Malays. Appl. Biol. (2014) 43(1): 41–51

SULFATE-REDUCING BACTERIA METABOLITE DETECTION USING GC-MS

NAWAWI, M.F.[1], SAHRANI, F.K.[1*], AZIZAN, K.A.[3], ALI, M.M.[1], AHMAD, A.[2] and USUP, G.[1]

[1]*School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, MALAYSIA*
[2]*School of Bioscience and Biotechnology, Faculty of Science & Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, MALAYSIA*
[3]*Intitute of Systems Biology (INBIOSIS) Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, MALAYSIA*
[*]*Email: fathul@ukm.edu.my*

ABSTRACT

This research was conducted to investigate and compare the various metabolite, produced from the metabolism of two different SRB strain, that were involved in the biocorrosion process. Stainless steel coupons were immersed in two strains of sulfate-reducing bacteria, A1H1 and EB3 (designated as SRB1 and SRB2 repectively) were isolated from Port Dickson marine vicinity that were grown in VMNI medium. The immersion period were set for eight days. Analysis of Gas Chromatography-Mass Spectrometry (GC-MS) was conducted using –trimethylsilyl (TMS) of *N*-methyl-*N*-trimethylsilylfluoracetamide (MSTFA). The data were than analyzed using Partial Least Squares Discriminant Analysis (PLS-DA) method to discriminate the unique metabolite according to each strain. The result showed that SRB1 generates less metabolite but high in concentration. Meanwhile, SRB2 shows a variety production of metabolites but less in concentration. Both strains share the same metabolism in the production of nitrogen based substance and production of norvaline and pentanoic acid. SRB1 shows a very distinct feature as the production of ribitol was spotted in its metabolism where it is usually associated with growth. SRB2 showed a very close usage of sulphur by production of methionine. These results suggest that different SRB strain produced different number and type of metabolites in the biocorrosion process.

Key words: Sulphate-reducing bacteria, Extra Polymeric Substances, biofilm, Atomic Force Microscopy

INTRODUCTION

SRBs are mostly classied as strict anaerobes, which are distributed in two domains, Archaea and Bacteria, and they are the most frequent microbial type associated with microbiological inuenced corrosion (MIC) and metallic dissolution processes (Castaneda & Benetton 2008). The biocorrosion process of SRBs mainly associated with the presence of bacteria biofilm. It typically consists of microbial cells and their metabolic products, referred to as metabolites, including extracellular polymers and inorganic precipitates (Beale *et al.,* 2010).

Metabolites are the product(s) resulting, from production or destruction via either physical and/ or chemical metamorphosis of an organism; while metabolomics refers to the study of these metabolite proles as produced in biological samples (Beale *et al.,* 2010). There is some evidence that pure cultures of sulfate reducers use amino acids directly in their metabolism (Hansen & Blackburn 1995). This metabolism activity consists of degrading and formation of various forms of substances from ptotein to amino acid, inorganic sulfur, hydrogen ion and many more that lead to the formation of biofilm (Anderson *et al.,* 2010, Cordas *et al.,* 2008).

Members of genera Desulfovibrio and Desulfotomaculum oxidize various single amino acids including L-alanine, serine and glycine, if sulfate is present, whereas the hyperthermophilic sulfur reducing members of the order Thermococcales, domain Archaea can grow on mixtures of amino acids. Some species can use amino acids using Stickland's reaction, where one amino acid acting as electron donor and the other as electron acceptor (Fardeau *et al.,* 1997). Reports have shown that the addition of thiosulfate to the growth medium enabled the utilization of amino acids by non sulfate-reducing bacteria such as *Thermoanaerobacter brockii* and *Dethiosulfovibrio*

* To whom correspondence should be addressed.

peptidovorans. Thus, these data suggest an important role of sulfate and thiosulfate in the degradation of proteinaceous compounds (Baena *et al.* 1998).

Combination of SRB metabolic activity with ferrous materials leads to the formation of aggressive corrosion products, such as hydrogen sulde (H_2S) (Castaneda & Benetton 2008). In temperate anaerobic sediments, fermentative and respiratory microorganisms cooperate to completely oxidize complex organic matter to carbon dioxide with Fe(III), Mn(IV), SO_4, and CO_2 serving as the terminal electron acceptor (Tor *et al.,* 2003, Wargin *et al.,* 2007). But The anaerobic degradation of proteins has not been studied as extensively as carbohydrate fermentation, though the input of proteins into anaerobic environments is large. Due to the presence of proteins in almost any ecosystem, the turnover of amino acids is a very important microbiological process (Baena *et al.,* 1998).

Thus this research was conducted to investigate and compare the various metabolite, produced from the metabolism of two different SRB strain, that were involved in the biocorrosion process.

MATERIALS AND METHODS

1.0 **SRB Isolation**

SRB isolates, recovered from biofilm formed on a metal that were immersed in seawater for 1 month were cultured anaerobically on solid and broth VMNI medium. Purification of these SRB isolates was performed using the spreading technique where 100 µmLof VMNI broth containing the sample were spread onto the VMNI plate. Single colony was then transferred into another VMNI plate using the streaking technique. SRB Bart kits were used to determine the culture content of SRB group. Incubation for both methods was done for 24 hours in 37ºC. Two purified strains were coded SRB1 and SRB2, respectively, were chosen.

2.0 **SRB Cultivation**

Purified A1H1 and EB3 SRB strains were grown in a static batch cultures with stainless steel coupons (1 x 1 x 1 cm) in VMNI medium at 37ºC together with a VMNI medium without SRB culture that act as a standard. These cultures were incubated for 8 days. Five biological replicates were prepared.

2.1 **Metabolite extraction**

Metabolite extraction were done according to Azizan *et al.,* 2012 and Beale *et al.,* 2010. VMNI samples, with and without SRB, were ltered using a 0.45 µm hydrophilic membrane and were transferred into 100 mL test tubes. The samples then were dried using a freeze drier at 50ºC. About 80 µL of methoxyamine hydrochloride dissolved in pyridine (2g/100mL) were added to the previously driedsamples and placed in a microwave instrument at 50% power for 2.8 min. MSTFA (80 µL) was then added to the samples and were incubated again in the microwave for 3 min. About 0.5 mL of this mixture were transferred to a 2.0 mL GC vial. Derivatized samples were then analyzed by GC–MS

2.2 **Gas chromatography–mass spectrometry (GC–MS) Metabolite Analysis**

GC-MS parameter was based on protocol by Azizan *et al.,* 2012 and Beale et al 2010. Analysis was carried out using GC-MS Turbo Mass Clarus 600, Perkin Elmer, USA system, equipped with quadrupole mass spectrometry (MS) and electron ionization (EI), operated at 70Ev. A 30 m Elite 5-MS (Perkin Elmer), i.d. 250 µm, lm thickness (df) 0.25 µm separation column was used for the analysis. The MS was operated in scan mode (start after 8.0 min, mass range 40-600 amu at 0.5s/scan). All injections were performed in split mode (1:50) with 1.0 µL volume. Briefly, the oven was held at an initial temperature of 70ºC for 2 min before increasing to 300ºC at 10 min and the final temperature was held for 5 min. Helium gas was set at 1.1mL/min. The GC column was equilibrated for 6 min prior to each analysis. Available pure standards (amino acid) were run to validate the retention time (RT) . VMNI medium were used as control.

