

INFLUENCE OF pH ON GROWTH AND NITROGEN FIXATION IN BACTERIAL STRAINS ISOLATED FROM ALTITUDINAL VEGETATION ZONES OF PARÂNG MOUNTAINS (ROMANIA)

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Abstract. The aim of present paper was to study the influence of different pH values on activity of nitrogen fixing strains isolated from five altitudinal vegetation zones of Parâng Massif (Central Romania). The effect of varying the pH on growth and development of *Azotobacter* strains as well as on the products of molecular nitrogen fixation was surveyed. The strains were cultivated on media with mannitol or sucrose at 35°C and continuous shaking at 150 rpm. The pH value for optimal growth of the *Azotobacter* strains isolated from mountain soils is around neutral pH and cell growth diminished at a slightly alkaline (pH=8) and an acid pH (pH=4). The molecular nitrogen fixation capacity by strains coming from mountain soils at the chosen pH values was determined indirectly, by extracellular proteins formation and ammonia secretion in culture media. The maximum value of extracellular proteins was obtained at the strains coming from the flood plain at pH 8 (21.452 mg/l). The extracellular proteins concentration on the studied media followed parallel and close lines which had a growing trend until the end of the studied interval. The ammonia secretion at each mountain zone was different on the two culture media taken into consideration. The level of the ammonia secretion attained a maximum of 6.02 mg/l at the strains from the beech zone at pH 8, on sucrose medium.

Keywords: *Azotobacter*, pH, nitrogen fixation, altitudinal vegetation zones

INTRODUCTION

Nitrogen fixation can be considered as one of the most interesting microbial activity as it makes the recycling of nitrogen on earth possible and gives a fundamental contribution to nitrogen homeostasis in the biosphere. Among the nitrogen cycle, biological nitrogen fixation takes the role of biological conversion of atmospheric dinitrogen to forms available for plant and microbial growth by a variety of prokaryotic microbes [12]. The bacteria belonging to genus *Azotobacter* play a remarkable role, being broadly dispersed in different environments, such as soil, water and sediments [1]. The ecological distribution of *Azotobacter* species is complicated and diverse environmental factors can determine their presence or absence in soil [5]. Soil populations of *Azotobacter* sp. rarely exceed several thousand cells per gram of neutral or alkaline soils, and in acid (pH < 5.0) soils these bacteria are generally absent or occur in very low numbers [3]. It is proven that environmental conditions and also the organic matter content, pH and the soil characteristics, in general, affect the distribution of these microorganisms [15]. The number and activity of nitrogen fixing microorganisms depend on the vegetation composition, soil type, micro- and macroclimate features of each sampling place [11, 14].

In order to avoid pH effect on the growth of nitrogen fixing strains and on nitrogen fixation products the pH of the culture medium was adjusted to 4, 5, 6, 7 and 8. Because the ingredient salts were added after sterilisation and the addition of salts lowers the pH, it was brought to the needed value after sterilisation using sterile NaOH 2 N. The pH interval from 5 to 8 studied in this experiment was selected in order to obtain the optimum growth pH for *A. chroococcum*, being known from the literature the fact that these species prefer a pH of 7-7.5 [8]. The behavior of *A. chroococcum* in an acid environment (pH = 4) was

also studied because pH 4 represents a value close to the one determined at the soil from coniferous zone.

MATERIALS AND METHODS

The strains used in this study were obtained from soils of Parâng Massif. In order to evaluate pH effect on the growth and formation of nitrogen fixing products in nitrogen-fixing strains, the pH of culture media were adjusted to 4, 5, 6, 7 and 8, respectively. Two different carbon sources were used mannitol in the „mannitol medium” (after Burk modified) and sucrose in the „sucrose medium” [2]. The strains were grown at 35°C, under continuous shaking at 150 rpm. The growth of microbial strains was followed as change in optical density at 600 nm.

The nitrogen fixing capacity of the studied strains was quantified indirectly by measuring the products of N-fixing activity: the extracellular proteins and the concentration of produced ammonia.

The extracellular proteins were measured by modified Lowry method [9]. The samples were taken from the culture medium and centrifuged at 15000 rpm for 15 minutes at 4°C and the supernatant was further analyzed by Lowry method. The absorbance of samples was read at 650 nm.

The ammonia concentration was determined colorimetrically with Nessler reagent (Nesslerization method) [6]. Absorbance of the samples was measured at 400 nm.

The statistical interpretation was realized based on the One-Way ANOVA (analysis of variance) test using a free version of GraphPad Prism 4 software. By applying the statistical analysis tests One-Way ANOVA and Bonferroni post-test the statistical significance for each altitudinal vegetation zone and each culture medium studied was established. One-Way ANOVA test was used for a general comparison of different samples. A value of $p < 0.05$ was considered statistically significant [13]. In order to compare the

samples from diverse pH intervals the Bonferroni post-test was applied.

