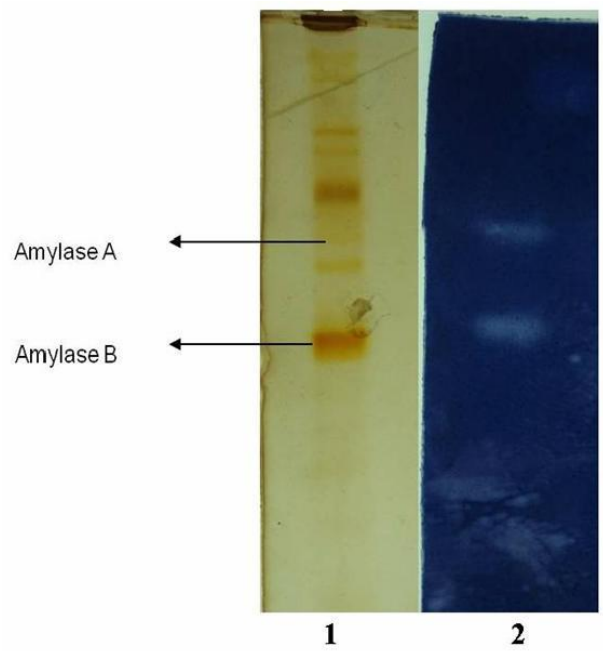

Fig. 7

Fig: 7. Silver stained PAGE gel electrophoresis of the crude extract of alkalophilic *Streptomyces sp* (Lane 1). Starch gel electrophoresis of the crude extract of *Streptomyces sp* (Lane 2).

3.7.2. Effect of additives on amylase activity

Alkaline amylases were found to vary in their response to the chelating agent EDTA [41]. In our studies the effect of EDTA was found to be interesting **[Supplementary Table–5]**. When EDTA was added at a concentration of 2 mM approximately 50% of the amylase activity was lost. However, with increasing concentrations of EDTA there was a progressive increase in activity and at 20 mM, 80% of the original activity was restored. These results were further corroborated by dialysis of the enzyme against EDTA. When the enzyme was dialysed overnight against 10 mM Tris-HCl, pH 8 containing 2 mM and 20 mM EDTA, the activities obtained were 52 and 80% respectively confirming that Ca^{+2} was essential for activity. Similar observations were made Bernhardsdotter et al [42]. The authors reported 30% enhancement of activity with 10mM EDTA as compared to 5 mM. Vishnu et al [43] too reported an enhancement of activity with EDTA at a higher concentration in the case of the amylases from *Lactobacillus amylophilus GV6*. Presently there is no explanation that can be given for these observations and must await the determination of the crystal structure. It has been observed that generally the saccharifying amylases are stable in their response to EDTA / EGTA as compared to the liquefying amylases [44]. The amylase activity decreased progressively with increasing concentrations of denaturing agents such as SDS and urea. The 6M urea treated enzyme when dialysed against buffer did not regain its activity but when dialysed against buffer containing 5 mM $CaCl_2$, 55% of the original activity was restored indicating that Ca^{+2} ions was essential for proper folding of the enzyme. The enzyme lost 15% activity as compared to the control when incubated with 0.2% Surf a commonly used detergent for 15 min at 40 to 50^0C, indicating that the amylases from this strain could have a potential application in laundry detergents. A progressive increase in activity was observed with increasing concentrations of reducing agents such as ß-mercaptoethanol and cysteine hydrochloride. The increase was threefold approximately with cysteine hydrochloride. Similar observations were made in case of xylanases where 10 mM cysteine and β-mercaptoethanol led to an increase in activity ranging from 47% to 116% [45-47].

3.7.3. Electrophoresis Pattern

The starch gel electrophoretic pattern is shown in **Figure–7**. Both, anodic as well as cathodic runs were carried out. In the anodic run, two bands of amylase activity were detected. No activity bands were detected in the cathodic run, suggesting that the amylases are anionic in nature.

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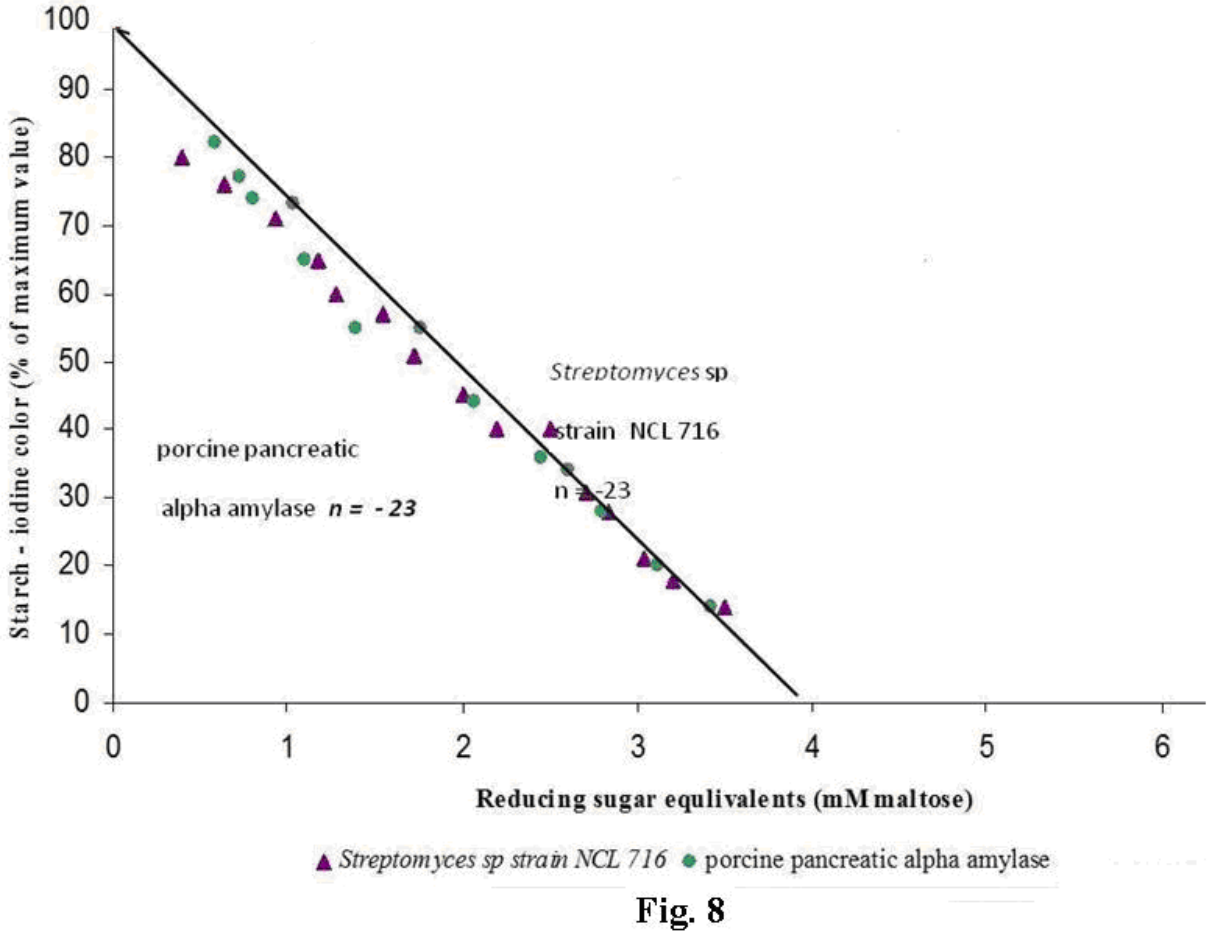

Fig. 8

Fig: 8. Determination of mode of action of the amylase. The values for the starch-iodine blue color are plotted against the production of reducing sugar equivalents in terms of maltose. The slope of the linear curve determines the endo- or exo-action of the enzyme.

3.7.4. Molecular weight and pI

Molecular weights of the amylases as determined by the mobilities in native gels of different concentration were found to be 36,920 and 36,300 daltons respectively. The pI of the major amylase was found to be 6.26 confirming the anodic nature of enzyme.

3.7.5. End product analysis

The slope of a plot of reducing sugar versus starch-iodine color could predict the mode of action of the amylase. A steep slope accounts for a fast reduction of the starch-iodine blue color due to random endo activity, while a comparatively flat slope indicates a prevalence of exo activity [48]. A slope of -26 was obtained for the endo α-amylase from *Bacillus amyloliquefaciens* while the maltohydrolase enzyme from *Pseudomonas stutzeri NRRLB 3389* showed a slope of -12. The *Bacillus stearothermophilus* exo-maltogenase showed a slope of -2. A comparative study of porcine pancreatic amylase with the amylases from *Streptomyces sp*. was carried out. The well characterized porcine pancreatic amylase gave a plot of –24 confirming that it is endo in action **[Figure– 8]**. The alkaline amylases from the *Streptomyces sp*. also gave a value of –24 indicating it to be also endo in action The HPLC profile of the starch hydrolysate for the *Streptomyces sp*. amylase showed the presence of maltohexaose (~ 48%) as the predominant end-product followed by almost equivalent amounts (~ 10%) of maltotetrose, maltotriose and maltose, even at the early stages of hydrolysis indicating it to be different from porcine pancreatic amylase **[Figure– 9]** The first report of amylases producing specific sugars was reported by Robyt et al from the culture filtrates of *Pseudomonas stutzerii* [49]. Subsequently, there have been several reports of amylases producing specific sugars ranging from G2 to G6, mainly from the genus Bacillus and

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Pseudomonas, most of which are active at neutral or alkaline pH. To date there are only two reports of maltotriose being produced by neutrophilic *Streptomyces* strains [7,8] and one report each of maltotetrose and maltohexaose being produced by *Streptomyces* strains [9,10].

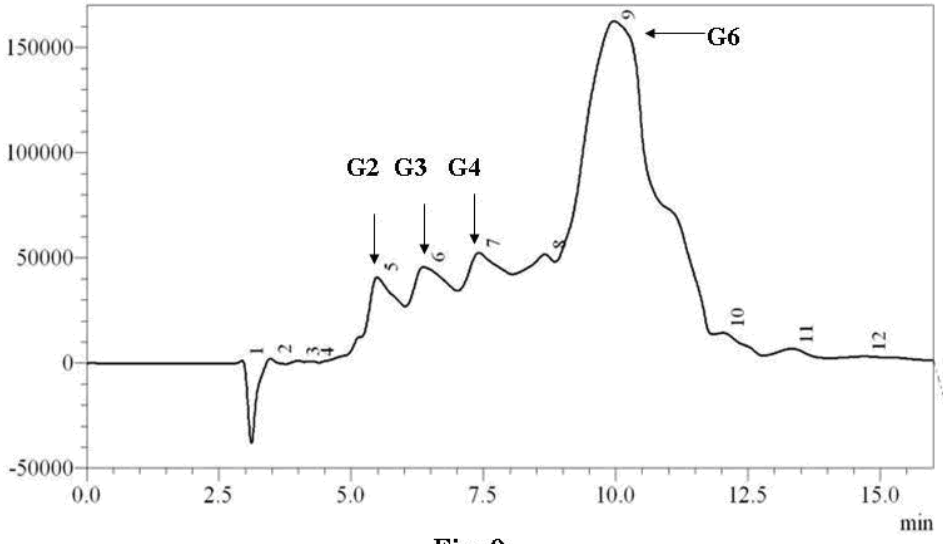

Fig. 9

Fig 9: HPLC analysis of the end products of hydrolysis of soluble starch. G2 – Maltose; G3 – Maltotriose; G4 – Maltotetraose; G6 : Maltohexaose

3.7.6. Raw starch determination

Amylases can be classified as endo–adsorbant and non-adsorbant [50]. The alkaline amylases from *Streptomyces sp* were unabsorbed at pH 7.0 indicating them to be of the non-adsorbant type.