2.3 **Data analysis and validation**

Data analysis was performed based on protocol by Azizan *et al.,* 2012 using Turbomass 4.1.1 software (Perkin Elmer Inc. USA) by extracting the height of GC peaks of the TMS derivatives. Signal to ratio was set to 3, followed by peak smoothing, before being aligned, deconvoluted and extracted. Identification of GC peaks was based on NIST mass spectral database library (2008) and available pure standard that were prepared and analyzed identically to sample. Roughly, a data matrix was rearranged in a way that the rows represent the identified metabolites and retention time (RT) and the column represent the GC height. Each dataset was normalized to the total sum of GC height and internal standard followed by log transformed. Visualization of data was carried out using PLS-DA and S-plot of OPLS-DA of Simca-P+ version 12.0 (Umetrics AB, Ume, Sweden). Data derived from the GC-MS analysis were used to construct a heatmap for visualization and clusteration of specific metabolite for each sample using the software from Metaboanalyst (Xia *et al.,* 2010)

RESULTS

Thirty one common metabolites were observed from the three samples analyzed. A sample chromatogram is presented in Fig. 1, and the peak identification is shown in Table 1.

GC-MS Analysis

About 17 metabolites were detected in VMNI, another 14 in SRB1 and 20 in SRB2. The different number of compound in each sample is closely related to the activity inside the sample which either the compound is use, degraded or produces.

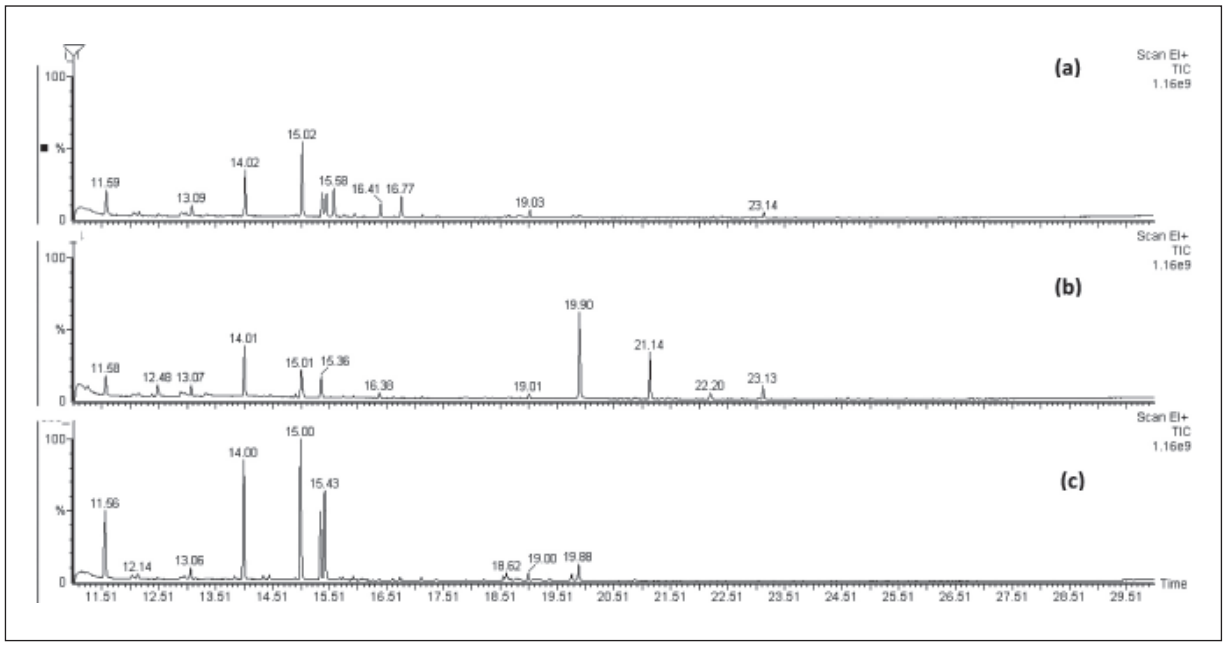

Fig. 1. GC-MS chromatogram for (a) control (VMNI), (b) SRB1 and (c) SRB2

Table 1. The retention time (RT) of each metabolite detected

Metabolite	RT (min)	Metabolite	RT (min)
Amino Acid		**Polyamine**	
Alanine	12.28	1,4-Butanediamine	21.15
Glycine	12.98	Cadaverine	28.74
Norvaline	14.02		
Valine	15.50	**Sugar Alcohols**	
Leucine	17.34	Glycerol	17.43
Proline	17.96	Arabinitol	22.21
Isoleucine	17.88	Ribitol	23.13
Serine	18.93		
Threonine	20.12	**Hydroxy Acid**	
Glutamine	25.63	2-Hydroxyisocaproic acid	23.02
Methionine	29.17	Ribonic acid	27.82
α-Amino Acid		**Asetyl Derivative**	
Aminobutyric acid	19.91	Acetyl-L-Lysine	29.52
Phenylalanine	23.55		
Aspartic acid	25.39	**Tetronic Acid**	
		Trihydroxybutyric acid	23.83
Carboxylic Acid			
Propanoic acid	10.84	**Other Organics Substances**	
Butanoic acid	13.05	Uracil	21.46
Pentanoic acid	15.45	Mercaptoacetic acid	28.42
Sugar Acid			
Threonic acid	19.02		

The heatmap shows the clustering of each sample. VMNI, clustered in the red line, and SRB1, in the green line showed a very distinct separation of group. However in SRB1, one of the sample was separated from its group. This anomaly is known as an ambient. This affect can be seen largely in SRB2 clustering as they were separated into two groups, consisting of two and three samples respectively. This was caused by the poor separation detection of metabolite in its group causing to create a bias between each sample as the group itself contains a wide variety of metabolites.