RESULTS

The strains were grown at the mentioned pH values, at 35 °C temperature and continuous agitation to 150 rpm. The carbon sources used were mannitol and sucrose. In these conditions the strains attained

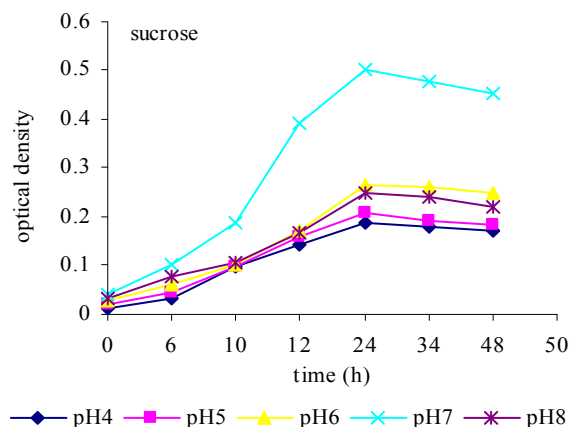
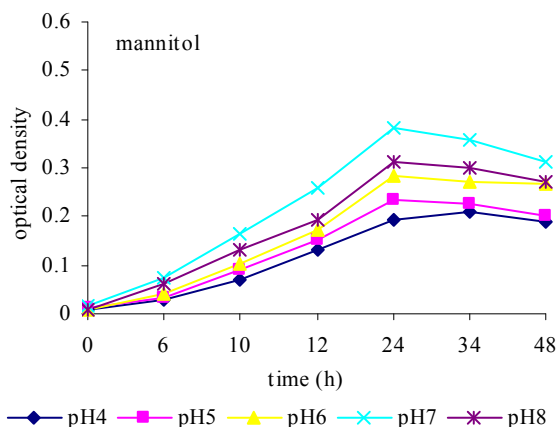


Figure 1. Effect of pH on growth of nitrogen fixing consortium isolated from alpine zone.

Surprisingly, growth of these strains was observed at pH 4, although the soil from the alpine zone does not attain a so strong acidity (pH = 4.49). Moreover, the growth measured as units of optical density was similar in the media with pH 4, 5, 6 and 8.

The strains from the subalpine zone, such as *Azotobacter* strains, are mostly neutrophilic and attain

the maximum/optimal growth on sucrose, to which they are familiarised due to the big amount of fruits specific to this zone (Fig. 2). There's also a growth at pH 4, because also in this zone the main species which edify plant associations are the coniferous *Juniperus communis* and *Pinus mugo*. These coniferous shrubs generate high acidity of soils.

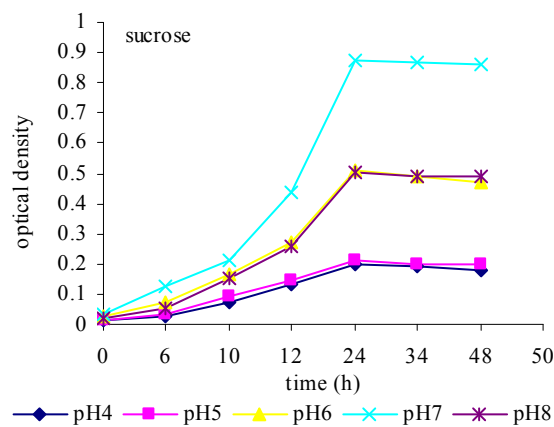
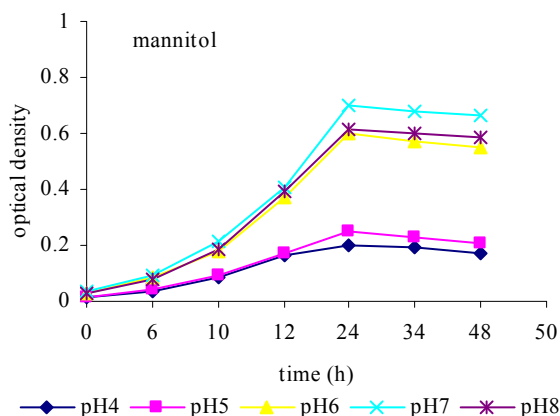


Figure 2. Effect of pH on growth of nitrogen fixing consortium isolated from subalpine zone.

In the coniferous zone it was observed that although the maximum is also attained at pH 7 after 24 hours of incubation, the values obtained at this pH are significantly lower than in other zones (Fig. 3). At all the chosen pH values the growth was close.

Because the strains from this zone had to survive in the soil with acid pH, which is specific for the soils where coniferous trees grow, these seem to have a lower preference for neutral pH. At pH 4, the closest value to the one of the natural environment from which the samples were taken, the maximum growth was obtained on mannitol.

In the beech zone it was observed that at pH 4 and pH 5 the growth is similar to the other zones but at neutral pH, especially on sucrose, growth is considerably higher (Fig. 4). This suggests that here there are both strains coming from the lower deciduous forests, which are not adapted at acid pH, and strains coming from the immediate higher zone, adapted to acidity.

At the samples from Maleia flood plain (Fig. 5) the same dichotomy as in the beech zone results. Also, here there are both strains specific to the deciduous forest and strains coming from the soil of coniferous

zone. The latter also develop at acid pH and have arrived here probably as cysts carried by the water.