3.7.7. Efficiency of the enzyme preparation in removing starchy stains

To increase the efficiency of detergents, a combination of proteases, amylases, cellulases and lipases are incorporated in the detergents. Alkaline amylases are especially used as additives in detergent formulations to remove starch based stains. The amylase from the *Streptomyces sp* lost 15% activity as compared to the control when incubated for 15 min at 40^0C to 50^0C with 0.2% Surf. From **Figure– 10** it is seen that the addition of the enzyme as an additive to the detergent Surf improved the washing and efficiency of removal of the starch based stain.

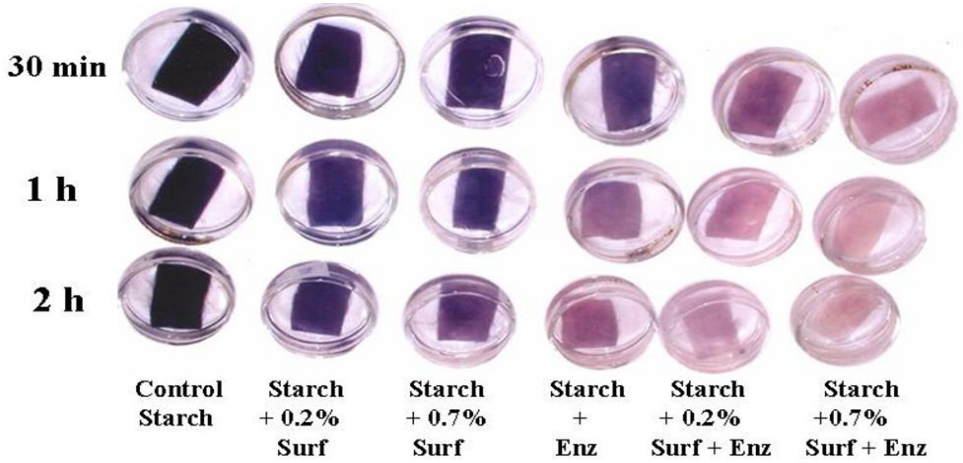

Fig: 10. Evaluation of the washing performance of the alkaline amylase preparation from the *Streptomyces sp* when added as an additive to the detergent Surf.

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[IV] CONCLUSION

The alkaline amylases from the alkalophilic *Streptomyces sp*. is the first report of a maltohexaose (G6) producing alkaline amylase from the genus *Streptomyces*. The distinguishing properties of the amylases of this strain are:

(i) The amylases break down starch to give maltohexaose (G6) as the predominant product. Malto-oligosaccharides with DP in the range of 3-6 are produced by only few amylases and such maltooligosaccharides are increasingly being used in food and pharmaceutical products due to their properties such as low sweetness, high water holding capacity and anti-staling capacity. The amylase activity from the *Streptomyces sp* is comparable with the activity of maltohexaose producing amylases from other organisms [10, 36, 44, 51-54] and hence would have a potential application in the food and pharmaceutical industry for the production of maltohexaose (G6).

(ii) Our results obtained using the amylase preparation as an additive with detergent is promising suggesting that the alkaline amylase preparation from this *Streptomyces sp* could also have an application in the detergent industry.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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SUPPLEMENTARY TABLES (As supplied by authors)

Supplementary Table: 1. Determination of optimum starch concentration on alkaline amylase production

Raw starch (%)	Maximum amylase activity* (IU/ml)/80h
1	4.0 ± 0.52
3	13.2 ± 1.42
4	10.7 ± 0.77
5	9.3 ± 0.49
7	8.7 ± 1.06

Control - yeast extract 0.5 %, peptone 0.5 %, potato raw starch 3 %, $MgS0_4$ – 0.02 %, K_2HPO_4 - 0.1 % , sodium carbonate 1 %. *n = 4
Significance 1% (ANOVA)

Supplementary Table: 2. Effect of various starch sources on alkaline amylase production

Raw starch (3%)	Maximum amylase activity* (IU/ml)/80h
Potato(control)	13.6 ± 0.84
Corn	13.5 ± 1.27
Wheat	12.6 ± 0.14
Tapioca	10.8 ± 0.28
Rice	8.7 ± 0.14
Refined wheat flour	7.5 ± 0.424

Control- yeast extract 0.5 %, peptone 0.5 %, potato raw starch 3 %, $MgS0_4$ – 0.02 %, K_2HPO_4 - 0.1 % , sodium carbonate 1 %
*n= 4, Signficance 1% (ANOVA)

Supplementary Table 3: Effect of combination of different nitrogen sources on alkaline amylase production

Nitrogen sources (%)	Maximum amylase activity * (IU/ml) / 80h
Control	9.9 ± 2.18
Soyabean (1%)	7.3 ± 0.69
Soyabean (2%)	6.5 ± 0.41
YE (0.5%) + Tryptone (0.5%)	5.9 ± 0.29
YE (0.5%) + Soyabean (1%)	5.3 ± 0.52
YE (1%) + Soyabean (1%)	4.3 ± 0.24

Control - yeast extract 0.5 %, peptone 0.5 %, potato raw starch 3 %, $MgS0_4$ – 0.02 %, K_2HPO_4 - 0.1 %, sodium carbonate 1 % *n = 4
Significance 1% (ANOVA)

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Supplementary Table: 4. Effect of metal ions on alkaline amylase production

Metals (%)	Maximum amylase activity*(IU/ml) / 80h
$MgS0_4$+ K_2HPO_4 (Control)	10.9 ± 0.98
$MgS0_4$	3.8 ± 0.29
K_2HPO_4	4.1 ± 0.18
$CaCl_2$	4.0 ± 0.81
K_2HPO_4+ CaCl2	7.4 ± 0.51
$MgS0_4$ + K_2HPO_4 +$CaCl_2$	7.7 ± 0.25

Control- yeast extract 0.5 %, peptone 0.5 %, potato raw starch 3 %, $MgS0_4$ – 0.02 %, K_2HPO_4 - 0.1 % , sodium carbonate 1 %. *n = 4
Significance 1% (ANOVA)

Supplementary Table: 5. Effect of various additives on the activity of alkaline amylases from alkalophilic *Streptomyces sp*.

Effectors	Concentration mM)	Retained activity(%)
Control	0	100 ± 2.9
$CaCl_2$ [a]	1	103 ± 1.8
$CaCl_2$ [a]	5	138 ± 2.3
EDTA [b]	2	48 ± 3.3
EDTA [b]	5	60 ± 1.6
EDTA [b]	10	70 ± 3.3
EDTA [b]	20	80 ± 1.6
SDS [b]	1 %	60 ± 2.9
SDS [b]	2 %	43 ± 2.6
SDS [b]	5 %	17 ± 1.8
Urea [b]	6 M	18 ± 1.8
Dithiothreitol [b]	10	132 ± 1.8
Cystine HCl [b]	10	343 ± 1.1
Surf [b]	0.2 %	48 ± 3.4 (30 min)
Surf [b]	0.2%	38 ± 1.8 (45 min)

[a] -Tris HCl buffer (50 mM), [b] -Glycine NaOH (50 mM) *n= 3 ,Signficance 1% (ANOVA)

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RESEARCH ARTICLE OPEN ACCESS

FLORA OF THE ORDER QUERCETALIA PUBESCENTIS BR.-BL. 1932 IN THE FOREST VEGETATION OF THE KOSOVO HILLY AREA

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ABSTRACT

This study is a part of floristic and synecological research into the forest vegetation of the Kosovo hilly area, which belongs to the order Quercetalia pubescentis. This order is comprised of two types of deciduous forests: thermophyles and mesophyles. The thermophyle forests belong to two alliances: Ostryo-Carpinion orientalis and Quercion farnetto, whereas the mesophyle forests belong to two other alliances: Carpinion betuli illyrico-podolicum and Fagion illyricum. In this study, the flora of 4 associations of the alliance Ostryo-Carpinion orientalis and 2 associations of the alliance Quercion farnetto were analyzed and the study mostly deals with the following associations: Carpinetum orientalis scardicum, prov., Dioscoro-Carpinetum orientalis B-ić, Colurno-Ostryetum carpinifolie B-ić, Seslerio-Ostryetum Horv.et H-ić, Quercetum farnetto-cerris scardicum, prov., and Quercetum montanum Čer. et Jov. A total of 140 relevés encompassing 6 forest communities were analyzed. The analysis of the floral composition of forest communities of Kosovo hilly area are revealed as many as 262 species, of which 130 or 49.61% belong to the association Carpinetum orientalis scardicum, which represents the largest number of species, whereas the smallest one belong to the association Seslerio-Ostryetum (93 species or 35.49%) in sociological sense. The biological spectrum of all associations and symbols of life forms of each species were also analyzed and are presented in Table 1.The percentages of species of all the associations are separately presented in Table 2. Also, in the analyses of floral geo-elements, 30 groups of these elements have been determined and presented its spectrum.

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KEY WORDS

Forest; vegetation; flora; synecology; Quercetalia pubescentis, Kosovo

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[I] INTRODUCTION

The present investigation deals with systematic synecological-vegetational study into the forest vegetation of the Kosovo mountain area, which was earlier has been studied by Krasniqi [1].

This is the most comprehensive study of this vegetation in Kosovo. In this study the following associations have been determined: *Carpinetum orientalis scardicum* with three subassociations (*Carpinetum orientalis scardicum seslerietosum*, *Carpinetum orientalis scardicum quercetosum* and *Carpinetum orientalis scardicum anemonetosum*); *Dioscoro-Carpinetum orientalis*, *Colurno-Ostryetum carpinifolie* with two subassociations (*Colurno-Ostryetum carpinifolie discoretosum*, and *Colurno-Ostryetum carpinifolie typicum*), *Seslerio- Ostryetum, Quercetum farnetto-cerris scardicum* with two sub-associations (*Quercetum farnetto-ceris scardicum moltkietosum*, and *Quercetum farnetto-cerris scardicum typicum*), *Quercetum montanum*, all of these thermophylic communities of the order *Quercetalia pubescentis*, as well as the two associations of the mesophyllic communities of order *Fagetalia illyrica Horv*.(associations *Querco-Carpinetum serbicum* and *Fagetum montanum*), are not included in this paper. These two last associations have been investigated previously by Nikolić [2], but not in the whole territory of Kosovo, i.e. only in a small part of the Kosovo plain, as part of a research of spores and pollen in lignite pool of Kosovo.

