MEDIUM OPTIMIZATION OF *Lactobacillus casei* CULTIVATION FOR CELL MASS PRODUCTION IN SEMI-INDUSTRIAL SCALE BIOREACTOR

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DEDICATION

This thesis is dedicated to the memory of my father, Mr.Eyahmalay. Although he is not here physically, I feel his presence that motivates me to achieve my goals in life. I also dedicate this thesis to my mother, Mdm. Jayseeli whom have been always showing unconditional love and care to me. I am blessed and thankful to have parents like them.

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ABSTRACT

Good health is an essential key for a happy life. Probiotics have numerous health benefits, hence it has gained a big global market for commercialization. The main challenge in the probiotic commercialization is the bioprocessing limitation to produce high cell mass, especially when involving the cultivation of lactic acid bacteria (LAB) which produces lactic acid as its byproduct. Low cell growth and lactic acid accumulation which leads to cell death are the challenges in large scale bioreactor cultivation. In this study, cultivation media of *Lactobacillus casei* strain WICC-B26 was optimized in shake flask through one factor at a time (OFAT) method and response surface methodology (RSM). The OFAT optimized medium consists of 50 $g.L^{-1}$ lactose, 60 $g.L^{-1}$ soybean meal, 6 $g.L^{-1}$ yeast extract and 0.4 g. L^{-1} $MgSO_4$. The OFAT optimized medium produced 6.9 g. L^{-1} cell mass of *Lactobacillus casei*, which was higher than the RSM optimized media (6.52 g. L^{-1}). Hence, the OFAT medium was selected for the fermentation to choose the best pH condition in 16 L stirred tank bioreactor. The uncontrolled pH condition produced higher cell mass (42.68%) compared to the pH controlled condition pH 6.5. After selecting the pH condition, the effect of different aeration condition was studied in the 16 L stirred tank bioreactor. The maximum biomass produced by the carbon dioxide sparging (0.2 vvm CO_2 gas) and cultivation with no aeration were 6.16 and 5.9 g. L^{-1} respectively. The results show that all the aeration conditions, no aeration, 0.2 vvm CO_2 sparging and microaerophilic condition (0.2 vvm air) produced similar outcome in terms of the biomass produced, which means *Lactobacillus casei* is able to grow in all 3 aeration conditions applied in this research. The outcome of this research is expected to serve as a basis for further research with complete anaerobic and fed-batch cultivation to improvise the production of *Lactobacillus casei* biomass in the future.

ABSTRAK

Kesihatan yang baik merupakan kunci utama kehidupan bahagia. Probiotik mempunyai banyak manfaat ke atas kesihatan dan oleh itu, probiotik telah mendapat pasaran global yang besar untuk pengkomersialan. Cabaran utama dalam pengkomersialan probiotik adalah kekangan bioprocess untuk pengeluaran jisim sel tinggi, terutamanya apabila melibatkan bakteria asid laktik yang mengeluarkan asid laktik sebagai produk sampingan. Pertumbuhan sel yang rendah dan pengumpulan asid laktik yang menyebabkan kematian sel adalah cabaran dalam pengkulturan pada bioreaktor skala besar. Dalam kajian ini, pengoptimuman media pengkulturan *Lactobacillus casei* jenis WICC-B26 pada skala kelalang goncang telah dilakukan mengunakan kaedah satu faktor pada satu masa (OFAT) dan kaedah sambutan permukaan (RSM). Medium optimum OFAT terdiri daripada 50 $g.L^{-1}$ laktosa, 60 $g.L^{-1}$ tepung kacang soya, 6 $g.L^{-1}$ ekstrak yis dan 0.4 $g.L^{-1}$ $MgSO_4$. Medium optimum OFAT menghasilkan jisim sel *Lactobacillus casei*, iaitu 6.9 $g.L^{-1}$, yang lebih tinggi daripada media optimum RSM (6.52 $g.L^{-1}$). Oleh itu, medium OFAT dipilih bagi proses penapaian untuk memilih keadaan pH terbaik dalam bioreaktor tangki teraduk 16 L. Keadaan pH yang tidak terkawal menghasilkan jisim sel yang lebih tinggi sebanyak 42.86% berbanding dengan pH yang dikawal pada pH 6.5. Selepas memilih keadaan pH, kesan keadaan pengudaraan yang berbeza telah dikaji dalam bioreaktor tangki teraduk 16 L. Jisim sel maksimum yang dihasilkan dalam pengkulturan dengan gas karbon dioksida (0.2 vvm) dan pengkulturan tanpa pengudaraan masing-masing adalah pada 6.16 dan 5.9 $g.L^{-1}$. Keputusan menunjukkan bahawa keadaan pengudaraan tanpa udara, dengan pengaliran 0.2 vvm CO_2 dan keadaan mikroerofilik (udara 0.2 vvm) menghasilkan biojisim yang sama dan ini bermaksud *Lactobacillus casei* mampu untuk berkembang dalam kesemua keadaan pengudaraan yang digunakan dalam kajian ini. Hasil penyelidikan ini dijangka menjadi asas untuk penyelidikan selanjutnya dalam pengkulturan penuh secara anaerobik dan pemberian makanan berkumpul bagi menambahbaik pengeluaran jisim sel *Lactobacillus casei* pada masa akan datang.