The influence of pH on the level of extracellular proteins. In the figures 6-10 a growing tendency is observed at the extracellular protein levels during incubation period at all pH values chosen (4, 5, 6, 7

and 8). In the incubation period even after 48 hours when the cultures are in the stationary phase the extracellular protein levels appear to be increasing.

In the alpine zone the maximum protein synthesis was observed at neutral pH and on sucrose as C-source. At the other pH values, the protein quantities are close.

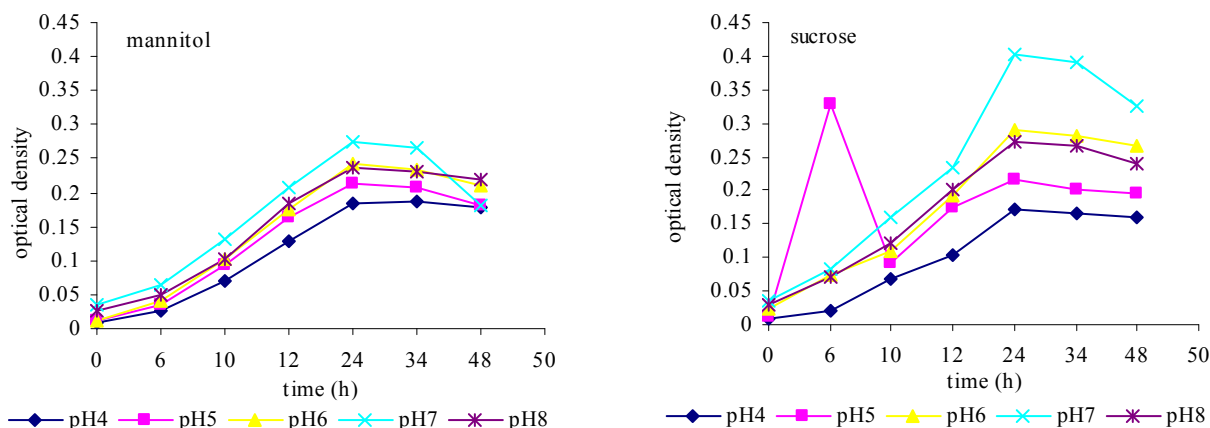


Figure 3. Effect of pH on growth of nitrogen fixing consortium isolated from coniferous zone.

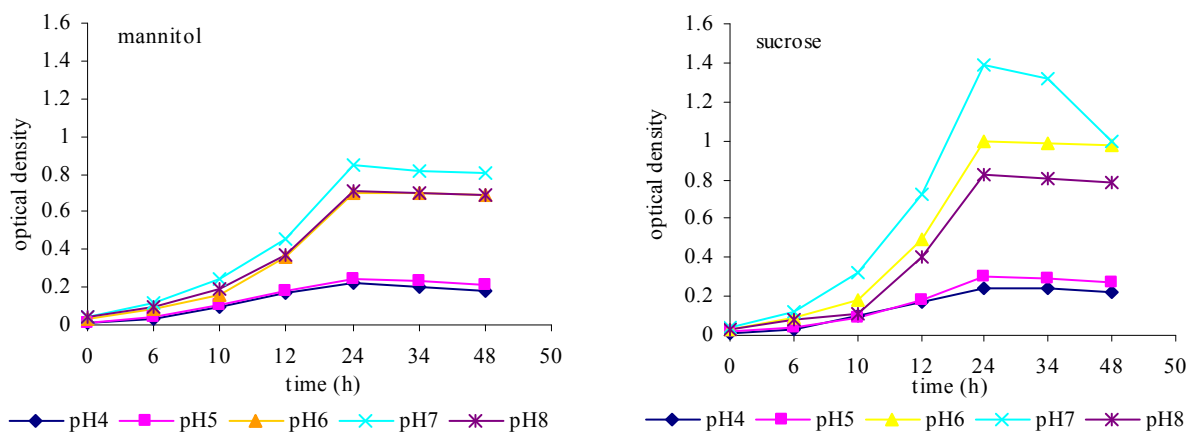


Figure 4. Effect of pH on growth of nitrogen fixing consortium isolated from beech zone.