Kosovo is located in the Balkans, with a surface of around 11.000 square kilometers. It is divided into two macro-regions: the Eastern part, which is comprises of The Plain of Kosovo, and the Western part, which comprises of The Plain of Dukagjini **[Figure–1]**.

The relief of Kosovo is young, with a high depth and density of fracturing. It is dominated by the mountainous parts, around 63% of overall surface of the territory. The average altitude of the whole territory is 810 meters. The highest peak is 2656 meters (the peak of Gjeravica) in the Bjeshkët e Namuna, whereas the lowest peak is 260 meters (the valley of Drini i Bardhë). According to their origin, they are divided into tectonic and volcanic mountains. The former, which are built from sedimentary rocks, occupy the largest part of the surface and are located mainly in the western, northern and southern parts. The mountains built from magma rocks are located mainly in the northern and eastern parts in Kosovo [3].

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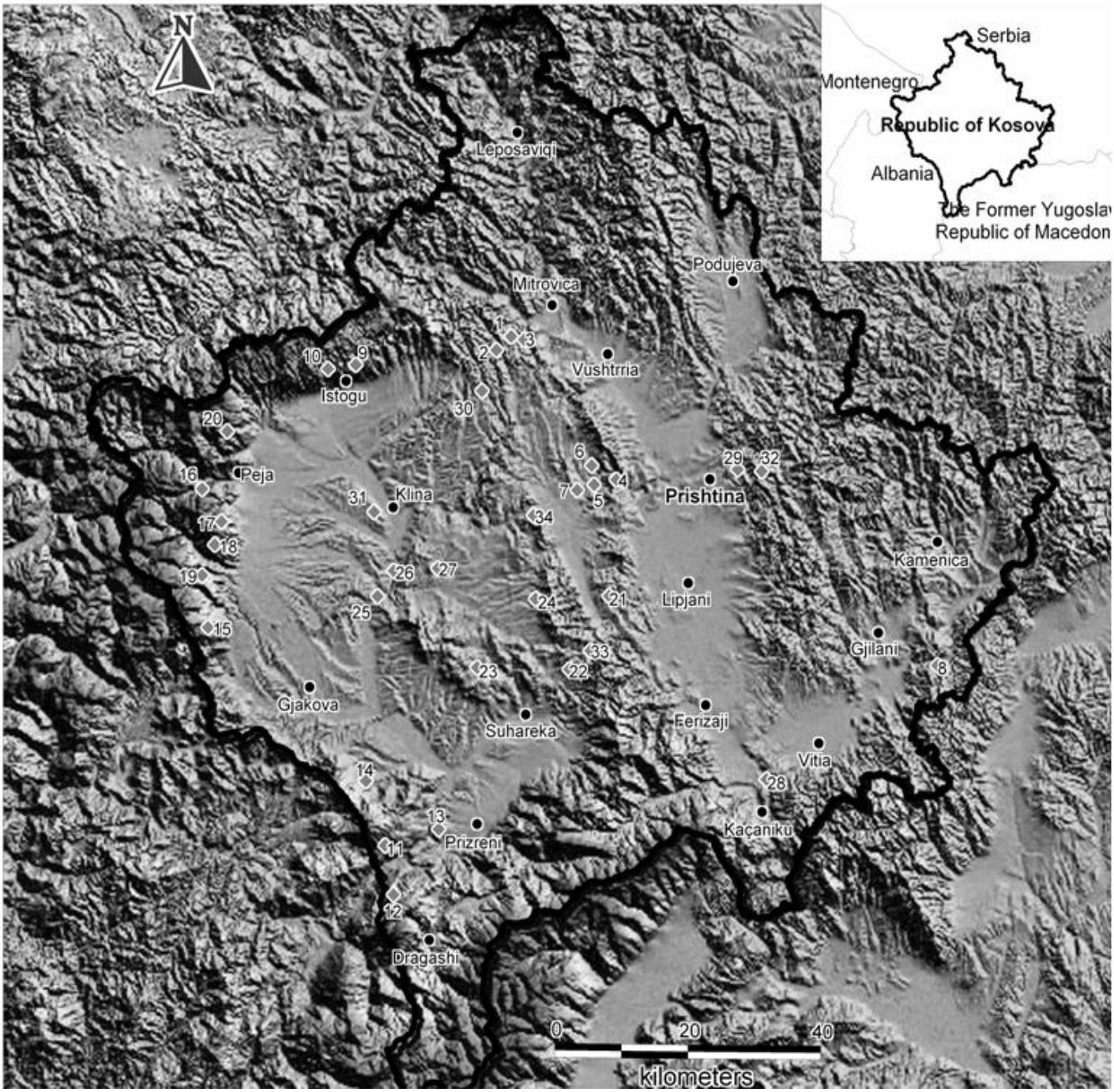

Fig: 1. Map of Kosovo and localities in which relevés of six communities have been registered: Carpinetum orientalis scardicum : 1 – Zmiq (Braboniq), 2 – Rosule (Gradevc, Braboniq), 3 – Koleno (Braboniq), 4 – Korriz (Grabovc), 5 – Korriz (Dobrashevc), 6 – Graode (Godanc), 7 – Seq (Murgë), 8 – Markov Kamen (Pogragjë), 9 – Selilo (Istog), 10 – Krshnica (Istog), 11 – Gorozhup (Prizren); Dioscoro-Carpinetum orientalis: 12 – Koritnik (Zhur), 13 – Goma Pole (Vllashnë); Colurno-Ostryetum carpinifolie: 12 – Koritnik (Zhur), 14 – Pashtrik (Kushnin), 15 – Shkoza (Koshare); Seslerio-Ostryetum: 16 – Koprivik (Pejë), 17 – Maja Lubeniq (Pejë), 18 – Bjeshkët e Strellcit (Pejë), 19 – Golobrdo (Pobërgjë), 20 – Peklen (Pejë); Quercetum farnetto-cerris scardicum: 21 – Lipovicë (Lipjan), 22 – Biraq (Duhle), 23 – Crni Llug (Prekorupë), 24 – Rreza (Lladrovc), 25 – Mali Dreni (Mrasor), 26 – Cerni Kamen (Volljakë), 27 – Pylli i Kijevës (Rigjevë), 28 Dushkaja (Kaçanik), 29 – Gërmia (Prishtinë), 30 – Çubrel (Skenderaj), 31 – Boshnjac (Klina); Quercetum montanum: 29 – Masivi i Gërmisë (Prishtinë), 32 – Bregu i Butovcit (Prishtinë), 21 – Lipovicë (Lipjan), 33 – Carralevë (Shtime), 34 – Mali Drenicë (Gllogovc).

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The relief of Kosovo was formed during the orogenic phase. The mountains appeared above water during Miocene, whereas ponds, valleys, Fushë-Kosova, during Pliocene. The region of Kosovo represents an important link of the south-eastern branch of Alpine range (Dinaric-Albanic-Hellenic mountain range). Within a small territory one can discern a variety of geological formations of different ages, from the Precambrian to the Quaternary periods [4].

As far as the climate is concerned, Kosovo belongs to the Mediterranean, with a slight influence of the continental climate. Moreover, in the Fushë-Kosova plain one can notice a small influence of the steppe climate. So Kosova in general has wet, relatively short and cold at winter, whereas summers are hot and relatively dry. The average year air temperature revolves from 9.5^{0}C (Prishtina) to 12^{0}C (Prizren). The average yearly rains revolve from 600 mm/year in the eastern region to 900 mm/year in the western ones (Peja and Gjakova), and over 1.500 mm/year in the Bjeshkët e Namuna [5].

Just like on the other parts of the Balkans, Kosovo has a diverse and rich flora and vegetation, which are mainly due to its geographical position, geological content, relief, climate and the historical development of flora and vegetation in its immediate geological past.

Viewed from its horizontal position, vegetation in Kosovo belongs mainly to the Euro-Siberian vegetative region. According to Horvatić [6], the hot valleys of the low parts of Kosovo belong to the Aegean province. The highest part of Kosovo belongs to the Moesic province, whereas smaller part in North-West belongs to the Illyrian one. The highest zones belong to the Nordic-Alpic region. Therefore, Kosovo is a cross- road of influences of three phyto-geographical regions.

In Kosovo about 2.400 species of vascular flora have been established [7]. If we have in mind that in the Balkans there are approximately 6.800 vascular species, then we can conclude that the flora of Kosovo represents around 35% of this flora. The flora of Kosovo appears to be even more interesting because of the participation of approximately 200 endemic and relict species. Even though the surface of its territory represents 2.3% of the Balkans, the endemic and relicts species represent 11% of these plants within the Balkan Peninsula [8].

[II] MATERIALS AND METHODS

The floral composition of these six forest communities in Kosovo`s hilly area was analyzed in detail in the synecological-vegetational research into the forest vegetation of Kosovo.

Forest vegetation was investigated according to the principles of the Zürich-Montpellier school. The scientific names of the species were left unchanged, they are used as registered in the original relevés of phytocenological tables.

In this study, a total of 140 relevés encompassing six communities were analyzed. The species were also analyzed according to biological forms according to Horvat [9]. The floral geoelements were determined according to Horvat, Glavač, Ellenberg [10] and partly to Flora of Albania [11] and Flora of Serbia [12].

[III] RESULTS

The analysis of the floral system of six forest communities in the hilly Kosovo area identified a total 262 species, (11species are not definite): *Carex sp*., *Cirsium sp*., *Crocus sp*., *Cynanchum sp*., *Hieratium sp*., *Galium sp*., *Iris sp*., *Orchis sp*.,. *Rosa sp*. div., *Rubus sp.* and *Thymus sp*. The analysis of their life forms and the belonging of their geofloristic elements were not done. In the **Supplementary Table-1** these species are marked with a question mark: *Cirsium silvaticum, Verbascum niger* and *Vicia sparssiflora*. Amongst them, *Cirsium silvaticum* is more problematic because of the fact that one cannot encounter it in the existing flora of the region. Because it has been in the association *Dioscoro-Carpinetum orientalis* and association *Quercetum farnetto-cerris scardicum,* perhaps the species *Ptilostemon strictum* is assumed, identified later precisely in the individuums of association with *Quercetum farnetto-cerris scardicum* in Braboniq (near Mitrovica) and Gërmia (near Prishtina). Nevertheless, this remains an arguable topic, since one might be talking also about a different, undetermined species of the genus *Cirsium sp*., (see the noted species in the analytical tables). Some other arguable species, noted on the analytical tables of these associations are the following, mentioned two synonyms: *Chrysanthemum corymbosum* and *Tanacetum corymbosum*, *Quercus petraea* and *Quercus conferta*, *Rhus cotinus* and *Cotinus coggygria*. In other words, here we are dealing with same species, but evidenced in two forms. Also, *Primula columnae* and *Primula verris* are noted, although the former are considered as subspecies of *Primula verris*. Similarly, problematic is the case with *Veronica chamaedryfolia*, which in current floristic literature does not exist under this name, but only as *Veronica hederifolia*.
From the analytical tables of these six communities,the followings were analyzed: the number of relives, the localities where this investigation took place **[Figure–1]**, number of species, the sea level, and the geological basis. So, in the association *Carpinetum orientalis scardicum* 33 releves have been noted, containing 130 species, at the sea level from 300 – 940 meters, and on the geological basis of limestone (24 relevés), serpentine (6 relevés) and silicates (3 relevés). In the association *Dioscoro-Carpinetum orientalis* it has been noted 22 relevés, containing 112 species, at the sea level from 260 – 960 meters, and on the geological basis of limestone. On the association *Colurno-Ostryetum carpinifolie* 19 relevés have been carried out, containing 101 species, at the sea level from 750 – 1120 meters, and on the geological basis of limestone. On the association *Seslerio-Ostryetum* only 12 relevés have been analyzed, 93 species have been registered, at the sea level from 700 – 1250 meters, and on the geological basis of limestone. On the association *Quercetum farnetto-ceris scardicum*, 34 relevés

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have been carried out, containing 128 species, at sea level from 360 – 760 meters, on silicate geological basis (28 relevés) and of limestone (6 relevés). On the association *Quercetum montanum* 20 relevés have been noted, containing 105 species, at the sea level from 630 – 960 meters, on the silicate (19 relevés), and on limestone basis (1 relevé).