Partial least square discriminant analysis (PLS-DA) was performed to filter out the main compound that separated these three groups. Variations of intracellular metabolites between conditions were visualized using PLS-DA model that is the contribution plot, showing the most expressed metabolite in respective sample, and the coefficient plot, showing the intensity of each metabolite that represent the concentration. The contributed metabolites were further tested using S-plot of PLS-DA to see how well it is separated. Two groups of test were carried out. The first one is between SRB 1 and VMNI and the second one is between SRB2 and VMNI. This was done to eliminate the metabolite that was present in the VMNI and act as control, and to identify which compounds were produced.

The contribution plot of VMNI vs SRB1 (Figure 3) shows that there are 24 compounds detected in both samples. The lower part of the graph represent compounds that were found in VMNI and the upper part is the compounds that were found in SRB1. Five metabolites that are unique to SRB1 based on the contribution plot is 1-4-butanediamine, cadaverine, norvaline, pentanoic acid and ribitol. Coefficient plot (Figure 4) showed that these compounds were detected in a small quantity as their bar graph were plotted on the bottom of the graph. The S-plot for this group was showed in (Figure 5). The separation of those 5 metabolites were located at the lower end of the graph and showed a very good separation from the other clustered metabolite. These strengthen the fact that those compounds were the unique compounds that separated SRB1 from SRB2.