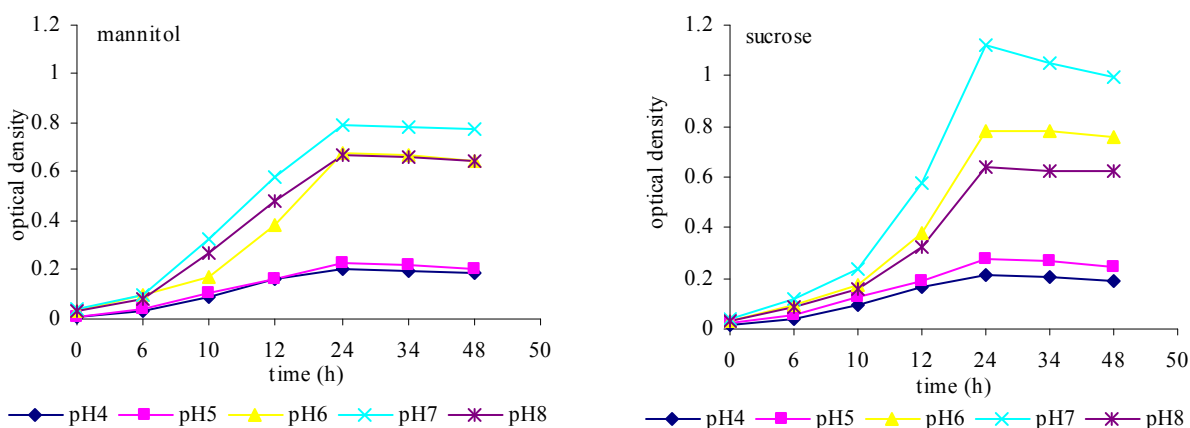


Figure 5. Effect of pH on growth of nitrogen fixing consortium isolated from Maleia flood plain.

The extracellular protein concentration on mannitol medium follows parallel and close tracks in all studied strains, with an increasing trend at the end of the studied interval (Fig. 6). The last two observations indicate the capacity of adaptation to the carbon source

and to different pH values that the strains from this harsh environment have.

Parallel growths are not visible in the subalpine zone. Although capable to develop and synthesize extracellular proteins at acidic pH values, typical for

the soil found at this altitudinal vegetation zone, the strains prefer neutral pH and sucrose (Fig. 7).

The lowest extracellular protein concentration was obtained at the strains originating from the coniferous

zone (Fig. 8). On mannitol medium, a similar protein secretion activity was observed at all studied pH because the strains from here had to adapt to a very acidic pH and to mannitol substrate.

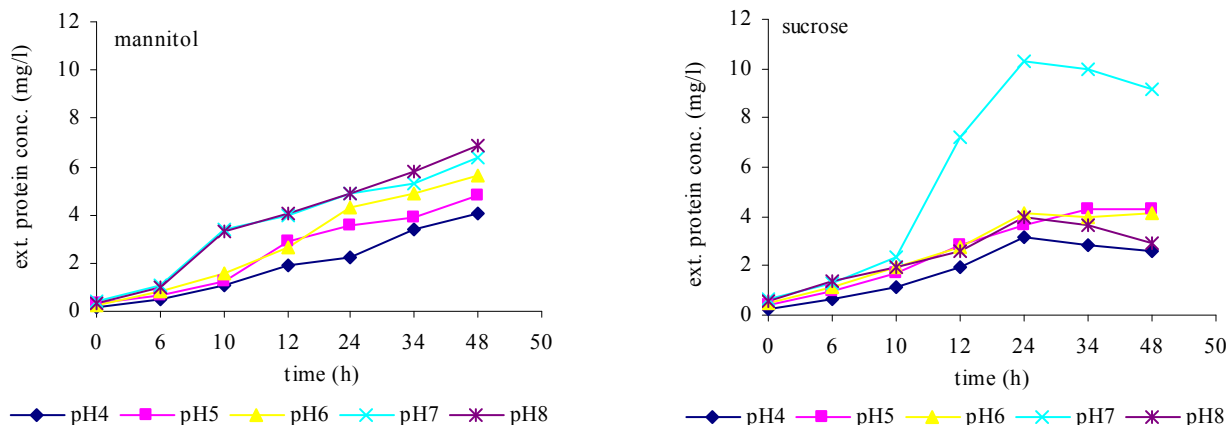


Figure 6. Effect of pH on extracellular protein levels at strains isolated from alpine zone.

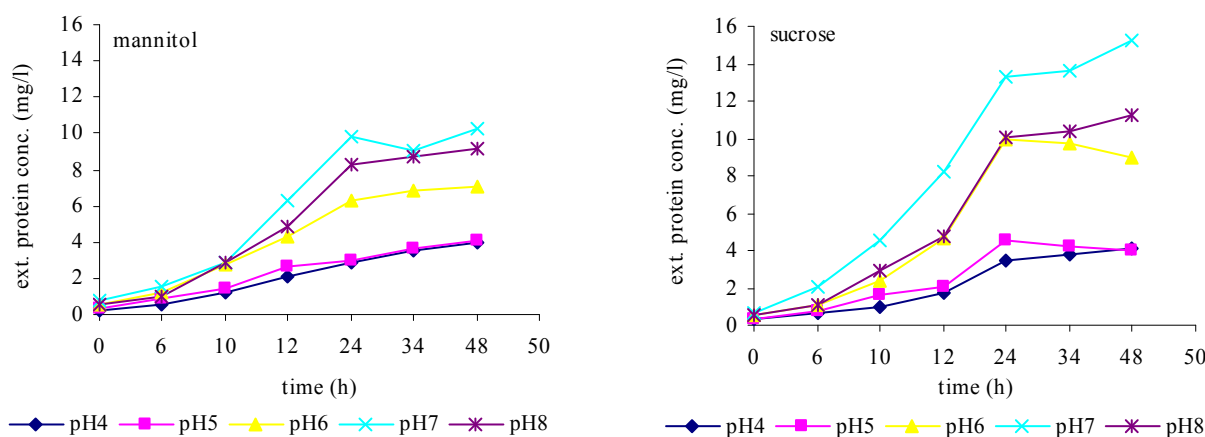


Figure 7. Effect of pH on extracellular protein levels at strains isolated from subalpine zone.