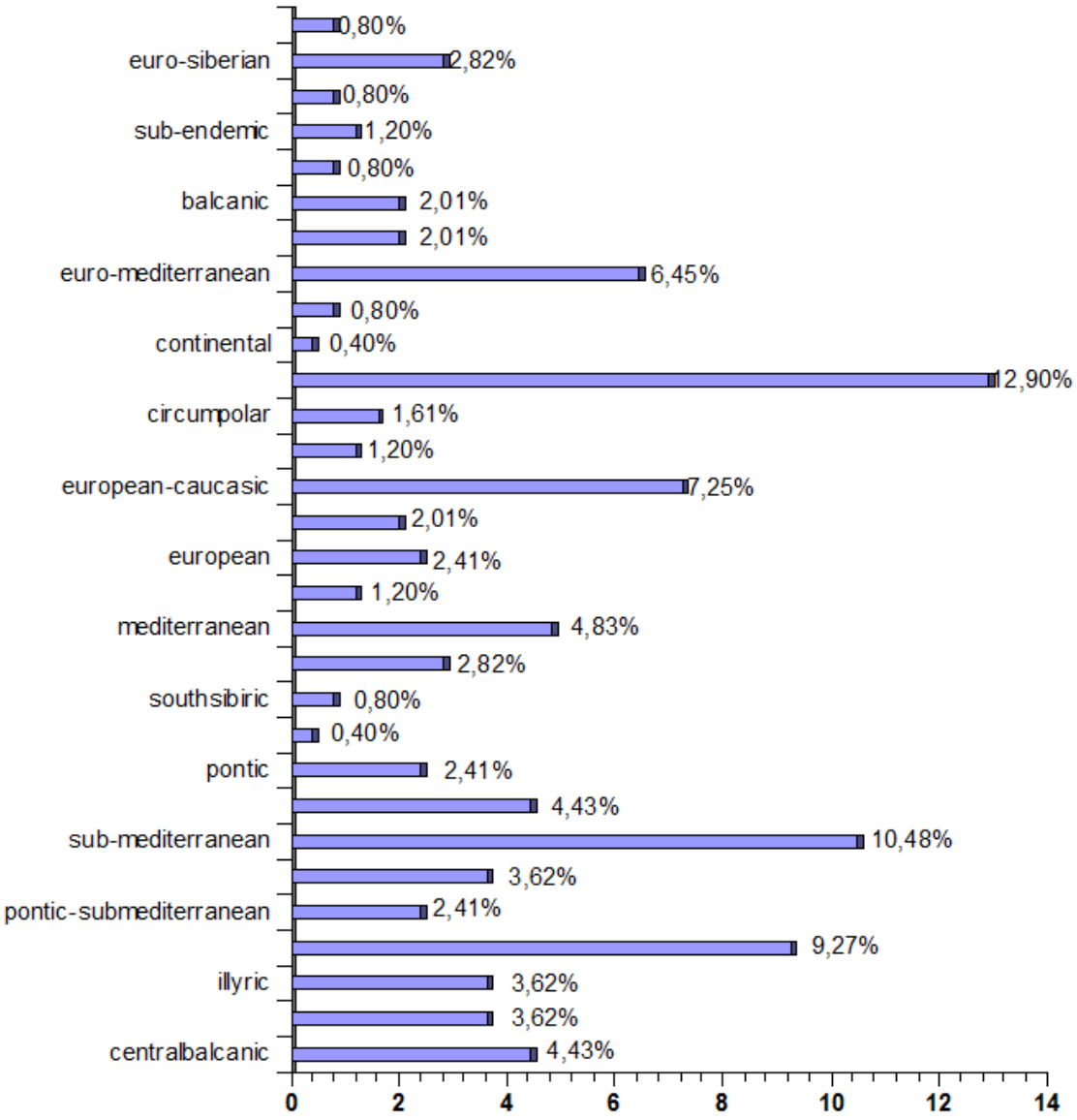

Fig: 2. Spectrum of floral geoelements

In order to shorten the table the species which are registered in one relevé are removed. The following species have been removed from the tree layer: Ph. *Fraxinus excelsior* I (+) of assoc. *Colurno-Ostryetum carpinifolie*; Ph. *Cornus mas* I (+ - 1), Ph. *Malus silvestris* I (+) of assoc. *Quercetum farnetto-cerris scardicum*; Ph. *Prunus avium* I (+) of assoc. *Quercetum montanum*.

The following species have been removed from the shrub layer: Ph. *Quercus macedonica* I (+ - 1), Ph. *Malus florentina* I (+), of assoc. *Dioscoro-Carpinetum orientalis*; Ph. *Evonymus latifolius* I (+) of assoc. *Colurno-Ostryetum carpinifolie*; Ph. *Juniperus oxycedrus* II (+ - 1) of assoc. *Seslerio-Ostryetum*; Ph. *Sorbus domestica* III (+), Ph. *Prunus spinosa* II (+ - 2), Ph. *Pirus amygdaliformis* I (+), Ph. *Ulmus campestris* I (+) of assoc. *Quercetum farnetto-cerris scardicum*; Ph. *Viburnum lantana* II (+ - 1), Ph. *Acer pseudoplatanus* II (+ - 1), Ph. *Staphylea pinnata* I (1 – 2) of assoc. *Quercetum montanum*.

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The following species have been removed from the layer of ground vegetation: H. *Galium purpureum* II (+ - 2), G. *Ornithogalum umbellatum* II (+ - 1), T. *Thlaspi perfoliatum* II (+ - 1), H. *Turritis glabra* I (+ - 1), T. *Bromus squarrosus* I (+ - 1), H. *Cynanchum huteri* I (+ - 1), H. *Polygala amara* I (+ - 2), H. *Waldsteinia geoides* I (+ - 2), T. *Sedum cepaea* I (+ - 2), H. *Hypericum barbatum* I (+), T. *Euphorbia falcata* I (+ - 1), T. *Filago germanica* I (+ - 1), T. *Veronica chamaedrifolia* I (+ - 1), H. *Convolvulus cantabricus* I (+), T. *Trifolium scabrum* I (1 – 2), Ch. *Teucrium polium* I (+ - 2), G. *Delphinium fissum* I (+ - 1), G. *Asphodeline liburnica* I (+ - 1), G. *Allium triquetrum* I (1 – 2), Ch. *Ruscus aculeatus* I (+) of assoc. *Carpinetum orientalis scardicum*; Ph. *Lonicera xylosteum* II (+), H. *Sanicula europaea* II (+ - 2), *Crocus sp*. II (+ - 2), G. *Anemone ranunculoides* I (1 – 1), H. *Hesperis matronalis* I (+ - 1) of assoc. *Dioscoro-Carpinetum orientalis*; H. *Tanacetum corymbosum* III (+), G. *Cystopteris fragilis* II (+), G. *Asphodelus albus* I (+), G. *Polystichum lobatum* I (+), G. *Asplenium adiantum-nigrum* III (+ - 2) of assoc. *Colurno-Ostryetum carpinifolie*; H. *Euphorbia agraria* II (+), H. *Luzula maxima* II (+ - 2), H. *Valeriana officinalis* II (+), H. *Calamintha grandiflora* I (1 – 1) of assoc. *Seslerio-Ostryetum*; H. *Galium silvaticum + schultessi* III (+ - 1), G. *Ranunculus millefoliatus* II (+ - 1), G. *Trifolium medium* II (+ - 2), *Thymus sp*. II (+ - 2), H. *Euphorbia cyparissias* II (+ - 1), T. *Vicia grandiflora* II (+), H. *Verbascum foeniceum* II (+), G. *Muscari botryoides* II (+), T. *Geranium robertianum* I (+ - 1), T. *Melampyrum cristatum* I (+), H. *Hypericum hirsutum* I (+), G. *Paeonia decora* I (+ - 2), H. *Campanula rapunculus* I (+ - 1), H. *Lathyrus silvestre* I (+), G. *Carex divulsa* I (+ - 2), H. *Lysimachia vulgaris* I (+), *Rubus sp*. I (+), H. *Saxifraga bulbifera* I (+), H. *Anthemis tinctoria* I (+ - 1), H. *Galium cruciata* I (+), H. *Vicia sparssiflora* (?) I (+ - 1), H. *Veronica officinalis* I (+ - 1) of assoc. *Quercetum farnetto-cerris scardicum*; *Galium sp*. IV (+ - 2), *Verbascum sp*. III (+ - 1), H. *Pulmonaria officinalis* III (+ - 1), *Cirsium sp*. III (+ - 2), G. *Cephalanthera alba* II (+ - 1), H. *Alliaria officinalis* II (+ - 2), H. *Hypericum hirsutum* II (+ - 1), G. *Asperula odorata* II (1 – 2), G. *Milium effusum* II (+ - 2), H. *Lathyrus pratensis* I (1 – 2), H. *Lamium luteum* I (+ - 1) of assoc. *Quercetum montanum*.

The biological spectrum of the communities has been presented in **Supplementary Table–2**, which shows that in all associations where the dominant group of life forms are hemicriptophytes (from 38.29% to 50.81%), then phanerophytes (20.50% - 28.87%), geophytes (17.21% - 27.83%), chamaephytes (1.03% - 9.38%), therophytes (0.94% - 7.81%) and nanophanerophytes (0.81% - 4.71%).

The spectrum of floral geoelements of 237 species confirms that the study area is located on the crossroads of highly diverse influences. This is clearly illustrated by a large number of floral geoelements and their combinations **[Figure– 2]**.

