UNIVERSITI PUTRA MALAYSIA

EFFECTS OF pH AND ALUMINIUM ON GROWTH OF RHIZOBIA AND THE RELATIONSHIP BETWEEN FATTY ACID COMPOSITION OF RHIZOBIA AND ITS TOLERANCE TO LOW pH

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BY

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h	:	hour
QO_2	:	Specific Oxygen Uptake (mmol O_2/g cell)
DOT	:	Dissolved Oxygen Tension
Al	:	Aluminium
P	:	Phosphate
ml	:	Millilitre
L	:	Litre

Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia as fulfilment of the requirements for the degree of Master of Science

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SHAHARAH MUHAMAD IDRIS

JULY 1996

Chairman : Associate Professor Zulkifli Hj. Shamsuddin,

Faculty : Food Science and Biotechnology

The effect of two different initial culture pHs on growth of six strains of rhizobia (TAL 102,

was first studied by using shake flask experiment. Only *Bradyrhizobium* TAL 102 grew better at pH 4.5 compared to 6.5 and this strain was chosen as an acid-tolerant rhizobia. This result is in agreement with the result of the experiment using the fermenter in which the culture pH was controlled at a constant value of 4.5 and 6.5 throughout the cultivation. However,

parameters such as maximum cell concentration attained (X_m) differed significantly.

A study on the effects of different culture pHs and aluminium (Al) concentrations on growth of acid-tolerant rhizobia (*Bradyrhizobium* TAL 102) was carried out using a 2 L stirred tank fermenter. A modified Gompertz equation was found to be sufficient in modelling growth of rhizobia at two different initial culture pHs (shake flask experiment) and also growth of TAL 102 at different pH levels and Al concentrations. The growth parameters (X_{max}, μ_{max} and λ) of acid-tolerant rhizobia under different culture pHs and Al concentrations were calculated using the model. Maximum cell concentration (X_{max}) value was highest at pH 4.5 with a drastic reduction at pH below 4. Although the maximum specific growth rate (μ_m) was reduced at pH 4 and below, the effect was not clear for growth at pH 4.5 and above. The presence of monomeric Al activity (Σa_{Almono}) reduced X_{max} significantly but the λ and μ_{max} were not significantly affected. The X_{max}, μ_{max} and λ for growth of TAL 102 at pH 4.5 with 45.6 μM Σa_{Almono} were 2.0 x 10^9 cfu/ml, 0.015 h^{-1} and 5 h, respectively.

The effect of Al concentration on growth of rhizobia (TAL 102) was also investigated using continuous (chemostat) culture. The relationship between Σa_{Almono} and steady-state cell concentration (X_s) can be presented in the form of ln $[\Sigma a_{Almono}]$ = 6.53 - $0.101[X_s]$, indicating a decrease in rhizobial cell concentration with increased in toxicity of Σa_{Almono}.

The composition of fatty acids of rhizobia was successfully separated and analysed using Gas Chromatography technique. The major proportions of fatty acids present in all rhizobia studied were C:16 to C:20. From this study it was observed that the tolerance of rhizobia to low pH can be related to high proportions of C:18 and C:20. The C:18 fatty acids of *Bradyrhizobium* TAL 102, which was the major proportion of fatty acid when grown at pH 4.5 in the presence of Al, increased significantly with increasing concentration of Al in the culture. Although the proportions of other fatty acids such as C:12 and C:20 changed with increasing Al level, a significant relationship to pH tolerance of rhizobia was not evident. It was suggested that C:18 fatty acids played a role in rhizobial tolerance to Al.

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PENGARUH pH DAN ALUMINIUM TERHADAP RHIZOBIA DAN PERKAITAN DIANTARA KOMPOSISI ASID LEMAK RHIZOBIA DENGAN TOLERANSI KEPADA pH RENDAH

OLEH

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JULAI 1996

Pengerusi : Profesor Madya Zulkifli Hj. Shamsuddin, Ph.D.

Fakulti : Sains Makanan dan Bioteknologi

Kesan dua kultura pH pemula ke atas pertumbuhan enam strain rhizobia (TAL 102, UPMR 29, CB 1809, NC 92, TAL 1826 dan TAL 1373) telah dikaji dengan menggunakan kelalang goncang. Hanya *Bradyrhizobium* TAL 102 menunjukkan pertumbuhan yang baik pada pH 4.5 berbanding pH 6.5 dan strain ini telah dipilih sebagai rhizobia toleran asid. Keputusan ini adalah bertepatan dengan keputusan yang diperolehi daripada eksperimen menggunakan fermenter di mana semasa pertumbuhan, pH dikawal pada pH 4.5 dan 6.5.