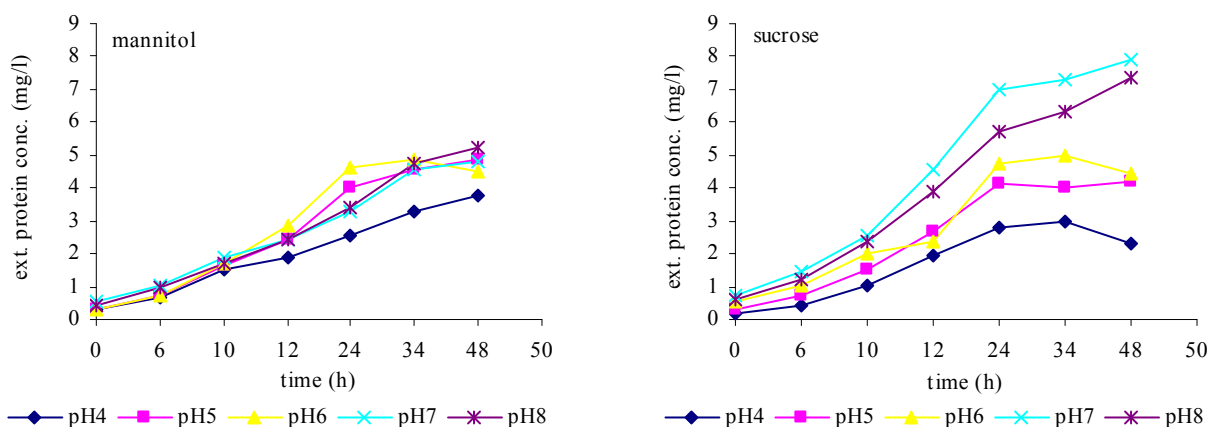


Figure 8. Effect of pH on extracellular protein levels at strains isolated from coniferous zone.

At the strains isolated from the beech zone, the secretion of protein is two times higher when grown on sucrose as compared to those grown on mannitol (Fig. 9).

The maximum value of the extracellular proteins was obtained at the strains isolated from the flood plain and attained 21.452 mg/l at pH 8 (Fig. 10). This maximum value recorded is due to the high microbiota

density from Maleia flood plain.

On mannitol medium, the maximum extracellular protein concentrations obtained were 4.042 mg/l at pH 4 at the alpine zone (Fig. 6) 4.893 mg/l at pH 5 at the coniferous zone and at pH 6, 7 and 8 the values were 8.022 mg/l, 12.234 mg/l and 10.328 mg/l, respectively, and were obtained at the beech zone (Fig. 9).

On sucrose medium, the maximum obtained

concentrations of the extracellular proteins were 4.912 mg/l at pH 4 at the beech zone (Fig. 9), 6.102 mg/l la pH 5 at the flood plain samples (Fig. 10), and at pH 6 and 7 the extracellular protein values were, respectively, 14.923 mg/l and 21.324 mg/l at the beech zone (Fig. 9).

The influence of pH on ammonia secretion. Considering the pH effect on ammonia secretion, a maximum secretion value of 6.02 mg NH_3/l was

observed at the flood plain strains, when grown at pH 8 on sucrose medium.

At the strains from the alpine zone, the ammonia secretion attains a maximum of 3.102 mg/l at pH 8 on sucrose medium. On the mannitol medium, the maximum concentration (1.822 mg/l) was recorded at pH 7, after 24 hours of incubation, afterwards decreasing.