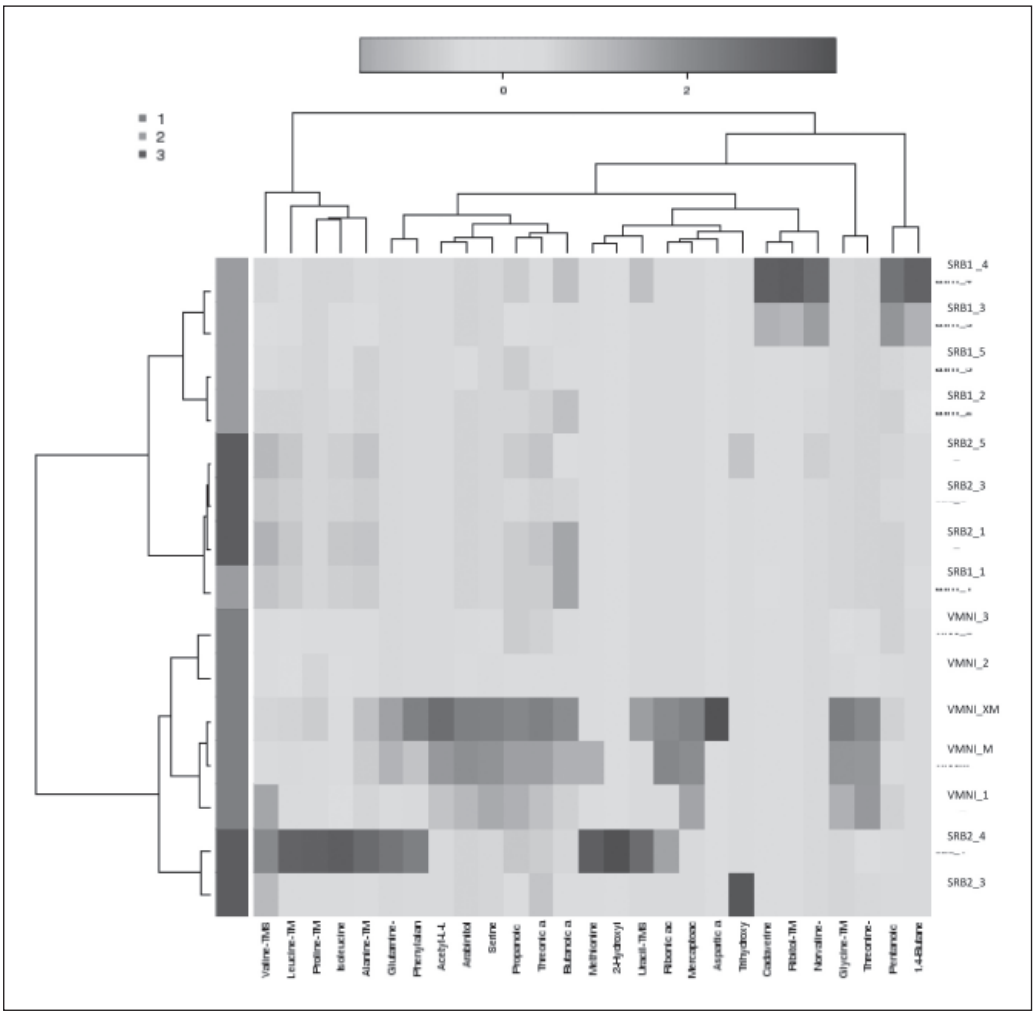

Fig. 2. Heatmap for VMNI, SRB1 and SRB2 metabolite

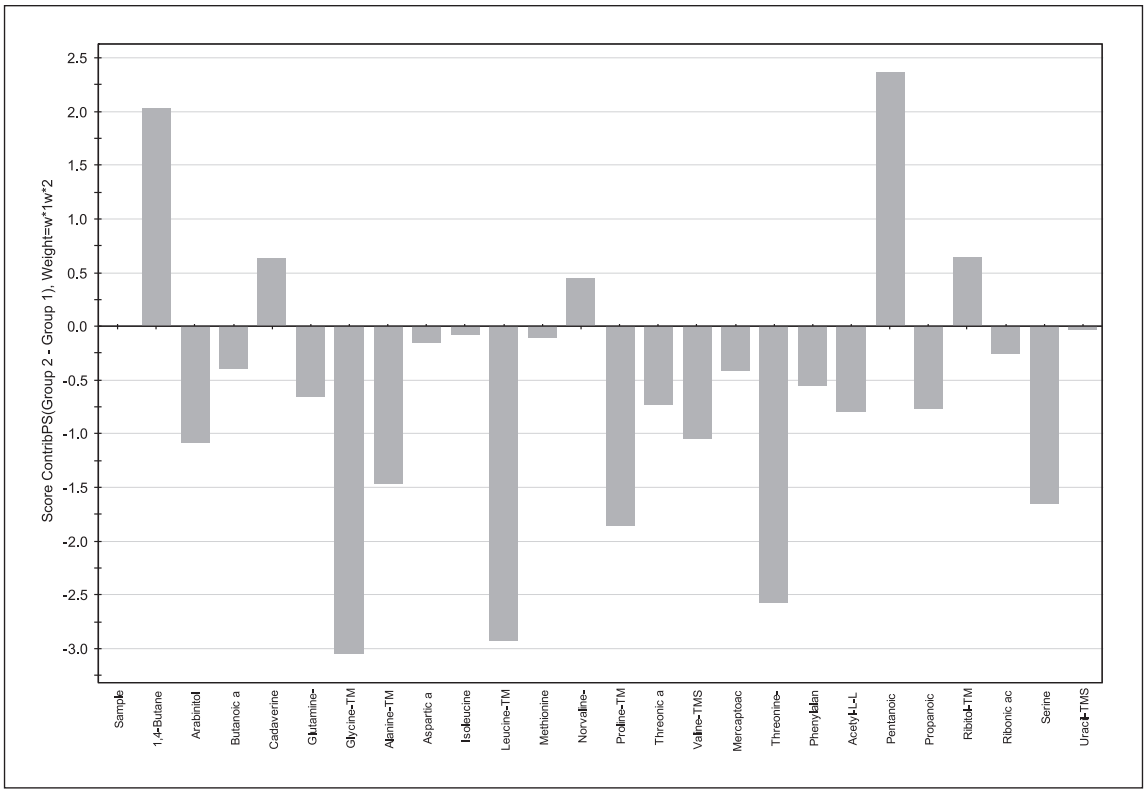

Fig. 3. Contribution plot of VMNI vs SRB1

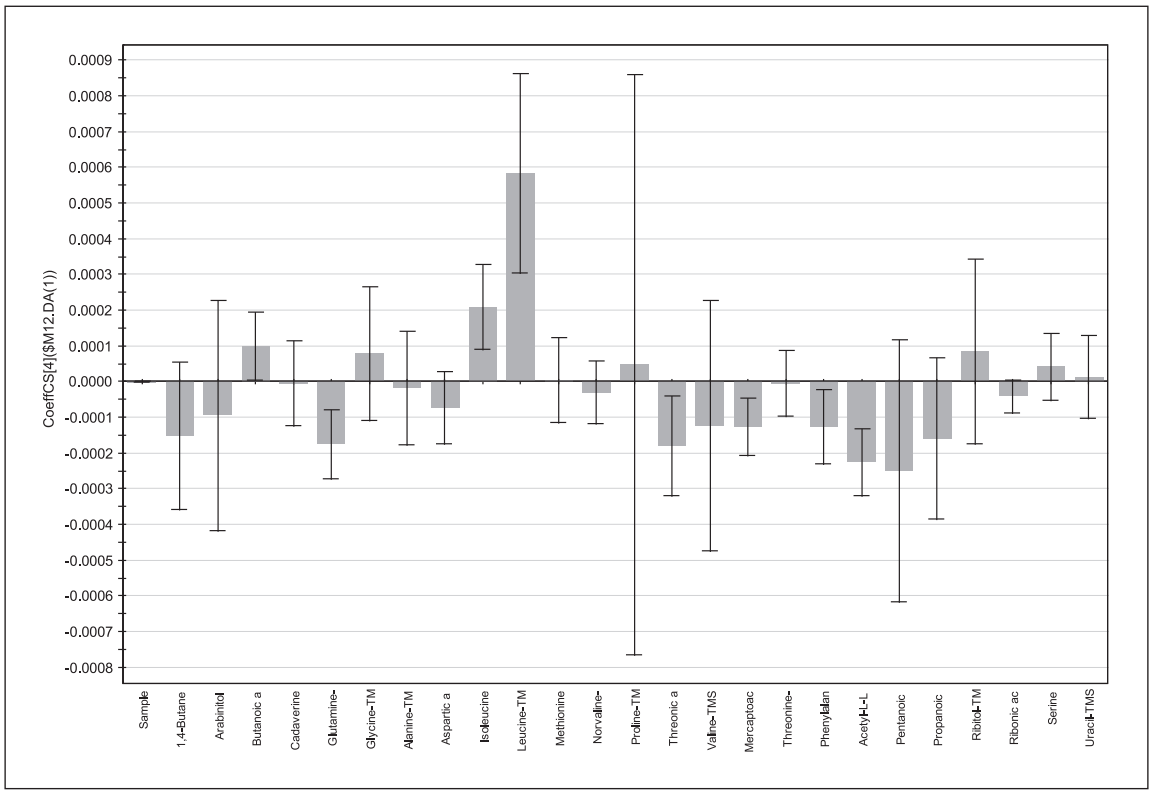

Fig. 4. PSLDA coefficient plot of VMNI vs SRB1

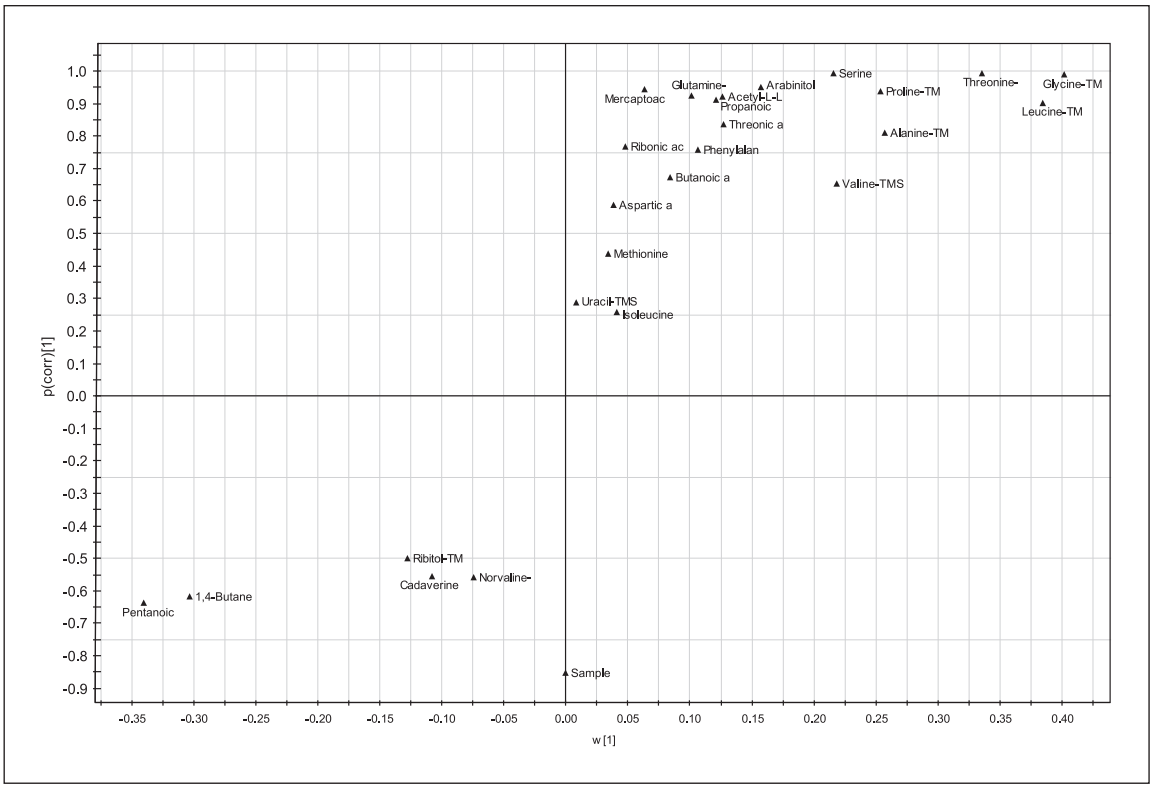

Fig. 5. The PSL-DA S-Plot for VMNI vs SRB1

The same analysis was done for SRB2 where the PLS-DA data is between VMNI vs SRB2. From the contribution plot (Figure 6), 6 metabolites; isoluecine, norvaline, pentanoic acid, proline, methionine and uracil, were showed to be express in SRB2. The yield result of these compounds is very small as shown in the coefficient plot (Figure 7). The S plot (Figure 8) for this group showed a good separation of the compound, same as SRB1.

DISCUSSION

The determination of formation of biofilm that occured in the sample can be constructed based on the metabolites detected. Comparison of higher productivity bacteria can be made through development of pathway of nutrients utilization and depletion by determination of energy producing pathway. Comparison between SRB1 and SRB2 can be made from the metabolites that were yield from their metabolisms activity, as these metabolites were produced or less used by each of them. SRB1 contain a lesser number of specific metabolites but yield a greater concentration compared to SRB2. On the other hand SRB2 generate a variety of compounds compared to SRB1. An overview on each metabolite production for both sample can be made using the KEGG Pathway Database.

