Screening & Identification of Oxalate Degrading Bacteria From Environmental samples

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INTRODUCTION

Oxalate degrading enzymes play an important role in maintaining bio-geo chemical cycles and are found to be useful in agriculture, therapeutics, and industries, therefore a potential candidate for bio economy. There are three oxalate degrading enzymes known, namely Oxalate decarboxylase (ODC), Oxalate Oxidase (OXO), and Oxyalyl CoA decarboxylase, which tend to only function at a low pH.
Humans lack oxalate degrading enzymes and there have been attempts to develop probiotic oxalate degrading bacteria for prophylactic use in managing kidney stones, However, with limited success. Other uses of oxalate degrading enzymes, primarily OXO works by removing oxalic acid from bleaching filtrates to prevent problems with scaling in paper and pulp industries. Identifying these enzymes which can function at a higher pH would have better application. As bacteria have adopted to different environments, there is a lot of scope to screen for oxalate degrading bacteria with different functional pH in Malaysia.

AIMS

- To develop a protocol that can screen for oxalate degrading bacteria.
- To Perform Agar well diffusion assay

Fig1. Root nodules of *Mimosa Pudica*

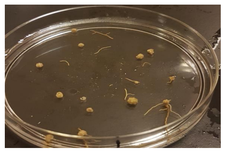

Fig2. Root nodules of *Mucuna Bracteata* in a petridish filled with distilled water..

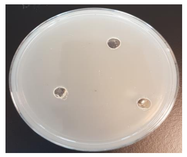

Fig3. Agar Plate with wells created using a cork borer

- To perform preliminary characterization tests for the samples

MATERIALS AND METHODS

To compare and contrast the effects of samples when subjected to an environment with and without oxalate, YEM agar plates were prepared[1] and 0.5% of di-ammonium oxalate monohydrate(A.O) was added to create an oxalate rich environment.

A wide range of environmental samples such as soil and root nodules from legumes were collected. Soil samples were collected by following a protocol[2] and diluted to 0.5% and spread on YEM and YEM+A.O agar plates. Root nodules from three different legumes were collected, namely *Mimosa pudica, Mucuna bracteata, and Crotolaria retusa* which underwent a series of washing steps.[3] Root nodules were cultured by streaking on YEM and YEM+A.O agar plates.

All samples were incubated at 28° C for two days in an incubator.

Agar well diffusion assay was performed by making wells in YEM+A.O agar plates using a cork borer, and 60ul of different nutritional media sets with isolated bacterial colonies were pipetted into them. The conditional media was made to force the isolated bacteria to maximize its utilization of oxalate, by restricting other available nutrients present in Yeast Extract Mannitol. They were pipetted into the wells prior to a 48 hour session in a shaking incubator at 30 ° C. Gram staining and Catalase test were performed for prelimenery characterization. [4,5]

RESULTS

All samples flourished when grown on YEM+A.O plates. This effect was most prominently observed in *Crotolaria retusa*. The bacterial colonies turned green in YEM+A.O plates and remained white on YEM plates. There was also a difference in colony diameter and growth rate, both of which were greater when grown on YEM +A.O plates

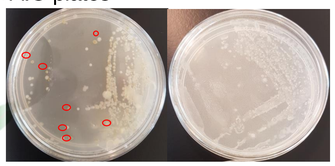

Fig.4,5 Green colonies of *Crotolaria retusa* observed on YEM+A.O plate (left) White colonies of *Crotolaria retusa* observed on YEM plate (right) Few green colonies are depicted with red highlights.

Fig 7. Agar well is surrounded with green colonies of *Crotolaria retusa*.The diffusion assay is with a YEM + A.O agar plate, showing growth of *Crotolaria retusa's* green colonies which was first cultured in conditional media 5, for 48 hours in a shaking incubator at 30 ° C. 60 ul of the cultured bacteria was pipetted into the agar well and incubated for 48 hours to measure zone diameter. However, results were inconclusive as no 'halo' was observed. Hence, zone diameter was not calculated.

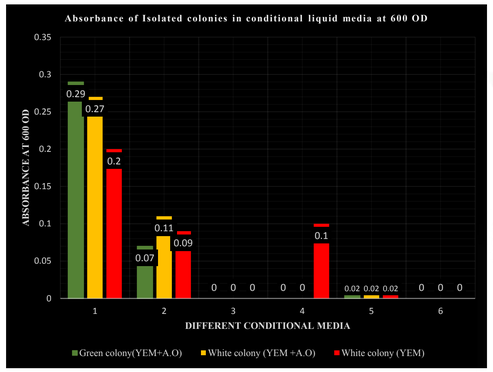

Fig 6.Absorbance of isolated bacterial colonies from Crotolaria retusa grown in different conditional media in shaking incubator at 30 ° C for 48 hours. Green and white colonies from YEM + A.O plate and white colonies from YEM plate. Components of the conditional media are as follows: (1. Magnesium sulfate heptahydrate, Sodium Chloride, Dipotassium hydrogen phosphate trihydrate,Sodium Gluconate and Di-ammonium oxalate monohydrate) (2. Sodium Chloride, Dipotassium hydrogen phosphate trihydrate,Sodium Gluconate and Di-ammonium oxalate monohydrate) (3. Dipotassium hydrogen phosphate trihydrate,Sodium Gluconate and Di-ammonium oxalate monohydrate) (4. Sodium Gluconate and Di-ammonium oxalate monohydrate) (5. Di-ammonium oxalate monohydrate and Yeast Extract) (6. Di-ammonium oxalate monohydrate) Results show that when all colonies were grown in conditional media 5, the absorbance reading were uniform and low which was suitable for the Agar well diffusion assay as the test was based on growing bacteria with limited nutrition and subjecting them to an environment with an abundance of nutrition, i.e YEM+A.O with wells to observe the formation of a 'halo' around the well which could demonstrate the utilization of oxalate.