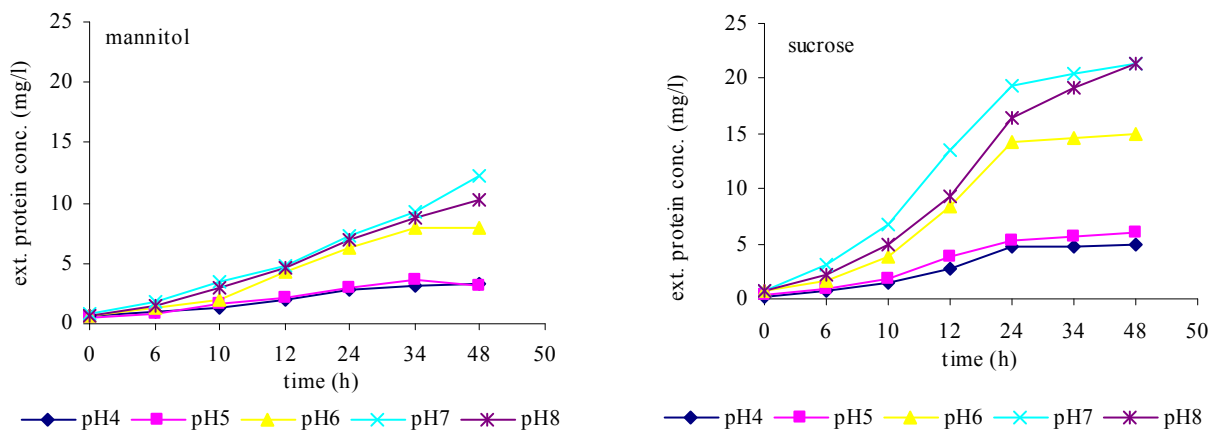


Figure 9. Effect of pH on extracellular protein levels at strains isolated from beech zone.

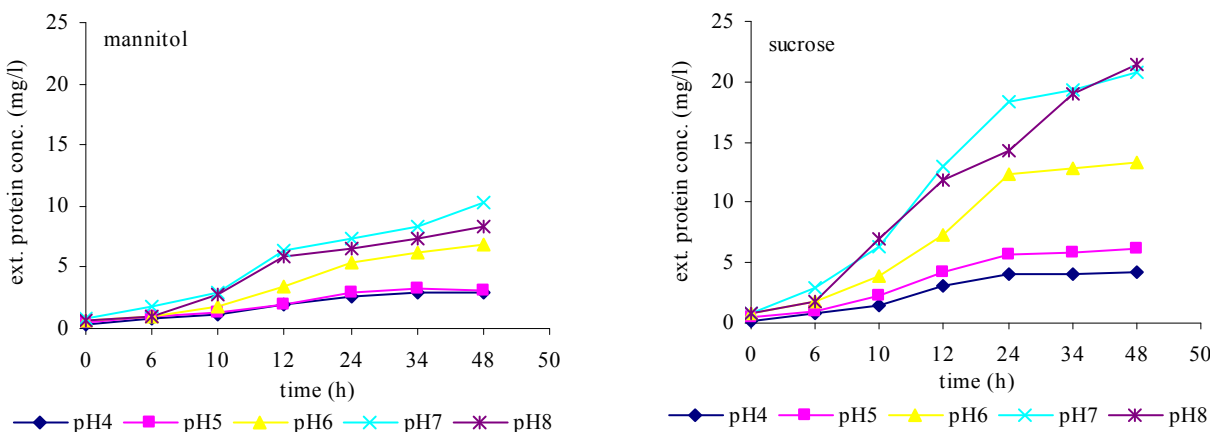


Figure 10. Effect of pH on extracellular protein levels at strains isolated from Maleia flood plain.

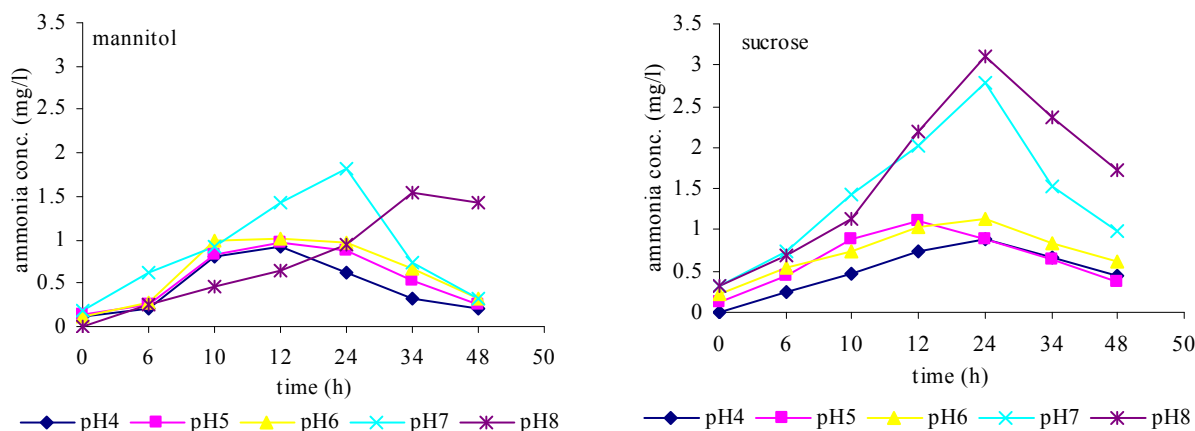


Figure 11. Effect of pH on ammonia secretion at strains isolated from alpine zone.