As mentioned earlier The PLS-DA-derived loading plot indicates norvaline and pentanoic acid to associate with SRB1 and SRB2. In general, norvaline is a non-usual amino acid analogs that may be formed under certain circumstances as byproducts of the branched-chain amino acid biosynthetic of Gram-negative microorganisms. This amino acid can accumulate and secreted under the depletion of oxygen (Soini *et al.,* 2008) where the accumulation of norvaline can inhibit urea synthesis (Grunnet & Joseph 1978). Norvaline can be intentionally incorporated into proteins either by semi-synthesis at designed positions or by feeding microorganisms expressing heterologous proteins with norvaline (Apostol *et al.,* 1997). It can also be found as by-product in isoleucine fermentation from threonine along with homoisoleucine. It is suggested that L-norvaline and L-homoisoleucine formation is closely related to the leucine biosynthesis (Kisumi *et al.,* 1976). Earlier experiments have convincingly shown that norvaline and norleucine are formed from pyruvate being an alternative substrate of α-isopropylmalate synthase. Norvalinewas found to be incorporated in minor amounts in heterologous proteins with a high leucine or methionine content (Soini *et al.,* 2008).

Pentanoic acid that is detected along with norvaline in both medium is actually a degradation product of norvaline. It had a 2-aminopentanoic

