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Metabolome profile of osmotolerant rhizobacteria under osmotic stress

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ABSTRACT

An osmotolerant rhizobacteria (*Enterococcus flavescens*), isolated from the weed rhizosphere, has been subjected to osmotic stress in broth media supplemented with varied concentrations of glucose and sodium chloride (NaCl). The aims of this work was to evaluate the cells' response and obtain a comprehensive view of metabolites profiles under osmotic stress. Cells were grown in rich medium (Luria Bertani, LB) supplemented with NaCl at varying concentrations (0.5 M – 1.8 M NaCl) and glucose (5% – 20%), both as a single or double stress condition. Physiological response was observed by measuring rhizobacterial growth and analysing metabolite synthesised using GC-MS. The results of this study demonstrated that different concentration of salt and glucose resulted in different metabolite profiles. It was also observed that cells cultivated in LB + 1,6 M NaCl and LB + 1,8 M NaCl medium resulted in only two different compounds detected, i.e amide and fatty acid. Under double stress, however, concentration of hexadecanoic acid, hexadecenoic acid, proline and glycerin was found increased, while under single-salt and glucose stress, hexadecanoic acid and 9- octadecenoic acidmethyl ester were detected. Those two acid were absent from the cells' metabolites under double stress. The results of this study thus suggested that osmotic stress provokes different pathway of fatty acid metabolism. The metabolome profile of the rhizobacteria under stress condition may also reveal specific mechanism of microbial tolerance to osmotic stress under different condition. The possible mechanism and pathway of osmotic stress in microorganisms will be discussed.

Keywords: *osmotolerant rhizobacteria, metabolome, osmotic stress*

1. INTRODUCTION

Environmental stress, such as osmotic and salt stress, has become a major issue in agricultural practices as it may hamper the plant cultivation and production. Soil salinity is a global and the most important issue in agriculture as it turns the agricultural land into unproductive areas about 1-2 % a year in arid and semi-arid zones. Salinity limits plant growth and development which in turn decreases plant production [1]. The use of plant growth promoting rhizobacteria (PGPR) for the improvement of plant growth and development has become a significant approach. In certain cases, such as in banana (*Musa acuminata*) plantation, the inoculation of banana with *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens* resulted in improved plant growth and altered the metabolome profile of the plant [2]. It was also observed that halotolerant bacteria *Kosakonia radicincitans* increased root-colonising capability and improved growth of radish plant [3]. The use plant growth promoting rhizobacteria, *Bacillus subtilis*, *B. amyloliqufaciens*, *B. cereus*, *Pseudomonas putida*, and *P. fluorescens*, were also shown to reduce the damage of tomato root by nematode [4]. Similarly, osmotolerant rhizobacteria has also been shown to improve rice growth under drought stress [5].

It is interesting to note that banana plant metabolome profile [2] altered when inoculated with halotolerant bacteria. Cytoplasmic membrane fluidity and fatty acid composition of

Acidithiobacillus ferrooxidans also changed when subjected to pH stress [6] suggesting that membrane permeability was compromised under extreme acidity (pH 1.5). It was also found that *Dunaliella salina* cultivated under high NaCl stress (1.5M) demonstrated the increase of 9,12,15-octadecatrienoic acid (Z,Z,Z) (C18:3 $^{\Delta 9,12,15}$) synthesis [7]. Similarly, in *Chlamydomonas reinhardtii* the synthesis of octadecatrienoic acid (C18:3) dan octadecadienoic acid (C18:2) also increased under NaCl stress [8]. In *Saccharomyces cerevisiae*, salt (NaCl) stress also resulted in the abundant synthesis of cis-9-hexadecanoic acid (C16:1$^{\Delta 9}$) which constitute 10-30% of the whole fatty acids [9].

In the work presented here we demonstrated and discussed the changes of metabolome profile. Our work demonstrated that osmotolerant rhizobacteria (*Enterococcus flavescens*), isolated from the weed rhizosphere, showed altered composition of fatty acid under single or double stress, suggested that osmotic stress provokes different metabolic pathway.

2. MATERIALS AND METHODS

2.1. Cell cultivation

Cells of *Enterococcus flavescens* were cultivated in Luria Bertani medium supplemented with 0,5; 1; 1,5; 1,8 M concentrations of NaCl and glucose at 5, 10, 15, dan 20% concentrations. Cells were incubated at 30^{0}C with shaking for 76 hours. Samples of cells were drawn in 4 hours period for growth analysis and metabolites extraction and analysis.