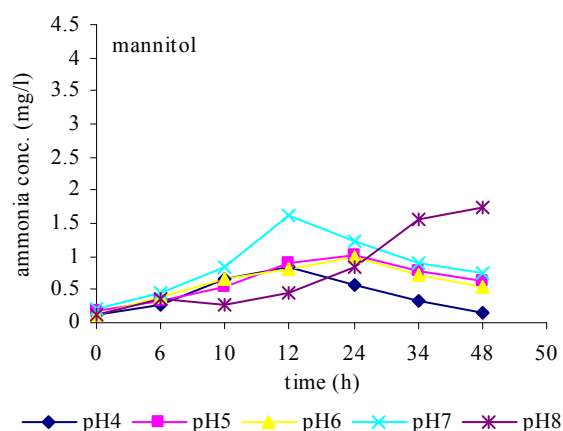
At the strains from the subalpine zone, the values of ammonia secretion obtained were higher than those from the alpine zone, the maximum ammonia secretion

(4.091 mg/l) being recorded at pH 8, on sucrose (Fig. 12).

The lowest values of ammonia concentration were

recorded at the strains originating from the coniferous zone (Fig. 13). Here the ammonia secretion attains a maximum of 0.98 mg/l on mannitol medium and 1.42 mg/l on sucrose, after 24 hours of incubation and then it decreases quite abruptly.

The highest values of the ammonia secretion were



obtained at the strains originating from the beech zone and the flood plain (Fig. 14 & 15). These were obtained at pH 8, on sucrose medium, after 24 hours of incubation and were 5.98 mg/l and 6.02 mg/l, respectively.

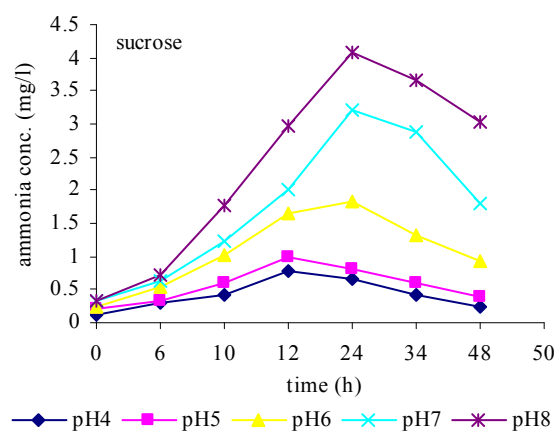


Figure 12. Effect of pH on ammonia secretion at strains isolated from subalpine zone.

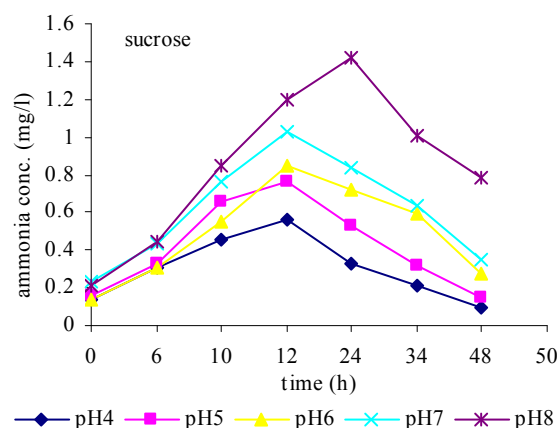
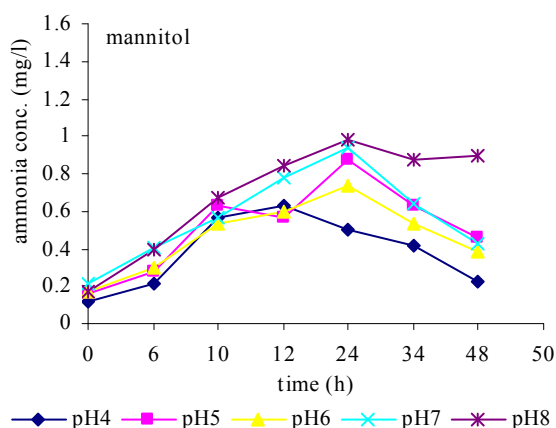


Figure 13. Effect of pH on ammonia secretion at strains isolated from coniferous zone.

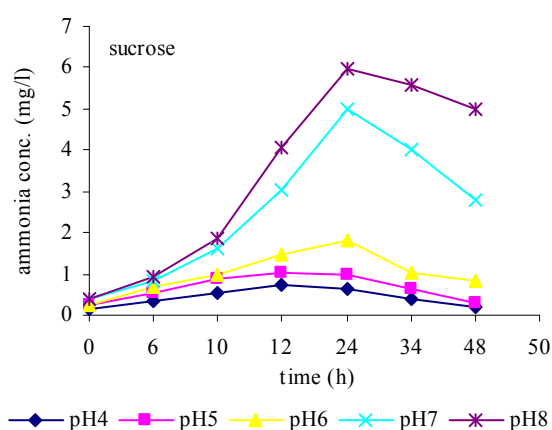
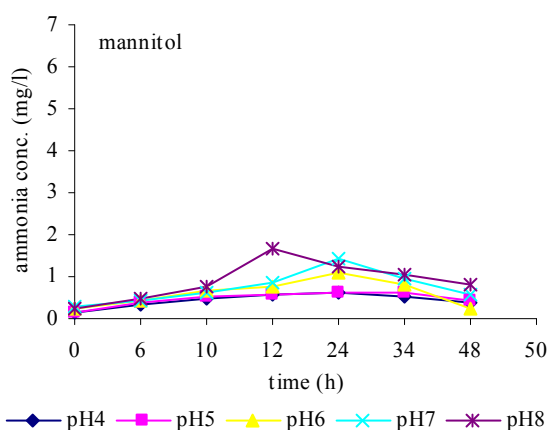


Figure 14. Effect of pH on ammonia secretion at strains isolated from beech zone.