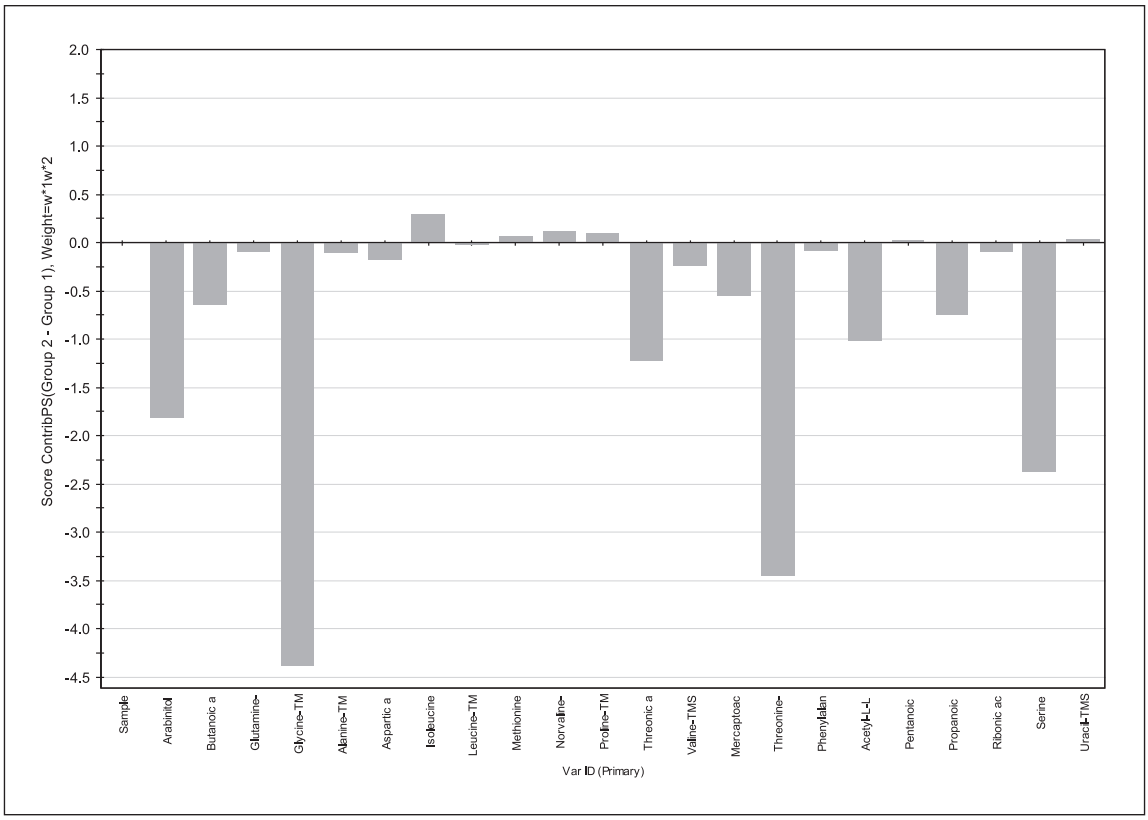

Fig. 6. PSL-DA contribution plot of VMNI vs SRB2

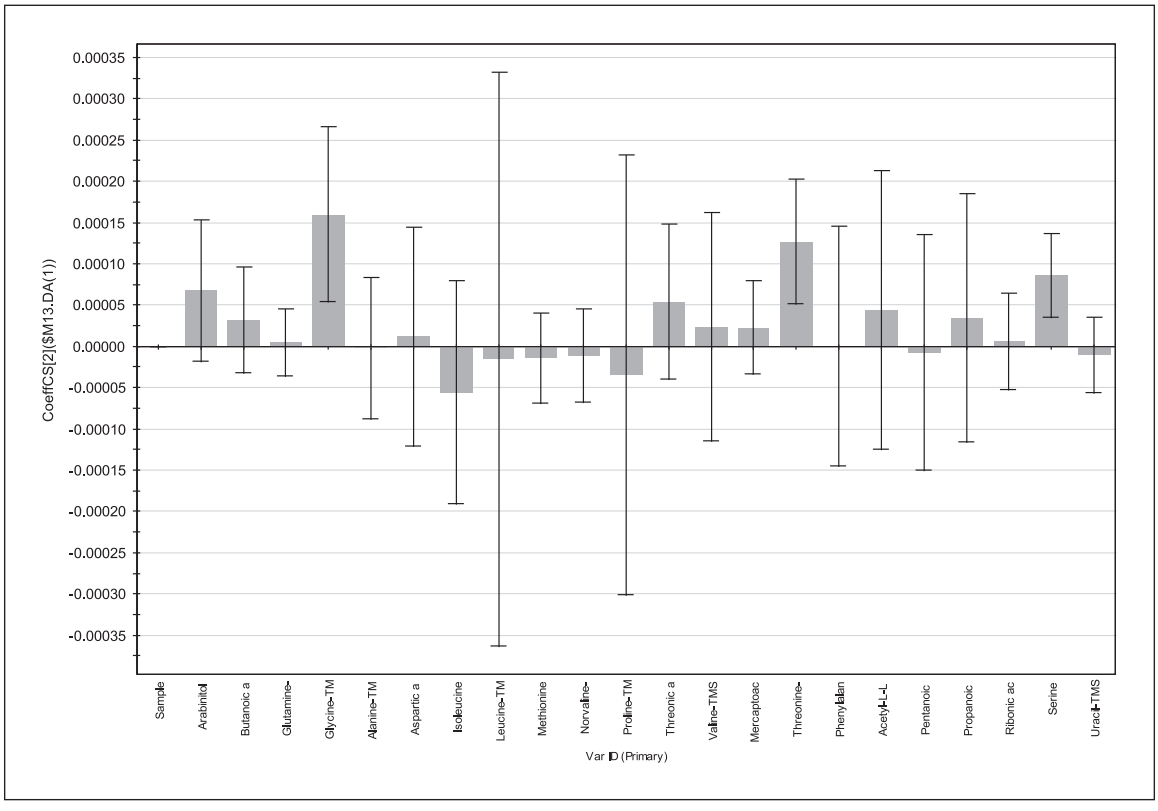

Fig. 7. PSLDA coefficient plot of VMNI vs SRB2

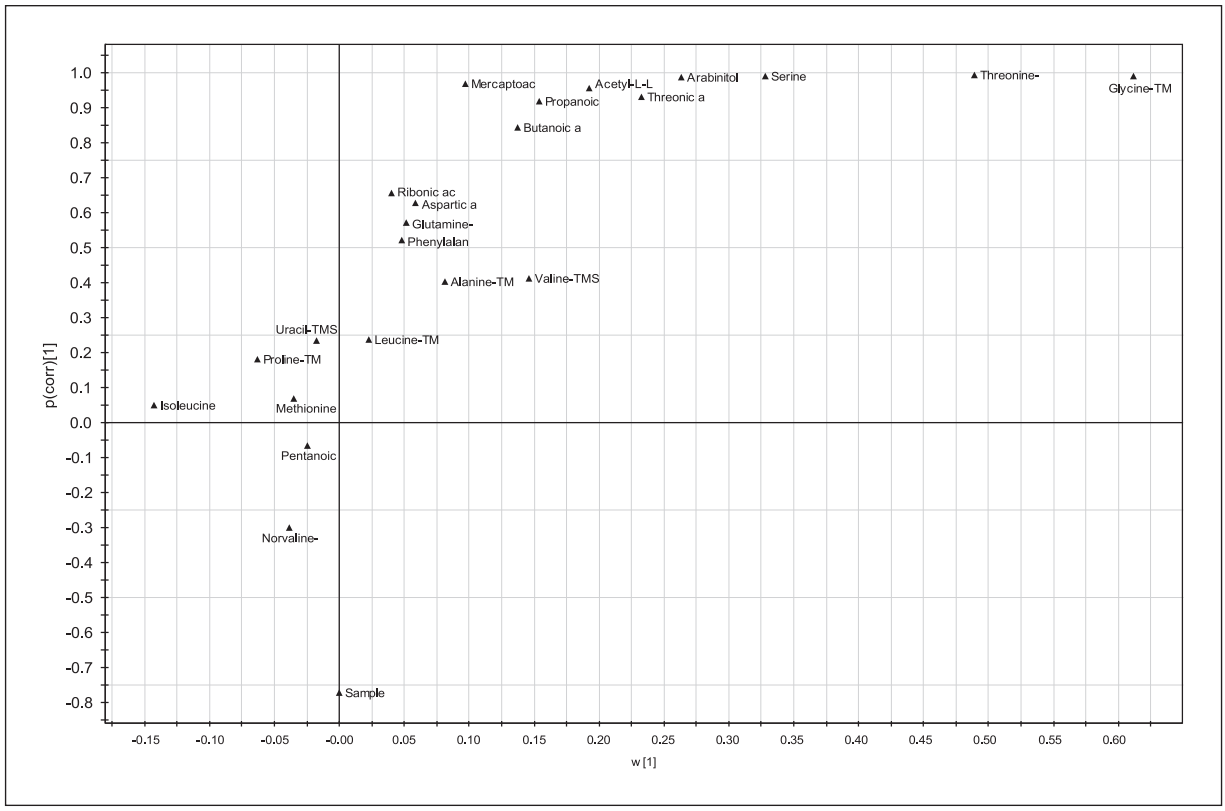

Fig. 8. The PSL-DA S-Plot for VMNI vs SRB2

acid structure (Kisumi *et al.,* 1976, Soini *et al.,* 2008). The separation of amino group from norvaline causes the accumulation of pentanoic acid. Other theory suggested that proline that undergoes reductive ring cleavage and deamination at the δ-position, form valeric acid also known as pentanoic acid (Dehoritya *et al.,* 1958).