2.2. Metabolite cell extraction

Fifty ml of cells grown under NaCl and/or glucose stress were harvested by centrifugation. Cell pellets were centrifuged at 10,000 *g* for 10 minutes. Cell pellets were then suspended in cold methanol and sonicated until the cells were broken up. Following sonication, cell suspension was kept at -80^{0}C for 24 hours. After incubation, cell suspension was centrifuged at 10,000 *g* for 10 min. Cell pellet was then discarded, and the supernatant was used for analysis using GC-MS.

2.3.Gas Chromatogaph-Mass Spectrometry (GC-MS).

Analysis of metabolites in the cell pellets was carried out by using GCMS-QP2010S SHIMADZU. Helium was used as the carrier gas, column temperature 70^{o}C. Samples were injected into the column at initial temperature of 70^{o}C and a maximum of 300^{o}C with splitless mode.

3. RESULTS AND DISCUSSION

3.1. Growth of E. flavescens under NaCl and glucose stress

Previous finding [10] suggested that rhizospheres of alfafa (*Medicago* sativa) plants exposed to differing watering-limiting conditions harbour distinct bacterial communities. It is interesting to see whether osmotic stress imposed by NaCl and glucose, which may also reflect water scarcity, changes rhizobacterial metabolites. Different response to osmotic stress may suggest the relationship between the bacterial population thrive in certain niche with their ability to withstand the environmental stress. In this study we found that, under different stress conditions, *E. flavescens* showed growth response (Figure 1) that reflects the shift in the metabolic pattern. It was observed that NaCl gave a more striking effect on growth of *E. flavescens* than glucose as evidenced by the fact that cells underwent longer adaptation under NaCl stress (Figure 1A). It is interesting to note that under double stress, glucose present in the medium, albeit at high concentration, reduced the stress effect of NaCl (Figure 1B) at higher concentration. Growth of cells under single, 1.8M NaCl, was lower than growth under double stress (1.8M NaCl + 15% glucose), suggesting that glucose may compensate the effect of NaCl at high concentration.

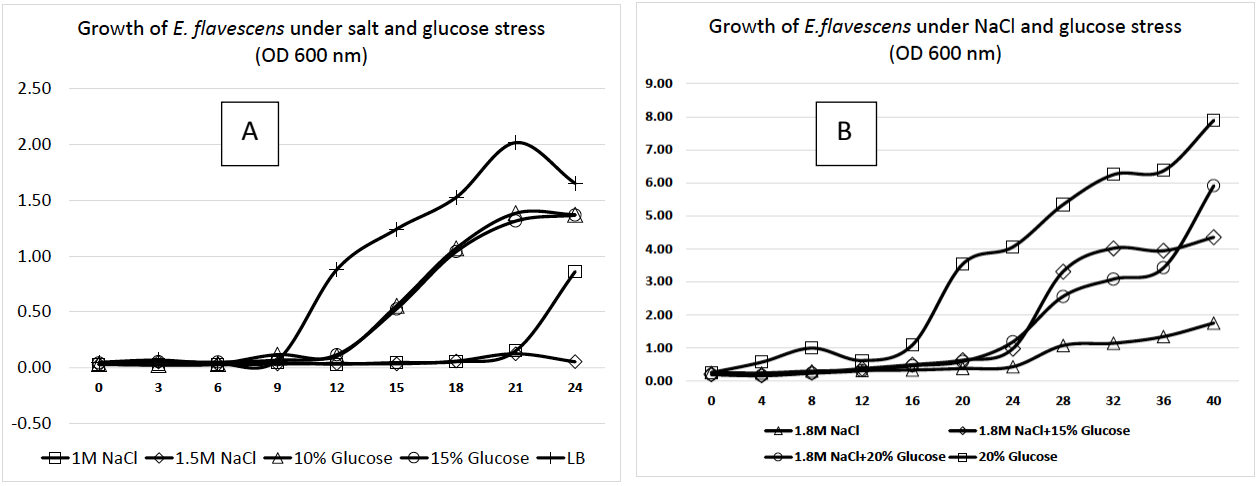

Figure 1. Growth of *E. flavescens* under different NaCl and glucose stress. A: 1M NaCl and 1.5M NaCl, 10% and 15% glucose; B: 1.8M NaCl, 20% glucose.