Below are presented the species of every floral geoelements group: **Central- Balcanic species**: *Acer hyrcanum, Coronilla elegans, Corylus colurna, Cotinus coggygria, Eryngium palmatum, Evonymus verrucosus, Lathyrus venetus, Mercurialis ovata, Paeonia decora, Tilia argentea, Waldsteinia geoides;* **Subatlantic-Mediterranean species**: *Acer monspesulanum, Anthericum ramosum, Asphodelus albus, Daphne laureola, Luzula forsteri, Physospermum aquilegifolium, Primula acaulis, Sedum cepaea, Tamus communis;* **Illyrian species**: Acer obtusatum, Aremonia agrimonoides, Epimedium alpinum, Erythronium dens-canis, Frangula rupestris, Genista ovata, Helleborus odoratus, Lonicera caprifolium, Oryzopsis virescens; **Central-European species**: *Abies alba, Acer pseudoplatanus, Anemone ranunculoides, Carex divulsa, Carpinus betulus, Dentaria bulbifera, Fagus moesiaca, Festuca heterophylla, Galium silvaticum, Genista saggitalis, Hedera helix, Luzula maxima, Melica uniflora, Melittis melissophyllum, Polygala amara, Prunus avium, Pulmonaria officinalis, Quercus conferta, Sedum maximum, Stellaria holostea, Thalictrum aquilegifolium, Tilia platyphyllos*; **Pontic-Sub-mediterranean species**: *Agropirum intermedium, Delphinium fissum, Dictamnus albus, Euphorbia falcata, Galanthus nivalis, Stachys germanica;* **Sub-Pontic species**: *Anthemis tinctoria, Astragalus glycyphyllos, Campanula persicifolia, Chrysanthemum corymbosum, Cynanchum vincetoxycum, Lathyrus niger, Melampyrum cristatum, Prunus spinosa, Trifolium alpestre;* **Sub-Mediterranean species**: *Arabis turrita, Aristolochia pallida, Asarum europaeum, Asparagus tenuifolius, Calamintha grandiflora, Campanula rapunculus, Carpinus orientalis, Ceterach officinarum, Convolvulus cantabricus, Cornus mas, Coronilla emeroides, Cotoneaster tomentosa, Euphorbia amygdaloides, Filago germanica, Fraxinus ornus, Galium purpureum, Lithospermum purpurocoeruleum, Lychnis coronaria, Ostrya carpinifolia, Quercus pubescens, Sedum glaucum, Sesleria autumnalis, Sorbus torminalis, Teucrium chamaedrys, Viburnum lantana*; **Sub-Boreal species**: *Fragaria vesca, Hypericum perforatum, Juniperus communis, Lonicera xylosteum, Luzula campestris, Melampyrum pretense, Milium effusum, Prunella vulgaris, Solidago virgaurea, Sorbus aucuparia, Veronica officinalis*; **Pontic species**: *Ajuga laxmani, Cytisus nigricans, Cytisus supinus, Lathyrus inermis, Linaria genistifolia, Verbascum phoeniceum*; **Boreal species**: *Geranium robertianum*; **South-Sibiric species**: *Lilium martagon, Polygonatum officinale*; **Pontic-Mediterranean species**: *Iris graminea, Muscari botryoides, Prunus mahaleb, Rhamnus catharcticus, Symphytum tuberosum, Thlaspi perfoliatum, Vicia grandiflora;* **Mediterranean species**: *Allium triquetrum, Amelanchier ovalis, Asparagus acutifolius, Asphodeline liburnica, Bromus squarrosus, Crataegus orientalis, Danaa cornubiensis, Evonymus latifolius, Juniperus oxycedrus,Pirus amygdaliformis, Pistacia terrebinthus, Teucrium polium;* **Westeuxynic species**: *Quercus cerris, Quercus farnetto, Quercus macedonica*; **European species**: *Hieratium murorum, Hypericum barbatum, Sorbus aria, Ulmus campestris, Valeriana officinalis, Vicia sparssiflora*; **Endemic-Balcanic species**: *Acanthus longifolius, Digitalis laevigata, Forsythia europaea, Moltkia doerfleri, Stachys scardica*; **European-**

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Caucasian species: *Acer campestre*, *Acer platanoides*, *Alliaria officinalis, Centaurea triumfetti, Crataegus monogyna, Doronicum collumnae, Elymus europaeus, Fraxinus excelsior, Geranium sanguineum, Helianthemum nummularium, Inula salicina, Lamium luteum, Lathyrus silvestris, Malus silvestris, Mercurialis perennis, Saxifraga rotundifolia, Stachys officinalis, Ulmus montana*; **Eastern-Submediterranean species**: *Anemone apenina*, *Cyclamen neapolitanum*, *Saxifraga bulbifera*; **Circumpolar species**: *Anemone hepatica*, *Anemone nemorosa*, *Arabis hirsuta*, *Turritis glabra*; **Euro-Asiatic species**: *Acer tataricum*, *Asperula odorata*, *Asplenium adiantum-nigrum, Brachypodium silvaticum, Calamintha officinalis, Cephalanthera rubra, Clematis flammula, Cornus sanguineus, Corylus avellana, Dactylis glomerata, Euphorbia cyparissias, Evonymus europeus, Galium aparine, Galium cruciata, Hesperis matronalis, Hypericum hirsutum, Lathyrus pratensis, Leucanthemum vulgare, Ligustrum vulgare, Pirus piraster, Polystichum lobatum, Primula verris, Rosa canina, Rosa spinosissima, Silene viridiflora, Silene vulgaris, Trifolium medium, Verbascum nigrum, Veronica hederifolia, Vicia cracca*; **Continental species**: *Asplenium trichomanes*; **Sub-Atlantic species**: *Campanula lingulata*, *Galium aristatum*; **Euro-Mediterranean species**: *Carex halleriana*, *Cephalanthera alba*, *Colutea arborescens*, *Geranium macrorhizum, Laburnum alpinum, Ornithogalum umbellatum, Potentilla micrantha, Ruscus aculeatus, Sanicula europaea, Scutellaria altissima, Siler trilobum, Smyrnium perfoliatum, Staphylea pinnata, Teucrium montanum, Thymus longicaulis, Trifolium scabrum*; **Circumboreal species**: *Clinopodium vulgare, Convallaria majalis*, *Epipactis palustris*, *Geum urbanum*, *Poa nemoralis*; **Balcanic species**: *Comandra elegans*, *Euphorbia agrarian*, *Peltaria alliecea*, *Ranunculus millefoliatus*, *Trifolium pignanti*; **Steno-Mediterranean species**: *Brachypodium distachyon*, *Convolvulus tenuissimus*; **Sub-Endemic species**: *Cynanchum huteri*, *Dioscorea balcanica*, *Malus florentina*; **Cosmopolitan species**: *Cystopteris fragilis*, *Polypodium vulgare*; **European-Siberian species**: *Campanula trachelium*, *Daphne mezereum*, *Lunaria rediviva*, *Platanthera bifolia*, *Serratula tinctoria*, *Veronica chamaedrys*, *Viola silvestris*; **Balcanic-Pontic species**: *Paeonia corallina*, *Veronica jacquini*.

[IV] DISCUSSION

The table also shows a high degree of participation of *Fraxinus ornus* in the tree layer, whereas amongst the shrub layer: *Cornus mas, Evonymus verrucosus, Sorbus torminalis, Pirus piraster and Quercus petraea*. On the layer of ground vegetation these species are present in all associations: *Veronica chamaedrys, Helleborus odorus, Lithospermum purpurocoeruleum, Fragaria vesca, Lathyrus venetus, Symphytum tuberosum, Stellaria holostea, Melica uniflora, Viola silvestris, Silene viridiflora, Stachys scardica, Lonicera caprifolium, Melitis melissophyllum, Primula acaulis, Brachypodium silvaticum, Polygonatum officinale* and *Dactylis glomerata.*

Some associations are differentiated into sub-associations. Hence, in the association *Carpinetum orientalis scardicum*, differential species of sub-association *Carpinetum orientalis scardicum seslerietosum* are: *Ostrya carpinifolia, Prunus mahaleb* and *Sesleria autumnalis*. Differential species of sub-association *Carpinetum orientalis scardicum quercetosum* are: *Quercus macedonica, Clematis flamula, Pistacia terebinthus, Carex halleriana, Aristolochia pallida* and *Asparagus acutifolius*, while the third sub-association *Carpinetum orientalis scardicum anemonetosum* characterized with these differential species: *Rhamnus catharctica*, *Viburnum lantana, Ligustrum vulgare, Acer campestre, Acer tataricum, Corylus avellana, Malus florentina, Carpinus betulus, Eryngium palmatum* and *Anemone apennina*. The association Colurno-*Ostryetum carpinifolie* is also differentiated into two sub-associations: *Colurno-Ostryetum carpinifolie discoretosum* with these differential species: *Dioscorea balcanica, Aristolochia pallida, Anemone nemorosa, Dentaria bulbifera*, and *Daphne laureola*, whereas the sub-asociation *Colurno-Ostryetum carpinifolie typicum* with species: *Corylus colurna* and *Sesleria autumnalis*. The association *Quercetum farnetto-cerris scardicum* is differentiated into two sub-associations: *Quercetum farnetto-cerris scardicum moltkietosum* with these differential species: *Moltkia doerfleri, Physospermum aquilegifolium, Inula salicina, Lychnis coronaria, Serratula tinctoria, Genista ovata* and *Trifolium pignantii*, whereas sub-association *Quercetum farnetto-cerris scardicum typicum* with species: *Eryngium palmatum, Cyclamen neapolitanum, Acanthus longifolius, Dictamnus albus, Coronila elegans* and *Comandra elegans*.

Association *Quercetum farnetto-cerris scardicum* presents the basic climazonal community of the lower zone of Kosovo. This is similar with the association *Quercetum farnetto-ceris serbicum* and association *Quercetum farnetto-cerris macedonicum*, which also belong to the *alliance Quercion farnetto* from the thermophyle order *Quercetalia pubescentis* [6]. This community has wide presence in Kosovo, with a higher concentration in eastern, central and southern parts of Kosovo. The comparisons which have been made between these three associations show clearer differences [1]. Therefore, in *Quercetum farnetto-cerris scardicum* the following species which are not met in associations *Quercetum farnetto-cerris serbicum* and *Quercetum farnetto-cerris macedonicum* have been determined: *Moltkia doerfleri, Eryngium palmatum, Siler trilobum, Coronila elegans, Dictamnus albus* and *Melampyrum cristatum*. On the other side, association *Quercetum farnetto-cerris scardicum* lacks the following species: *Crataegus orientalis, Helleborus cyclophyllus, Asphodelus albus, Hieratium bauchini* and *Stachys germanica*, which are present in the associations *Quercetum farnetto-cerris serbicum* and *Quercetum farnetto-cerris macedonicum* [1]. Due to the differences Krasniqi [1] differentiates this association into two sub-associations. One of them (*Quercetum farnetto-cerris scardicum moltkietosum*) is very particular, because amongst

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other things, it is unique due to its endemic species *Moltkia doerfleri*. This species grows mainly in the area of Lipovica massif (around 15 km southwest of Prishtina). It has a geological basis that is built in mainly from metamorphic and less limestone rocks. Because of this unique composition, Krasniqi [13] identifies in this material the association *Quercetum frainetto-cerris scardicum* as a particular geographical variant. The characteristic species of this association are as follows: *Quercus conferta, Quercus cerris, Fraxinus ornus, Sorbus torminalis, Pirus piraster, Physospermum aquilegifolium, Cytisus supinus, Silene viridiflora, Genista tinctoria, Iris graminea*, which have a high degree of presence.