SRB2 consist of high amount of Isoleucine and methionine that correlate with the production of norvaline. Isoleucine is synthesizes from the valine, leucine and isoleucine pathway where it was derived from threonine and synthesize by 5 reactions which are catalyzed by threonine dehydratase (Eikmanns, Eggeling & Sahm 1993, Tang *et al.,* 2009). While most bacteria employthe threonine pathway to form isoleucine, some anaerobic bacteria and archaea, such as *Methanococcus jannaschii* and *Geobacter sulfurreducens*, can synthesize isoleucine from citramalate via condensation of acetyl-CoA and pyruvatecatalysed by citramalate synthase (CimA) (Tang *et al.,* 2009, Wu *et al.,* 2010). Methionine is a sulfur containing amino acid (Rosen *et al.,* 2009). There are two alternative pathways of methionine synthesis in microorganisms. The transsulfuration pathway involves cystathionine as an intermediate and utilizes cysteineas the sulfur source while the other one is the direct sulfhydrylation pathway by passes cystathionine and uses inorganic sulfur instead (Rodionov *et al.,* 2004). Sulde is produced from sulfate during assimilatory sulfate reduction for the synthesis of cysteine and methionine (Wang *et al.,* 2000).

Both of isoleucine and methionine are interrelated as homoserine becomes the common precursor for these amino acids from the aspartate family (isoleucine, threonine and methionine) (Rodionov *et al.,* 2004). Homoserine is converted to methionine through homocysteine in the cysteine and methionine metabolism pathway, or it can be converted straight to threonine and enter the valine, leucine and isoleucine pathway.

In this study, both SRB1 and SRB2 showed the utilization of nitrogen in their metabolism. SRB1 show a production of biogenic amines (BA), putrescine and cadaverine, that are low-molecular nitrogenous basic compounds (Wunderlichová *et al.,* 2012). The diamines putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) are found in high concentrations(mmol/ L) in all major groups of marine organisms (Landete *et al.,* 2010) because they are part of a group of natural polyamines which serve as stabilizing cations of the macromolecular structure of DNA and RNA (Hofled 1984). Several authors had classified cadaverine and putrescine among polyamines (Wunderlichová *et al.,* 2012). Sulfate Reducing bacterias have been related to

producing these two substances by dissimilatory of sulfur with the degradation of amino acid in an anaerobic environment (Willis *et al.,* 1999). The source for these substances is the decarbocylation of proteins and aminoacids (Wunderlichová *et al.,* 2012). Deamination of lysine or ornithine results in cadaverine or putrescine, respectively (Hofled 1984, Landete *et al.,* 2010) in the arginine and proline metabolism, where a traced of proline can be seen in SRB2.

Proline is a proteinogenic amino acid with an exceptional conformational rigidity, and is essential for primary metabolism (Szabados & Savoure 2009) as well as bioenergetics (Tanner 2008). Proline is associated with the proline and arginine metabolism that occured in the urea cycle. The involvement of glutamine in the metabolism linked proline to uracil. Uracil was detected as one of SRB1 metabolism product. Production of uracil is usually associated with the pyrimidine metabolism. The deamination of cystosine to uracil produced ammonia which leads to precipitation of metal. The identification of proline and uracil is interconnected as both represent the nitrogen base metabolite. Glutamine degradation in arginine and proline metabolism forming Carbonyl-P can be utilized in the pyrimidine metabolism that yields uracil production.

Ribitol is a metabolite that was formed in SRB1 metabolism. According to Kegg pathway, ribitol is associated with the Riboflavin metabolism.There are some bacteria recover ribitol (adonitol) as a carbon source from riboflavin (Reiner 1975, Phillips et al. 1999). Some bacteria use free ribitol to be converted back to riboflavin. Ribitol can be converted to ribulose in the penthose glucornate pathway. This reaction is vice versa. It was reported that certain bacteria growth is intensify with the free ribitol applied into the medium of growth compare to ribose and ribulose where the sudden increase in the number of bacteria at the early stage of growth curve occur (Helanto *et al.,* 2007, Mehta *et al.,* 1972).

An organism produces sufficient amount of different amino acids during its normal growth, to meet its needs for proteins synthesis. If in certain cases a particular amino acid is required, the production of other amino acids being produced at the same time ceases through a complex regulatory control (Bajwa *et al.,* 2010). The different production of metabolites for SRB1 and SRB2 indicates different type of requirement for their growth.

CONCLUSIONS

There are five metabolites that is unique for SRB1 that is 1-4-butanediamine, cadaverine, norvaline, pentanoic acid and ribitol, and six metabolites for SRB2, isoluecine, norvaline, pentanoic acid, proline, methionine and uracil. Both strain do share the same metabolism activity that involve the same group of substances; the usage of nitrogen base compound and production of norvaline. SRB1 showed a production of ribitol that act as carbon source, indicating the growth activity of SRB1. SRB2 showed a distinct usage of sulfate when it produced methionine. Overall, metabolites produce by both SRB strain is slightly different form each other.

ACKNOWLEDGEMENTS

This work was supported by FRGS grant (Vot UKM-ST-07-FRGS0040-2009) and DIP-2012-0023. The authors would like to thank all the staff from Marine Microbiology Lab and INBIOSIS for their helps and cooperation.

REFERENCES

Apostol, I., Levine, J., Lippincott, J., Leach, J., Hess, E., Glascock, G.B., Weickert, M.J. & Blackmore, R. 1997. Incorporation of norvaline at leucine positions in recombinant human hemoglobin expressed in *Escherichia coli*. The Journal of Biological Chemistry. **272**(46): 28980-28988.

Andersen, T.E., Kingshott, P., Palarasah, Y., Alei, M. & Kolmos, H.J. 2010. A ow chamber assay for quantitative evaluation of bacterial surface colonization used to investigate the inuence of temperature and surface hydrophilicity on thebiolm forming capacity of uropathogenic Escherichia coli. Journal of Microbiological Methods. **81**: 135-140.

Azizan, K.A., Baharum, S.N., Ressom, H.W. & Nor, N.M. 2012. GCMS analysis and PLS-DA validation of the TMS derivation techniques. American Journal of Applied Science.

Baena, S., Fardeau, M.L., Labat, M., Ollivier, B. & Patel, B.K.C. 1998. *Desulfovibrioaminophilus* sp. nov., a novel amino acid degrading and sulfate reducing bacterium from an anaerobic dairy wastewater lagoon system. Application Microbiology. **21**: 498-504.

Bajwa, M.A., Zahoor, T., Butt, T.M., Atiq, M. & Sahi, S.T. 2010. Microbial production of L-isoleucine from different substrates using locally isolated bacteria. International Journal of Agriculture & Biology. 12(5).