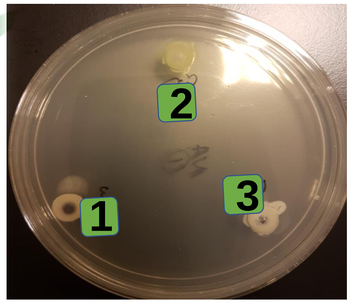

Fig8. Bacterial colonies isolated from *Crotolaria retusa* grown on a Nutrient agar + A.O and Incubated at 30 ° C for 48 hours. No 'halo' observed. 1 represents the white colonies of Crotolaria retusa found on the YEM plate, 2 and 3 represents the green and white colonies of Crotolaria retusa found on the YEM +A.O plate

Fig 11. Catalase test being carried out. 1.Green colony *Crotolaria retusa* (YEM +A.O plate) 2.White colony *Crotolaria retusa*(YEM plate) 3. White colony *Crotolaria retusa* (YEM +A.O plate) 4.Soil samples from Gunung Pulai,Johor,Malaysia. 5.*Mucuna Bracteata* (Yellow bacterial colonies) 6.*Mucuna Bracteata* (white colonies). This test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate those bacteria that produces an enzyme catalase, such as *staphylococci*, from non-catalase producing bacteria such as *streptococci*. Sample 5, *Mucuna Bracteata* (Yellow bacterial colonies) showed very strong effervescence of 7cm high. Samples 2,3, and 6 showed no effervescence whereas samples 1 and 2 showed little effervescence.

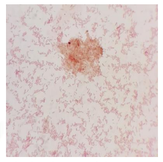

Fig 9. Picture of Green colony of *Crotolaria retusa* Gram stained and under 100X oil immersion. Bacteria is rod shaped and appears red. Hence gram negative. The clumped cluster at the centre has a green residue

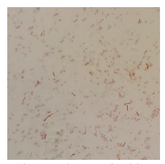

Fig 10. Picture of White colony of *Crotolaria retusa* Gram stained and under 100X oil immersion. Bacteria is rod shaped and appears red. Hence gram negative.

CONCLUSION

Table 1. The objectives, outcomes and inferences of this project.

OBJECTIVES	OUTCOME	INFERENCE
To develop a protocol that can screen for oxalate degrading bacteria	There was progress, in terms of the difference in appearance of the bacterial colonies when subjected to an environment with and without oxalate, however more data is required to prove the presence of oxalatrophs	Different conditional media assays might prove the presence of oxalatrophs
To collect a wide range of environmental samples such as soil and root nodules from legumes.	Root nodules of three legumes namely *Mimosa Pudica, Mucuna bracteata, Crotolaria retusa* and soil samples from 'Gunung Pulai, Johor,Malaysia' were also collected.	Prominent changes were observed in *Crotolaria retusa* as a few bacterial colonies of the legume turned green when subjected to an environment with oxalate and remained white in the absence of it.
To compare and contrast the effects of samples when subjected to an environment with and without oxalate.	Bacterial colonies were observed to flourish in an environment with oxalate. Growth rates and colony diameter were prominently greater.	
To force the isolated bacteria to maximize its utilization of oxalate, by restricting other available nutrients present in the recipe of Yeast Extract Mannitol media.	The various conditional medias prepared did give different OD readings and they managed to restrict the growth of colonies in media 3, 4, and 6.	Media 5 with least OD readings of available colonies and was used for the agar well assay. By restricting nutrition initially and then furnishing nutrition present in YEM+A.O was done to get a 'halo', which would signify oxalate utilization.
To Perform Agar well diffusion assay	This Assay was executed using YEM+A.O and Nutrient agar + A.O	Results were inconclusive as no 'halo' was obtained. There was only the growth of bacterial colonies surrounding the wells.
To perform preliminary characterization tests for the samples	Gram staining and a Catalase test were performed for all the samples collected	All samples turned out to be rod-shaped and red, Gram-negative bacteria.

FUTURE WORK

DNA of all samples were isolated and stored for future use. If a screening method were to be developed, new oxalatrophs which operated at a higher pH could be developed into a probiotic through genetic engineering for prophylactic use in managing kidney stones.

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REFERENCES

1. Untergasser A. "Media - YEM (YEast extract - Manitol)" *Untergasser's Lab*. Summer 2008. http://www.untergasser.de/lab/media/media_yem_v1_0.htm
2. YouTube. (2017). *How to Collect a Soil Sample for Lab Analysis*. [online] Available at: https://www.youtube.com/watch?v=eo2pSRmSZ58 [Accessed 17 Jul. 2017].
3. YouTube. (2017). *Isolating rhizobia from root nodules*. [online] Available at: https://www.youtube.com/watch?v=I2faIR7qB3Q [Accessed 1 Jul. 2017].
4. Iwase, T., Tajima, A., Sugimoto, S., Okuda, K., Hironaka, I., Kamata, Y., Takada, K. and Mizunoe, Y. (2017). *A Simple Assay for Measuring Catalase Activity: A Visual Approach*. [online] Available at: https://www.nature.com/articles/srep03061 [Accessed 18 Aug. 2017].
5. Uphs.upenn.edu. (2017). *How to perform a Gram stain*. [online] Available at: http://www.uphs.upenn.edu/bugdrug/antibiotic_manual/Gram2.htm [Accessed 11 Aug. 2017].