The other communities of the alliance *Quercion farnetto* (*confertae*), such as association *Quercetum montanum* in Kosovo has also climazonal character, but with a limited presence. This community has been researched mainly in Fushë Kosovë (Gërmia, Lipovica, Carraleva, Mali Drenicë) **[Figure–1]**.

The communities of the alliance *Ostryo-Carpinion orientalis* in Kosovo present orographic and climacteric conditioned groupings. Such associations are as follows: *Carpinetum orientalis scardicum, Dioscoro-Carpinetum orientalis, Colurno-Ostryetum carpinifolie* and *Seslerio-Ostryetum*. The individuums of these associations have been researched mainly in the central parts of Kosovo and in the Dukadjin **[Figure–1]**.

[V] CONCLUSION

This study is a part of systematic synecological-vegetational research into the forest vegetation of the hilly area in Kosovo. The flora of 6 communities of the hilly region of Kosovo, which belongs to the thermophilic order *Quercetalia pubescentis,* among which, 140 relevés, with 262 species, registered in the analytical table of these associations have been analyzed: *Carpinetum orientalis scardicum*, *Dioscoro-Carpinetum orientalis*, *Colurno-Ostryetum carpinifolie*, *Seslerio-Ostryetum*, all of them of the alliance *Ostryo-Carpinion orientalis*, as well as the associations: *Quercetum farnetto-cerris scardicum* and *Quercetum montanum*, of the alliance *Quercion farnetto* (*confertae*). From the sinecological Table we can see that the species with a higher presence in most of the associations are: *Fraxinus ornus*, *Quercus pubescens*, *Quercus cerris*, *Carpinus orientalis*, *Ostrya carpinifolia*, *Quercus petraea*, *Tilia platyphyllos*, *Carpinus orientalis*, *Fagus moesiaca*, *Acer hyrcanum*, *Acer campestre*, *Acer obtusatum*, *Sorbus torminalis*, *Crataegus monogyna*, *Cornus mas*, *Evonymus verrucosus*, *Pirus piraster*, *Juniperus communis*,, all of them in the tree and shrub layer, whereas in the layer of ground vegetation are following species: *Veronica chamaedrys*, *Fragaria vesca*, *Lathyrus venetus*, *Melica uniflora*, *Dactylis glomerata*, *Stachys scardica*, *Lathyrus niger*, *Polygonatum officinale*, *Symphytum tuberosum* **[Supplementary Table–1]**.

The spectrum of life forms is dominated by hemicryptophytes (38.29% - 50.81%), then phanerophytes (20.50% - 28.87%) and geophytes (17.21% - 27.83%). Other life forms represented with a small number of species in these associations **[Supplementary Table–2]**. The association with a larger number of species is *Carpinetum orientalis scardicum* (130 species or 49.61%) followed by the associations *Quercetum farnetto-cerris scardicum* (128 species or 48.85%), *Dioscoro-Carpinetum orientalis* (112 species or 42.74%), *Quercetum montanum* (105 species or 40.07%), *Colurno-Ostryetum carpinifolie* (101 species or 38.54%) and *Seslerio-Ostryetum* (93 species or 35.49%) **[Figure–2]**.

From the analysis of floral geoelements of the 248 species, the highest number belongs to the following groups: Euro-Asiatic (32 species or 12.90%), Sub-Mediterranean (26 species or 10.48%), Central-European (23 species or 9.27%), European-Caucasic (18 species or 7.25%), Euro-Mediterranean (16 species or 6.45%), Mediterranean (12 species or 4.83%), Sub-Boreal (11 species or 4.43%) and Central-Balcanic (11 species or 4.43%). Other floral geoelements are represented with a small number of species (from 1 – 9) **[Figure–1]**.

The most widespread communities are the associations *Quercetu farnetto-cerris scardicum* and *Quercetum montanum*, which have a climazonal character, whereas the associations *Carpinetum orientalis scardicum*, *Dioscoro-Carpinetum orientalis*, *Colurno-Ostryetum carpinifolie* and *Seslerio-Ostryetum* have a limited presence and present orographic and climateric grouping **[Figure–1]**.

CONFLICT OF INTEREST

Author declares no conflict of interest.

FINANCIAL DISCLOSURE

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REVIEW ARTICLE OPEN ACCESS

IMPLICATIONS OF BACTERIAL RESISTANCE AGAINST HEAVY METALS IN BIOREMEDIATION: A REVIEW

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ABSTRACT

Intensification in industrialization, agricultural practices and various anthropogenic activities add a significant amount of metals in the soils. The continuous magnification in metal concentrations in soil ecosystem beyond the threshold limit leads to the deleterious effect on the microbial communities and their functional activities in soils. Some microorganisms under heavy metals stress may develop resistance against the elevated levels of these toxic metals and evolve various strategies to resist against the metal stress. Therefore, the metal resistant microorganisms including bacteria can be exploited as bioremediation agents. This review deals with bacteria resistance mechanisms against heavy metals (zinc and copper) comprehensively. In addition, biosorption and bioaccumulation processes with reference to the metal resistant bacteria are also explicitly described.

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Bioremediation; copper; heavy metal resistance; soil; zinc

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[I] INTRODUCTION

Environmental contamination owing to the anthropogenic activities and the natural resources is increasing progressively on account of an unabated increment in population, industrialization and urbanization [1, 2]. An enigma for the scientists is how to tackle the contaminants that jeopardize the environmental health. Advancement in science and technologies parallel to industrial revolution has enabled to exploit the inherent traits of natural resources to overcome the pollutants mediated environmental damage. An idyllic process for pollution abatement is 'bioremediation'. The term bioremediation has been introduced to describe the process of exploiting biological agents to eliminate toxic waste from environment. At present, bioremediation is the most effective management tool to manage the polluted environment and recover contaminated environment [3]. In other words, bioremediation is an alternative that offers the possibility to destroy or render harmless various contaminants including heavy metals using natural biological activity. As such, it uses relatively low-cost techniques which generally, have a high public acceptance and can often be carried out on site [4]. Compared to other methods, bioremediation is a more promising and less expensive way for cleaning up contaminated soil and water [3, 5]. Bioremediation uses biological agents, mainly microorganisms, e.g. yeast, fungi or bacteria to clean up contaminated soil and water [6, 7].

Most of the bioremediation systems are generally, operated under aerobic conditions. However, running a system under anaerobic environment may permit microbial organisms to degrade the most recalcitrant pollutants. The most essential parameters required for bioremediation are the nature of pollutants, soil structure, temperature, pH, moisture content, hydrogeology, the nutritional state, redox-potential, and microbial diversity of the site [8, 9]. In bioremediation processes, microorganisms use the contaminants as nutrient or energy sources [10, 11]. Bioremediation activity through microbe is stimulated by supplementing nutrients (nitrogen and phosphorus), electron acceptors (oxygen), and substrates (methane, phenol, and toluene), or by introducing microorganisms with desired catalytic capabilities [12, 13]. Plant and soil microbes develop a rhizospheric zone (highly complex symbiotic and synergistic relationships) which is also used as a tool for accelerating the rate of degradation or to remove contaminants [2, 14].

Generally, the higher concentration of these metals above threshold levels has deleterious impact on the functional activities of microbial communities in soils. Otherwise, microorganisms exposed to the higher concentrations of toxic heavy metals may develop resistance against the elevated levels of these metals [15]. In addition, microorganisms inhabiting in metal polluted soils have evolved various strategies to resist themselves against metal stress [16]. Such metal resistant microorganisms can be used as successful bioremediation agents [17]. This review highlights the general resistant mechanisms of microbes specifically bacteria against the selective heavy metals (zinc and copper) in detail.

[II] SOURCES OF HEAVY METAL CONTAMINATION IN SOILS

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Heavy metals such as lead, arsenic, cadmium, copper, zinc, nickel, and mercury are discharged from industrial operations such as smelting, mining, metal forging, manufacturing of alkaline storage batteries, and combustion of fossil fuel. Moreover, the agricultural activities like application of agrochemicals, and long-term usage of sewage sludge in agricultural fields also add a significant amount of metals in the soils [18, 19]. Various anthropogenic sources of metal contamination of soils have been shown in **Figure– 1**.

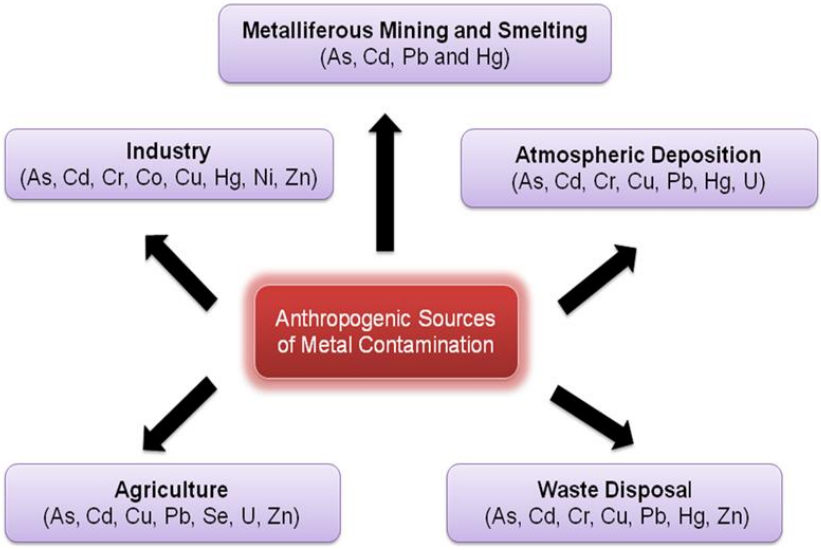

Fig: 1. Anthropogenic activities leading to the contamination of soils with heavy metals

[III] BIOAVAILABILITY OF METALS IN SOILS

Heavy metals exist both in bioavailable and non-bioavailable forms. Their mobility depends on two factors: (i) the metallic element that precipitates as positively charged ions (cations) and (ii) the one, which makes up negatively charged component of salt [20]. Physico-chemical properties of soils, such as cation exchange capacity (CEC), organic matter, clay minerals and hydrous metal oxides, pH and buffering capacity, redox potential and extent of aeration, water content and temperature, together with root exudates and microbial activities determines the metal availability in soils [17, 21]. The toxicity of metals within soils with high CEC is generally low even at high total metal concentrations. Under oxidized and aerobic conditions, metals are usually found in soluble cationic forms while in reduced or anaerobic conditions, as sulphide or carbonate precipitates. At low soil pH, the metal bioavailability increases due to its free ionic species, while at high soil pH it decreases due to insoluble metal mineral phosphate and carbonate formation. The mobility and bioavailability of certain metals in soils is usually in the order: Zn > Cu > Cd > Ni [17, 22]. However, the concentration of heavy metals within all components of the ecosystems varies considerably. Coexistence and persistence of metals in soils as multiple contaminants facilitate the entry and accumulation of these pollutants into food webs and ultimately into the human diets. Contamination of agricultural soils with heavy metals (both by single or combination of metals) has thus become a global threat to the sustainability of the agro-ecosystems and therefore, is receiving considerable attention from the environmentalists. Therefore, assessment of heavy metal bioavailability helps to evaluate the impact of metals on soil microbes and in predicting the application of bioremediation technologies that could be used to clean up metals from the polluted soils [17].