3.2. Metabolite profiles of E. flavescens under NaCl and glucose stress

Cells of *E. flavescens* grown under NaCl and glucose stress were extracted and analysed using GC-MS to reveal metabolites synthesised under certain stress condition (Figure 2). It was observed that several compounds were found in cells under all stress conditions, i.e.: *Cholesteryl myristate*; *Pentadecanoic acid; 14-methyl-, methyl ester; 11-Octadecenoic acid, methyl ester;* and *Dodecanoic acid*. However, different stress condition resulted in the synthesis of different metabolites. It was observed that, several compounds were only detected when the cells were grown under single stress (1.8M NaCl or 15% glucose), i.e.: *9-Octadecenoic acid, methyl ester; Butanedioic acid, 2,3-dimethoxy-, dimethyl ester; 6- Octadecenoic acid, methyl ester; 2-Dodecenyl(-)succinic anhydride; Oxiraneundecanoic acid, 3-pentyl-, methyl ester, cis-; 4-Piperidinamine, N,1-dimethyl-; Glycerin; Phenylethylamine; 2,6,10-Dodecatrienol, 3,7,11-trimethyl-; Butyrolactone; Germacrene B; Cyclopropanedodecanoic acid, 2-octyl-, methyl ester; delta.-Guaiene; Glycolic acid, methyl ester;* and *Carbamic acid, methyl ester*.

Under double stress (1.8M NaCl + 15% glucose), 17 compounds were detected which fall into groups of aldehyde, alkana, alcohol, amide, fatty acids, lipid, terpene, while under single stress the metabolites synthesis were mostly lipid, fatty acids, amine, and amide. These compounds: *Decanedioic acid, ethyl decenoate phthalic acid, tridecane, tridecanoate, undecane, decanedione, decenol, hexylvinyl sulfide, dioxolanone, L-Proline, heptanone, pentanone, aminopropanol, glycolic acid, carbamic acid, Glycerin, dodecanetrienol, dodecenyl, germacrene*, dan *azulene* were detected under double stress of 1.8M NaCl + 15% glucose, suggesting that metabolites may be grouped into fatty acids, osmoprotectant, and other compounds.

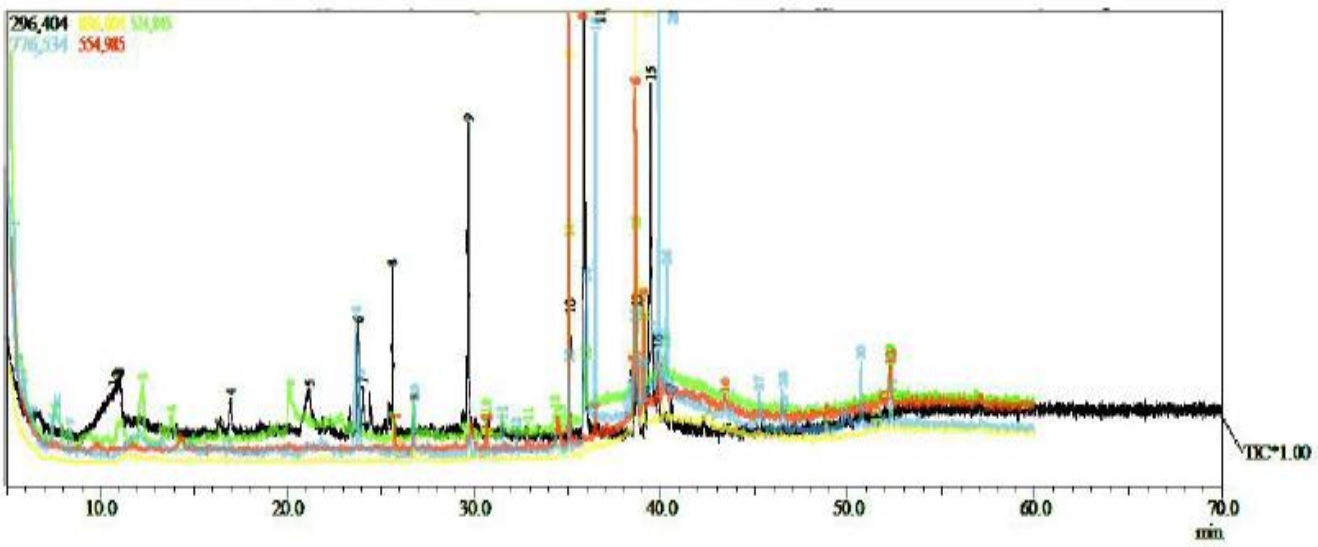

Figure 2. Metabolites profiles of *E. flavescens* under NaCl and glucose stress. Cells were grown in medium LB (green); 1.8M NaCl + 15% glucose (black); 1.8M NaCl + 15% glucose (extracted using diethyl ether) (blue); 15% glucose (red); 1.8M NaCl (yellow).