Regarding the influence of the pH on ammonia secretion a similar tendency to the one from the growth was noticed, his concentration growing until stationary phase was reached, followed by a slight

decreasing during the stationary phase. This decreasing may be due to the fact that ammonia is released in the atmosphere or included in other compounds.

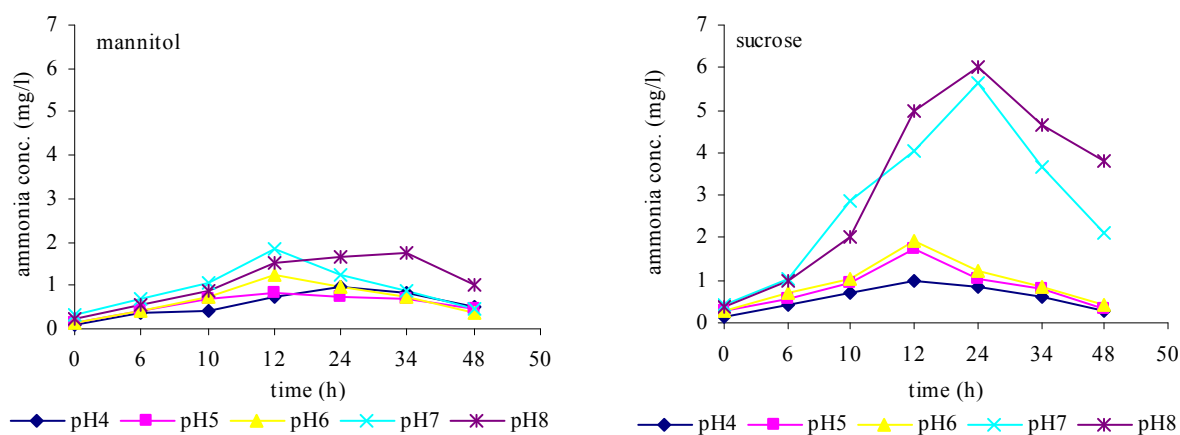


Figure 15. Effect of pH on ammonia secretion at strains isolated from Maleia flood plain.

DISCUSSIONS

In these experiments the pH influence on **growth** and nitrogen fixation products was evaluated, the culture medium pH being adjusted to 4, 5, 6, 7 and 8. This pH interval was chosen in order to obtain the pH for optimal growth considering that the *Azotobacter* strains were obtained from acid environments. The optimum pH value for growth (pH 7) is in a good agreement with data existing in literature regarding the *Azotobacter* strains [10].

An explanation to the similar growth obtained in the alpine samples at pH 4, 5, 6 and 8 could be that as the alpine meadow formed it was colonized with bacterial strains originating in the coniferous zone.

Because the temperature, humidity and pH conditions from the soil are not so different between the alpine and subalpine zone, here specific strains to both zones can be found, which implicitly also grow at strongly acid pH.

At the coniferous strains the maximum growth was on mannitol. This substance is found in nature especially in the coniferous forests because these contain mannose which reduces to mannitol [4].

It can be concluded that the pH value for optimal growth is around neutral pH and the cellular growth diminished at an pH slightly alkaline (pH 8) and also at an acidic one pH 4. The data mentioned above back up the results according to which the optimum pH for *Azotobacter* is situated within the interval from 7.2 to 8.2 [10] and the growth is reduced both in the acid and in the alkaline range of pH [7].

All the above data represent the mean of three independent measurements. Regarding the growth of the strains isolated from the mountainous soils there is between the two culture media a statistically significant difference of the results obtain ($p < 0.0001$).

The **extracellular protein** level varied not a little on the two culture media, at different pH values. The maximum value of extracellular proteins was obtained at the strains coming from the flood plain at pH 8 (21.452 mg/l). The extracellular proteins concentration on the studied media followed parallel and close lines which had a growing trend until the end of the studied interval.

The extracellular protein levels varied not a little on the two culture media, at different pH values. In order to emphasize this difference the One-Way ANOVA statistical analysis test, completed by Bonferroni post-test, was applied. For each altitudinal vegetation zone it was noticed that the extracellular protein levels attained statistically significant differences ($p < 0.0001$) for all the cases studied.

The **ammonia secretion** at each mountain zone was different on the two culture media taken into consideration. The level of the ammonia secretion attained a maximum of 6.02 mg/l at the strains from the beech zone at pH 8, on sucrose medium.

In order to emphasize the differences regarding the ammonia produced in the two culture media for each altitudinal vegetation zone the statistical analysis test was applied. It was noticed that the produced ammonia values on the two culture media show statistically significant differences ($p < 0.0001$) for all the bacterial consortia studied.

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