Beale, D.J., Dunn, M.S. & Marney, D. 2010. Application of GC–MS metabolic proling to 'blue-green water' from microbial inuenced corrosion in copper pipes. Corrosion Science. **52**: 3140-3145.

Cordas, C.M., Guerra, L.T., Xavier, C. & Moura, J.J.G. 2008. Electroactive biolms of sulphate reducing bacteria. Electrochimica Acta. **54**: 29-34.

Castaneda, H. & Benetton, X.D. 2008. SRB-biolm inuence in active corrosion sites formed at the steel-electrolyte interface when exposed to articial seawater conditions. Corrosion Science. **50**: 1169-1183.

Dehoritya, B.A., Johnsona, R.R., Bentleya, O.G. & Moxon, A.L. 1958. Studies on the metabolism of valine, proline, leucine and isoleucine by rumen microorganisms in vitro. Archives of Biochemistry and Biophysics. **78**(1): 15-27.

Eikmanns, B.J., Eggeling, L. & Sahm, H. 1993. Molecular aspects of lysine, threonine, and isoleucine biosynthesis in *Corynebacterium-glutamicum*. Antonie van Leeuwenhoek. **64**: 145-163.

Fardeau, M.L., Patel, K.C., Magot, M. & Ollivier, B. 1997. Utilization of serine, leucine, isoleucine, and valine by *Thermoanaerobacter brockii* in the presence of thiosulfate or *Methano-bacterium sp*. as electron acceptors . Anaerobe. **3**: 405-410.

Grunnet, N. & Joseph, K. 1978. Effects of Ammonia and Norvaline on Lactate Metabolism by Hepatocytes from Starved Rats. Journal of Biochemical. **172**: 595-603.

Hansen, L.S. & Blackburn, T.H. 1995. Amino acid degradation by sulfate-reducing bacteria: Evaluation of four methods. Limnology and Oceanography. **40**(3): 502-510.

Helanto, M., Kiviharju, K., Leisola, M. & Nyysso¨la¨ A. 2007. Metabolic Engineering of Lacto-bacillus plantarum for Production of L-Ribulose. Applied and Environmental Microbiology. **73**(21): 7083-7091.

Hofled, M.G. 1984. Degradation of Putrescine and Cadaverine in Seawater Cultures by Marine Bacteria. Applied and Environmental Micro-biology. **47**(4). 843-849.

Kisumi, M., Suguira, M., Kato, J. & Chibata, I. 1976. L-Norvaline and L-Homoisoleucine Formation by Serratiamarcescens. Journal of Biochemical. **79**(5): 1021-1028.

Landete, J.M., Arena, M.E., Manca de Nadra, M.C., Pardo, I. & Ferrer, S. 2010. The role of two families of bacterial enzymes in putrescine synthesis from agmatine via agmantine Deceased. International Microbiology. **13**: 169-177.

Mehta, S.U., Matoo, A.K. & Modi, V.V. 1972. Ribitol and Flavinogenesis in Eremothe-ciumashbyii. Journal of Biochemical. **130**: 159-166.

Phillips, D.A, Joseph, C.M., Yang, G.P., Martínez-Romero, E., Sanborn, J.R. & Volpin, H. 1999. Identification of lumichrome as a Sinorhizobium enhancer of alfalfa root respiration and shoot growth. Agricultural Journal. **96**(22): 12275-12280.

Reiner, A.M. 1975. Genes for Ribitol and D-Arabitol Catabolism in Escherichia coli: Their Loci in C Strains and Absence in K-12 and B Strains. Journal of Bacteriology. **123**(2): 530-536.

Rodionov, D.A., Vitreschak, A.G., Mironov, A.A. & Gelfand, M.S. 2004. Comparative genomics of the methionine metabolism in Gram-positive bacteria: a variety of regulatory systems. Nucleic Acids Research. **32**(11).

Rosen, H., Klebanoff, S.J., Wang, Y., Brot, N., Heinecke, J.W. & Fu, X. 2009. Methionine oxidation contributes to bacterial killing by the myeloperoxidase system of neutrophils. PNAS. **106**(44): 18686-18691.

Soini, J., Falschlehner, C., Liedert, C., Bernhardt, J., Vuoristo, J. & Neubauer, P. 2008. Norvaline is accumulated after a down-shift of oxygen in *Escherichia coli* W3110. Microbial Cell Factories. **7**(30).

Szabados, L. & Savoure, A. 2009. Proline: a multifunctional amino acid. Trends in Plant Science. **15**(2).

Tang, Y.J., Yi, S., Keasling, J.D., Zhuang, W.Q., Zinder, S.H. & Alvarez-Cohen, L. 2009. Investigation of Carbon Metabolism in "Dehalococcoidesethenogenes" Strain 195 by Use of Isotopomer and Transcriptomic Analyses. Journal of Bacteriology. **191**(16): 5224-5231.

Tanner, J.J. 2008. Structural biology of proline catabolism. Amino Acids. **35**: 719-730.

Tor, J.M., Amend, J.P. & Lovley, D.R. 2003. Metabolism of organic compounds in anaerobic, hydrothermal sulphate-reducing marine sediments. Environmental Microbiology. **5**(7): 583-591.

Wang, C.L., Maratukulam, P.D., Lum, A.M., Clark, D.S. & Keasling, J.D. 2000. Metabolic Engineering of an Aerobic Sulfate Reduction Pathway and Its Application to Precipitation of Cadmium on the Cell Surface. Applied and Environmental Microbiology. 4497-4502.

Wargin, A., Olañczuk-Neyman, K. & Skucha, M. 2007. Sulphate-Reducing Bacteria, Their Properties and Methods of Elimination from Groundwater. Polish Journal of Environmental Study. **16**(4): 639-644.

Willis, C.L., Gibson, G.R., Holt, J. & Allison, C. 1999. Negative correlation between oral malodour and numbers and activities of sulphate-reducing bacteria in the human mouth. Archives of Oral Biology. **44**: 665 - 670.

Wu, B., Huang, R., Zhang, B., Hicks, L.M., Feng, X., Pakrasi, H.B., Rubens, J.R. & Tang, Y.J. 2010. Alternative isoleucine synthesis pathway in cyanobacterial species. Microbiology. **156**: 596-602.

Wunderlichová, L., Buòková, L., Koutný, M. & Buòka, F. 2012. The Posibilities of detection of Putrescine Production on Gram-Negative Bacteria. Journal of Microbiology, Biotechnology and Food Sciences. **1**: 848-854.

Xia, J., Psychogios, N., Young, N. & Wishart, D.S. 2009. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucl. Acids Res. 37, W652-660.