Other interesting finding was that metabolites *oxirane* and *propylheptanol* were detected only under single stress imposed by 1.8M NaCl, while *piperidinamine* was detected only under 15% glucose and not in other stress conditions. It was also found that under non-stress condition (in LB medium), most of the metabolites synthesised were of long chain of alkana (C14 – C44) and alkena (C21 – C44). Under single stress (NaCl), most of the metabolites were of medium chain length of fatty acids (C13 and C18). The changes of metabolite profiles of *E. flavescens* under NaCl and glucose stress suggest the shift of metabolic pathway of fatty acids, as found in *Saccharomyces cerevisiae* [9], *Dunaliella salina* [7], and *Chlamydomonas reinhardtii* [8].

Under non-stress condition, concentration of fatty acids reached 46% consists of fatty acids with C10 to C25. On the other hand, under single stress (NaCl or glucose) condition, fatty acids of C10 to C12 were synthesised abundantly up to 92.5%, while under double stress fatty acids lowered to 62.8% of C11 to C25. This observation suggests that under stress condition, *E. flavescens* increased the synthesis of long chain fatty acids. The increase and accumulation of long chain fatty acids helps the cells in responding to osmotic stress and changes cell membrane permeability and fluidity [11]. The lower fatty acid concentration under double stress than under non-stress may be attributed to the fact that under double stress, the cells shifted its metabolic pathway to synthesis other compounds. It was also found that decomposition derivatives of fatty acids, *3- (Chloromethyl)heptane; 2-Ethylbutyraldehyde; 3,3-dimethyl-Pentane; 4-methyl-1,3- Dioxolanone; 3-Decenol*; and *methyl heptanone*, were detected under double stress condition. It is interesting to note that Bambara garoundnut rhizobacteria synthesised several volatile compounds that demonstrated antibacterial activity [12].

In addition to changes of the composition of fatty acids, alkena, alcohol, amide, ketone were among compounds synthesised by cells under different stress (Table 1). Interestingly it was observed that osmoprotectant compounds were detected only in the cells subjected to double stress (NaCl and glucose). This observation clearly suggests the metabolic pathway shift occurred in the cells under double stress by synthesising osmoprotectant to ease the environmental pressure. It is, therefore, of interest to pursue further the implication of environmental pressure on the metabolic pathway of the cells, not only in microbial cells but also in higher cells.

Table 1. Several compounds other than fatty acids synthesised under stress.

Compounds	1,8 M NaCl + 15% Glucose	1,8 M NaCl	15% Glucose	LB
Alkena	1H-Cycloprope azulene, decahydro-1,1,7-trimethyl-4-methylene	ND	ND	ND
Alcohol	ND	2-Propylheptanol	ND	Hexadecanol Phenylethyl Alcohol
Amide	ND		9-Octadecenamide	9-Octadecenamide
Ketone	ND	ND	ND	Dihydro-2(3H)-Furanone
Osmoprotectant	Glycerin Germacrene B delta.-Guaiene	ND	ND	2,8,4,6-(Epoxyethanediylidenoxy) 1,3 dioxino ND
Miscellaneous	2,8,4,6-(Epoxyethanediylidenoxy) 1,3 dioxino	ND	ND	2,8,4,6-(Epoxyethanediylidenoxy) 1,3 dioxino
		Cholesteryl myristate	Cholesteryl myristate	Cholesteryl myristate
				Aziridine, 2-ethyl-
				Aziridine, 2-heptyl-3-methyl-
	2-Dodecenyl(-)succinic anhydride	Oxiraneundecanoic acid, 3-pentyl-, methyl ester, cis- (C19)	4-Piperidinamine, N,1-dimethyl-	Piperazine (CAS) R22
	Carbamic acid, methyl ester	ND	ND	Phenylethylamine
	2,6,10-Dodecatrienol, 3,7,11-trimethyl-	ND	ND	Butyrolactone
	Piperazine (CAS) R22	ND	ND	Cyclopropanedodecanoic acid, 2-octyl-, methyl ester (C24)
	Glycolic acid, methyl ester	ND	ND	ND

ND: not detected

Data presented in this study thus provide an insight to the complexity of cellular metabolism under environmental stress which is reflected on the growth pattern as well as the metabolism shift. Changes in fatty acids composition are not the sole implication of the metabolic shift as it was observed

that other compounds, including osmoprotectants were also synthesised under certain stress condition.

4. CONCLUSION

The results of this study demonstrated that osmotolerant rhizobacteria, *Enterobacter flavescens*, responded differently in terms of its metabolites synthesised under different stress conditions (NaCl and glucose stress) as evidenced by the appearance of specific metabolites under specific stress condition, suggesting that different stress provokes metabolic shift.

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