Walaubagaimanapun, parameter pertumbuhan contohnya kepekatan sel maksimum tercapai (X_m) didapati mempunyai perbezaan yang ketara.

Kajian ke atas kesan pH kultura dan kepekatan aluminium (Al) terhadap pertumbuhan rhizobia toleran asid (*Bradyrhizobium* TAL 102) telah dijalankan menggunakan fermenter berpengaduk 2 L yang dilengkapi dengan sistem kawalan pH. Persamaan Gompertz terubahsuai didapati sesuai dalam memodelkan pertumbuhan rhizobia dalam keadaan pH pemula kultura yang berbeza (eksperimen kelalang goncang) dan juga pertumbuhan TAL 102 pada tahap pH dan kepekatan Al yang berbeza. Parameter pertumbuhan (X_{max}, μ_{max} dan λ) oleh rhizobia toleran asid dalam kultura pH dan kepekatan Al yang berbeza, telah dikira menggunakan model tersebut. Nilai kepekatan sel yang maksima (X_{max}) adalah tertinggi pada pH 4.5 dan menurun secara mendadak di bawah pH 4. Walaupun kadar pertumbuhan maksima (μ_m) adalah rendah pada pH 4 dan ke bawah, kesannya adalah tidak jelas pada pH 4.5 dan ke atas. Kehadiran aktiviti Al monomerik (Σa_{Almono}) telah menurunkan X_{max}, dengan ketara, tetapi tidak memberi kesan yang bererti kepada λ dan μ_{max}. Nilai X_{max}, μ_{max} dan λ untuk pertumbuhan TAL 102 pada pH 4.5 dengan Σa_{Almono} pada 45.6 μM adalah 2.0×10^9 cfu/ml, 0.015 j^{-1} dan 5 j berturutan.

Kesan kepekatan Al ke atas pertumbuhan rhizobia (TAL 102) juga telah dikaji menggunakan kultura selanjar. Perhubungan antara Σa_{Almono} dan fasa pegun bilangan sel (X_S) boleh ditunjukkan dalam bentuk ln [Σa_{Almono}] = 6.53-0.101 [X_s], di mana menunjukkan penurunan bilangan sel dengan meningkatnya ketoksikan Σa_{Almono}.

Komposisi asid lemak rhizobia telah dianalisis dengan menggunakan teknik Kromatografi Gas. Asid lemak utama yang hadir dalam semua rhizobia yang telah dikaji didapati dari C:16 hingga C:20. Daripada kajian ini didapati toleransi rhizobia pada pH yang rendah boleh dikaitkan dengan peningkatan kandungan asid lemak C:18 dan C:20. Asid lemak C:18 *Bradyrhizobium* TAL 102, yang paling tinggi apabila dikulturkan pada pH 4.5 dengan kehadiran Al, mengalami peningkatan dengan meningkatnya kepekatan Al di dalam kultur. Walaupun kandungan rantaian karbon lain contohnya C:12 dan C:20 mengalami perubahan dengan pertambahan Al, perkaitan bererti dengan toleransi rhizobia kepada pH adalah tidak nyata. Adalah dicadangkan bahawa rantaian asid lemak C:18 memainkan peranan bererti dalam toleransi rhizobia kepada Al.

CHAPTER I

INTRODUCTION

Soil acidity is a major factor which limits legume growth and nitrogen fixation because of its adverse effects on growth of the host plant, root nodule bacteria namely *Rhizobium* and *Bradyrhizobium* and the symbiotic process (O'Hara et al., 1989). Successful symbiotic associations between legumes and their root nodule bacteria are of immense importance both in agriculture and forest ecosystem. *Rhizobium*-legume symbiosis is a highly specific interaction (Glenn and Dilworth, 1991) and is influenced by the environment, such as soil pH and Al concentration. Soil acidity is a complex of high proton concentration and its interaction with various mineral ions. It was found that as the pH decreases below 5 the concentration of soluble Al increases. The degree of Al tolerance in rhizobia appears to be a stable genetic character, implying some underlying physiological or biochemical differences between Al-tolerant and Al-sensitive strains which may be attributed to the synthesis of specific enzymes.

Growth of rhizobia is influenced by the physical properties and chemical composition of soils (Ayanaba et al., 1983; Keyser and Munns, 1979(a,b)).