[IV] GENERAL MECHANISMS OF BACTERIAL RESISTANCE AGAINST HEAVY METAL STRESS

Accumulation of heavy metals in the soil environment and their uptake by plants is a matter of growing environmental concern. Unlike many other pollutants, which can undergo biodegradation and produce less toxic, less mobile and/or less bio-available products, heavy metals are difficult to be removed from contaminated environment [7]. These metals cannot be degraded biologically, and are ultimately indestructible, though the speciation and bioavailability of metals may change with variation in the environmental factors [23].

Some metals such as, zinc, copper, nickel and chromium are essential or beneficial micronutrients for plants, animals and microorganisms [24] while others (e.g., cadmium, mercury and lead) have no known biological and/or physiological functions [25]. However, the higher concentration of these metals has great effects on the microbial communities in soils in several ways- (1)

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it may lead to a reduction of total microbial biomass [18] (2) it decreases numbers of specific populations [26] or (3) it may change microbial community structure [27]. Thus, at high concentrations, metal ions can either completely inhibit the microbial population by inhibiting their various metabolic activities **[Figure– 2]** or organisms can develop resistance or tolerance to the elevated levels of metals. Generally, tolerance may be defined as the ability to cope with pollutant-toxicity by means of intrinsic properties of the microorganisms. In contrast, resistance is the ability of microbes to survive in higher concentrations of toxic substances by detoxification mechanisms, activated in direct response to the presence of the same pollutant [28]. Toxic heavy metals therefore, need to be either completely removed from the contaminated soil, transformed or to be immobilized, producing much less or non-toxic species. However, in order to survive and proliferate in metal contaminated soils, tolerance has to be present both in microbes and their associative hosts.

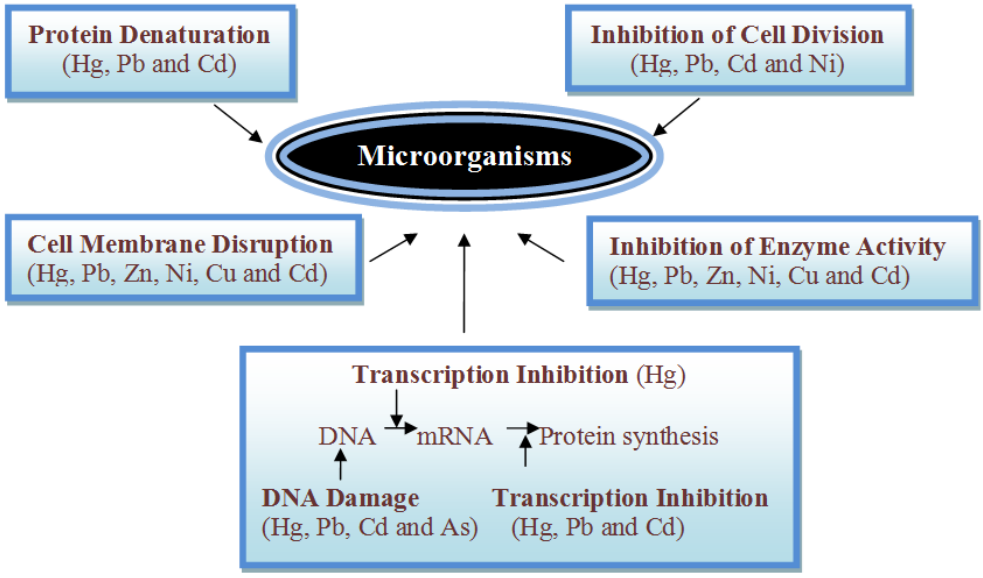

Fig: 2. Heavy metal-toxicity mechanisms to microbes [17]

For survival under metal-stressed environment, bacteria have evolved several mechanisms by which they can immobilize, mobilize or transform metals rendering them inactive to tolerate the uptake of heavy metal ions [29]. These mechanisms include (1) exclusion-the metal ions are kept away from the target sites (2) extrusion-the metals are pushed out of the cell through chromosomal/plasmid mediated events (3) accommodation-metals form complex with the metal binding proteins (e.g. metallothienins, a low molecular weight proteins) [30, 31] or other cell components (4) bio-transformation-toxic metal is reduced to less toxic forms and (5) methylation and demethylation. One or more of these defense mechanisms allows these microorganisms to function metabolically in environment polluted by metals. These mechanisms could be constitutive or inducible. The bacterial resistance mechanisms are encoded generally on plasmids and transposons, and it is probably by gene transfer or spontaneous mutation that bacteria acquire their resistance to heavy metals. For example, in Gram-negative bacteria (e.g. Ralstonia eutropha), the czc system is responsible for the resistance to cadmium, zinc and cobalt.

The czc-genes encode for a cation-proton antiporter (CzcABC), which exports these metals [32]. A similar mechanism, called *ncc* system, has been found in *Alcaligenes xylosoxidans* which provides resistance against nickel, cadmium and cobalt. In contrast, the cadmium resistance mechanism in Gram-positive bacteria (e.g. *Staphylococcus*, *Bacillus* or *Listeria*) is through Cd-efflux ATPase. Plasmid encoded energy dependent metal efflux systems involving ATPases and chemiosmotic ion/proton pumps are also reported for arsenic, chromium and cadmium resistance in other bacteria [33]. The exploitation of these bacterial properties for the remediation of heavy metal-contaminated sites has been shown to be a promising bioremediation option [34]. Though, the threshold limit of metal toxicity to soil microorganisms is not conclusive, yet the interaction between heavy metals and microbes do occur in nature. Microorganisms can interact with metals via many mechanisms **[Figure– 3]**, some of which may be used as the basis of potential bioremediation strategies.

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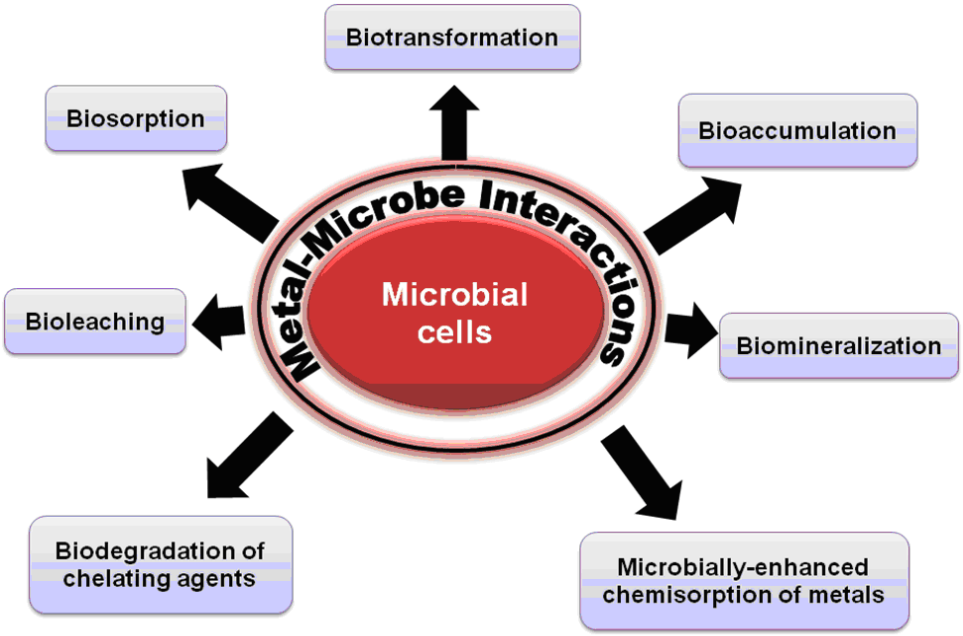

Fig. 3: Metal-microbe interactions affecting bioremediation

[V] BIOSENSORS

Metal resistance trait in microorganisms is regulated by genes which are organized in operons. Generally, the genes for heavy metal resistance are found on extrachromosomal circular DNA i.e. plasmid carried by metal resistant bacteria. The heavy resistance genes are expressed and induced in the presence of specific metals in the vicinity of bacterial niche. Since the regulation of the metal resistant gene expression is specific for each heavy metal and is dependent upon metal species concentration, the promoters and regulatory genes from the bacterial operons responsible for resistance attribute can be used to create metal-specific biosensors (promoter-reporter gene fusions). The metal specific bacterial sensors coupled with tools for chemical analyses can be used to differentiate the bioavailable metal concentration from the total metal concentration of the samples [35].

Various metal-specific sensor strains have been developed and applied in many laboratories. These sensor strains are all based on the same concept: a metal responsive regulation unit regulates the expression of a sensitive reporter gene. Reporter genes include those that code for bioluminescent proteins, such as bacterial luciferase (*luxAB*) and firefly luciferase (*lucFF*) or for β-galactosidase, which can be detected electrochemically or by using chemiluminescent substrates. The light produced can be measured by a variety of instruments, including luminometers, photometers and liquid-scintillation counters [35].

[VI] METAL RESISTANCE MECHANISMS

When the bacterial cells are exposed to the high concentrations of heavy metals, the metals react within cells with various metabolites and form toxic compounds [29, 36]. It is well known that some of heavy metals are essential for functioning of cellular enzymes and for the bacterial growth and metabolism, therefore, mechanisms for uptake of these metal species is present in the bacterial cell through which heavy metals enter the cell. Generally, there are two types of uptake mechanisms for heavy metals: one of them is quick and unspecific which is driven by a chemiosmotic gradient across the cell membrane and consequently, does not require ATP. Although, this mechanism is an energy efficient process, it is responsible for the influx of a number of heavy metals. Obviously, when these metals are present at high concentrations exterior to the bacterial cell, they are more likely to produce noxious impact on bacterial growth metabolisms if entered the cell cytoplasm. In contrast, the second process of metal uptake is comparatively, slower and more substrate-specific and is dependent upon the energy released from ATP hydrolysis [32, 36]. Moreover, most of the bacterial genera have evolved numerous mechanisms to tolerate the uptake of heavy metal ions and to protect their cell homeostasis against the heavy metal induced damage to survive in heavy metal stress. These mechanisms for instance, are the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state [29]. As typical examples, bacterial resistance mechanisms against copper and zinc are discussed as follows:

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6.1. Bacterial resistance against copper

Heavy metal, copper is utilized by bacterial cells in small quantities in biosynthesis of metabolic enzymes like, cytochrome c oxidase. However, bacteria in different ecosystems including soil and water, are exposed to very high concentration of this metal as high levels of copper exists in soil ecosystem due to its wide application in mining, industry processes, and agricultural practices [37]. Consequently, bacteria have evolved several types of mechanisms to defend against the high copper concentration and copper induced biotoxicity [36].

Regarding the prevalence of copper resistance in bacteria, Lin and Olson [38] isolated copper resistant bacteria from a copper corroded water distribution system and studied resistance pattern against copper. They observed that 62% of the total isolates exhibited substantial resistance against copper. Among these resistant bacteria, 49% isolates had *cop* or *cop*-like gene systems as well as both compartmentalization and efflux systems [39]. In other study, Cooksey [40] reported that resistance against copper in the plant pathogen *Pseudomonas syringae* was because of the copper accumulation and compartmentalization in the cell's periplasm and the outer membrane and concluded that the protective mechanism against copper in *P. syringae* was due to four types of proteins (CopA, CopB, CopC and CopD). These proteins are encoded by the *cop* operon present on bacterial plasmid and proteins are found in the periplasm (CopA and CopC), the outer membrane (CopB), and the inner membrane and work together to compartmentalize copper away from bacterial cells [36].

In contrast, copper resistance in *E. coli* is dependent upon efflux mechanism to overcome copper stress. The efflux proteins are expressed by plasmid-borne *pco* genes, which are in turn rely upon the expression of chromosomal *cut* genes [39]. Moreover, two *cut* genes (*cutC* and *cutF*) encode a copper binding protein and an outer membrane lipoprotein [41]. Most bacterial species in the metal stressed environment have acquired at least one of the abovementioned protective mechanisms. In addition, the evolution of the bacterial copper resistance occurred through the modification of copper uptake genes found on chromosomes [36].

6.2. Bacterial resistance against zinc

Zinc, an essential trace element is not biologically redox reactive. Hence, it is not used in cellular metabolisms like respiration. However, it is structurally, a vital constituent of several cellular enzymes. Furthermore, it also forms complexes in cells for instance, zinc fingers in DNA [29, 36]. In addition, zinc actually, displays comparatively less toxicity to bacterial cells than other heavy metals and it is generally occurs in higher concentrations within bacterial cells. That is why bacteria in heavy metal polluted environment accumulate zinc by a fast but unspecific uptake mechanism [29]. Generally, uptake of zinc ions by bacterial cells is coupled with magnesium, and both ions may be transported by similar mechanism [32, 36].

The zinc resistance in bacteria is achieved through the two general efflux mechanisms: (i) mediated by a P-type ATPase efflux system and (ii) mediated by an RND-driven transporter system [36]. As a matter of fact, a P-type ATPase catalyzes the reactions by ATP hydrolysis forming a phosphorylated intermediate [32] whereas, the term RND belongs to a family of proteins involved in the heavy metal transport [29]. The P-type ATPase efflux system transports zinc ions across the cytoplasmic membrane by the energy released from ATP hydrolysis. In this regard, Beard et al. [42] isolated a chromosomal gene, *zntA*, from *E. coli* K-12 and inferred that the gene *zntA* might be accountable for the zinc and other cations transporting ATPase across cell membranes. In contrast to P-type ATPase efflux system, the RND-driven transporter system does not derive energy through ATP hydrolysis to transport zinc within the bacterial cells. As an alternative, it is powered by the proton gradient across the cell wall specifically, in gram-negative bacteria [29, 36].

[VII] BIOSORPTION AND BIOACCUMULATION OF HEAVY METALS

Fundamentally, biosorption of heavy metals by bacterial cells is based on non-enzymatic processes such as, adsorption. Adsorption is characterized by the non-specific binding of metal ions to extracellular/ cell surface associated polysaccharides and proteins [43, 23]. In a nut shell, biosorption is defined as an attribute of the inactive or dead microbial biomass to bind and concentrate heavy metals even from highly dilute solutions [44].

The metal uptake by the microbial biosrobent may be an active or passive process or exhibit both active and passive processes depending upon the microbial species. Moreover, passive uptake is a rapid and reversible process and is independent of cellular metabolisms, physical conditions such as pH and ionic strength. However, the passive process is relatively nonspecific with respect to the metal species. Conversely, the active process is comparatively slow method and depends on the cellular metabolism. In this process, heavy metals form complexes with specific proteins like metallothionins. However, it is affected by metabolic inhibitors, uncouplers and temperature. Both the active and passive mode may occur simultaneously [45].

Generally, many microbial species with high cell wall chitin contents act as an effective biosorbent in addition to the chitosan and glucans. Furthermore, the walls of fungi, yeasts, and algae, are also efficient metal biosorbents. Moreover, the cell walls of the Gram-positive bacteria attach higher concentrations of metals than that of the Gram-negative bacteria [23]. Therefore, bacteria, waste fungal biomass derived from several industrial fermentations are considered the cost-effective and efficient sources of biosorptive materials. These biosorbents loaded with

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charged metal species may be regenerated with the treatment of acid or some chelating agents [23].

Bioaccumulation is an active process dependent upon metabolic energy of microorganisms. In other words, bioaccumulation is an energy-dependent heavy metal transport system [46]. Besides, potential bioaccumulation mechanisms of heavy metal influx across the bacterial membranes include ion pumps, ion channels, carrier mediated transport, endocytosis, complex permeation, and lipid permeation. This active mechanism has been reported to be associated with the transport of heavy metals like, mercury, lead, silver, cadmium and nickel. Assessment of heavy metal accumulation in the microbial cells can be done by transmission electron microscopy (TEM). In a study, TEM analysis of *P. putida* 62BN demonstrated intracellular and periplasmic accumulation of cadmium [23]. Similarly, heavy metal transport through bioaccumulation has been reported in many bacterial genera like, *Citrobacter sp.* (lead and cadmium), *Thiobacillus ferrooxidans* (silver), *Bacillus cereus* (cadmium), *Bacillus subtilis* (chromium), *Pseudomonas aeruginosa* (uranium) *Micrococcus luteus* (strontium) *Rhizopus arrhizus* (mercury), *Aspergillus niger* (thorium), *Saccharomyces cerevisiae* (uranium) [23, 47].

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A NORMAL 46 XX KARYOTYPE DOES NOT ALWAYS REPRESENT FEMALE PHENOTYPE

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[I] KARYOTYPE

A karyotype generally refers to the number and the appearance of metaphase chromosomes in a diploid cell. Generally, G-banding (Giemsa) is used to stain the chromosomes for karyotyping. A Karyotype analysis is done to investigate chromosomal aberrations to diagnose various blood cancers where in general translocation occurs and syndromes such as Down syndrome, Turner syndrome among others where the number of chromosome varies. Therefore, karyotype analysis has great importance in prenatal diagnosis and medical genetics. Another important aspect of karyotype analysis is underlying in sex determination. The normal human male karyotype shows 46 XY and female 46 XX. However, the genotype-phenotype does not match always and there are phenotypically males with 46 XX and females with 46 XY. Such instances are very rare. The G-banding generally fails to correlate such genotype-phenotype correlation.

1.1 46 XX males

Males with 46 XX karyotype are referred as XX male syndrome or de la Chapelle syndrome. These individual are phenotypically male but are genetically female and the phenotype is clinically termed as XX male syndrome. The incidences are very rare (4 in 1 lakh). Individuals exhibit feminine characteristics, develop small testes, develop gynecomastia, without Müllerian tissue (that develop fallopian tubes, uterus, cervix, vagina etc in female), and are sterile. The SRY (Sex determining Region Y) on Y chromosome is responsible for male sex determination in an individual. In XX male syndrome, the X chromosome contains the SRY gene due to unequal crossing over between the X and Y chromosomes in the father during the meiosis. The abnormal X chromosome containing the SYR gene when transmitted to an individual with a normal X chromosome, it gives rise to 46, XX karyotype (female karyotype); however, due to presence of the SYR gene, the individual is phenotypically male [1].

1.2. 46 XY female

Similarly, an individual having 46 XY does not always represent male characters; instead it may represent XY complete gonadal dysgenesis (CGD) or Swyer syndrome. Although, in these genetically male fetuses, the Y chromosome is present; gonads do not differentiate into active testes due to loss of function mutation in SYR gene. In such case the gonads represent as streak gonads without having ovarian or testicular tissue. Testosterone or antimüllerian hormone (AMH) does not produce due to lack of testis and therefore male sexual organs do not develop. Instead it develops female sexual organs such as uterus, fallopian tubes, cervix, and vagina from Müllerian ducts. Such individuals are phenotypically female at infant stage and fail to develop secondary sex characteristics such as breast development and menstrual periods etc. in the later age. The diagnosis is difficult at early age; however a delay in puberty, 46 XY karyotype, presence of a

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pubic hair and uterus but no ovaries, and absence of breasts along with female sexual organs confirm Swyer syndrome [2].

[II] TESTS

The conventional karyotyping using G-banding is not always helpful in diagnosing these disorders. Fluorescence in situ Hybridization (FISH) based karyotypying using SRY gene probes and additional gene tests are recommended. The following tests can be used to determine 46 XX male and 46 XY female genotypes.

2.1. Tests for 46 XX males

Clinical diagnosis: 46 XX karyotype, male external genitalia, two testicles, absence of germ cells in testis, azoospermia, no Müllerian structures. **Hormone tests:** Elevation of serum LH and FSH, decreased or lack of serum testosterone. **Cytology:** Conventional karyotyping. However, sensitivities of these tests are low and may not conclude genotype-phenotype correlation. Therefore, **molecular genetic tests** are required. Such tests are- Karyotyping using FISH method: for presence of SRY gene in X chromosome. **Gene tests** for SRY gene (80% cases) using PCR amplification for presence of SYR gene are recommended [1].

2.2 Tests for 46 XY females

Clinical diagnosis: 46 XY karyotype, normal female external genitalia, streak gonads, no sperm production, presence of Müllerian structures. **Hormone tests:** Elevation of serum LH and FSH, decreased or lack of serum testosterone. **Diagnostic imaging:** USG, MRI, laparoscopy etc. shows uterus but no ovaries. However, the sensitivities of these tests are low. Therefore, karyotyping using **FISH method** for deletion of SRY gene and additional genetic tests are advised. Such **gene tests** are- SRY gene (15% cases) -FISH or deletion /duplication analysis; **DNA Sequencing:** DHH gene (50% cases)-sequencing for heterozygous mutations; NR5A1 (SF1), NR0B1 (DAX1), and WNT4 (rare), FISH analysis for deletion/duplication analysis of these genes [2].

[III] CONCLUSION

Conventional G-banding based karyotype sometimes failed to explain genotype-phenotype correlation in respect to 46 XX male and 46 XY female. Therefore, additional gene test are recommended to precisely characterize the sex of an individual while the phenotype of an individual varies from its karyotype.

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