Acidity and Al toxicity are the major soil factors which limit growth of rhizobia. The influence of pH (Date and Halliday, 1978; Shamsuddin, 1987), Al (Ayanaba et al., 1983; Keyser and Munns, 1979 (a,b); Shamsuddin, 1987), phosphate (Ayanaba et al., 1983; Cassman et al., 1981) and calcium (O'Hara et al., 1989) concentrations on growth of rhizobia have been extensively studied. The relationship between Al toxicity and culture pH; and also its interaction with other minerals in soils have also been investigated (Ayanaba et al., 1983; Cassman et al., 1981; Date and Halliday, 1978; Keyser and Munns, 1979(a,b); O'Hara et al., 1989). However, interpretation of data available from these types of experiments is difficult because it involved many variables and parameters. In addition, many sets of experiment have to be carried out in order to get more realistic result on the effect of each chemical component and other growth variables. Furthermore, if the result is not presented in a concise form or in the form of quantifiable values, comparison of data with others available in the literature is difficult.

Most studies on the effect of pH and Al on growth of bradyrhizobia have been carried out in batch culture using shake flask without automatic pH control system. In many cases, the culture pH was presumably controlled using biological buffers. Once growth proceeded the buffers may be metabolised and compound such as ammonia will be liberated which leads to a reduction in the buffering capacity and a significant increase in culture pH . Interpretation of data available from this type of experiment is difficult because comparison was normally made between cultures cultivated at different pH levels with or without Al, whereby the culture pH and hence, monomeric Al has changed during growth. Small changes in culture pH alone could significantly affect the growth of rhizobial cultures (Richardson and Simpson, 1989; Thornton and Davey, 1983 (a,b)). In order to get more realistic result, the experiment should be conducted using the fermenter with an automatic pH control system.

Microorganisms can adapt to different changes in the environment by modifying their membranes (Heipieper et al., 1994). Changes in the fatty acid composition of membrane lipids are the most common reaction of bacteria against membrane active substances. Currently there is no documented evidence on the identification of specific fatty acid induced or repressed by acidity. Several possible starting points are evident in the literature including the production of shorter carbon chain fatty acid in acid-tolerant strains of rhizobia (Shaharah, 1993). The significance of fatty acid composition in characterising acid-tolerant and intolerant strains is still unknown. If the relationship between pH tolerance and fatty acid composition is known, this will assist in developing simple techniques for screening of acid-tolerant rhizobial strain.

The objectives of this research study, based on the problems related to the relationships between pH and Al concentration on growth and survival of rhizobia and the possible methods overcoming these problems, are as follows:

1) To investigate the effect of pH on growth and survival of several rhizobial strains in shake flask culture without the addition of buffer to control culture pH at the required level.
2) To investigate the effect of pH and Al on growth of acid-tolerant rhizobia (selection based on results from shake flask experiment) in batch cultivation using fermenters with automatic pH controls.
3) To develop a simple mathematical model for describing growth of rhizobia in biological terms under different cultivation conditions.
4) To investigate the effect of monomeric Al activity (Σa_{Almono}) on growth and survival of rhizobia in continuous culture with pH control in which steady state of nutrients in the culture medium can be achieved.
5) To determine the relationship between fatty acid compositions of rhizobia and their tolerance to low pH.

In the present study, a quick selection of acid-tolerant bradyrhizobia was carried out using shake flask cultures. The selected strain was then used for the subsequent experiment to study growth of acid-tolerant rhizobia at different pH levels and Al concentrations using a fermenter with automatic pH control system.

The experimental data obtained was used to explain the mechanism of the interaction between pH and Al on growth of rhizobia. The identified acid-tolerant strain was then used for subsequent continuous cultivation experiments in which the culture pH and Al concentration were controlled at required values while other cultivation conditions such as organic materials and phosphate levels were kept constant. This highly controlled cultivation conditions enabled investigations on the effect of Al toxicity on growth of *Bradyrhizobium* under different conditions which could be used to extrapolate results from laboratory to soil conditions. The Gompertz equation was used to verify the experimental data under different cultural conditions in which various parameter values such as maximum cell population, maximum specific growth rate and lag phase were calculated. The modelling was aimed at simulating the growth pattern so that a prediction of growth under different cultural conditions could be logically constructed. Although work on the influence of pH and Al on growth of rhizobia have been studied extensively, no models has been proposed to describe the result in a concise form.

The composition of free fatty acid in all rhizobia employed in this study was analysed using gas chromatography. The changes in fatty acid composition under different cultivation conditions (different pHs and Al concentrations) was also investigated.