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LIST OF ABBREVIATIONS

ATP	-	Adenosine triphosphate
C/N	-	Carbon to nitrogen ratio
CDW	-	Cell Dry Weight
CO_2	-	Carbon Dioxide
CP	-	Crude Protein
DNA	-	Deoxyribonucleic acid
DO	-	Dissolved Oxygen
EMP	-	Embden-Meyerhof Pathway
EPS	-	Exopolysaccharide
GI	-	Gastrointestinal
GRAS	-	Generally recognised as safe
GOS	-	Galactaoligosaccharides
HCl	-	Hydrochloric acid
H. pylori	-	*Helicobacter pylori*
K	-	Potassium
K_2HPO_4	-	Dipotassium hydrogen phosphate
LAB	-	Lactic acid bacteria
MRS	-	Man Ragosa Sharpe
$MgSO_4.7H_2O$	-	Magnesium sulfate heptahydrate
$MnSO_4.7\ H_2O$	-	Manganese sulphate heptahydrate
Na	-	Sodium
NaOH	-	Sodium hydroxide
NH_4Cl	-	Ammonium chloride
$(NH_4)_2HPO_4$	-	Ammonium phosphate
$(NH_4)_2SO_4$	-	Ammonium sulphate
O_2	-	Oxygen
OD	-	Optical density
RNA	-	Ribonucleic acid
RPM	-	Rate per minute
RSM	-	Response Surface Methodology

OFAT	-	One factor at a time
LAB	-	Lactic acid bacteria
L. casei	-	*Lactobacillus casei*
LOS	-	Low oxygen supply
NOS	-	No oxygen supply
vvm	-	Volume of air per working volume of bioreactor per minute
WICC	-	Wellness Industry Collection Culture

LIST OF SYMBOLS

%	-	Percentage
°C	-	Degree Celsius
g	-	Gram
$g.L^{-1}$	-	Gram per litre
min	-	Minutes
h	-	Hour
ml	-	Millilitre
mg	-	milligram
μ	-	Specific growth rate
μ_{max}	-	Maximum specific growth rate
L	-	Litre
X	-	Biomass concentration
S	-	Substrate concentration
PX	-	Biomass productivity
P_{max}	-	Maximum production of lactic acid
$Y_{x/s}$	-	Biomass yield
Q_P	-	Lactic acid production rate
Q_{Lac}	-	Lactose consumption rate

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CHAPTER 1

INTRODUCTION

1.1 Research Background

"Let food be thy medicine and medicine be thy food". This famous quote by the great philosopher Hippocrates could be significantly related to the consumer's preference nowadays when it comes to choosing the food they eat. They are giving more importance to the food choices they make, and they seem to opt for a food product that satisfies their hunger and improves their health as well. This preference and increasing health awareness among consumers created a niche for functional food among the conventional food market and thus paved way for the rise of functional food as a billion-dollar market globally.

"Functional foods are natural or processed foods that contains known or unknown biologically-active compounds; which in defined amounts provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease" Martirosyan and Singh (2015).According to Vergari *et al.,* (2010), the global functional food market was worth of USD80 billion in the year 2008, with US, Japan and European market as the dominant players and South East Asian and Middle East nations as the emerging markets.

Jones and Jew (2007) mentioned that probiotics is one of the blooming clusters of functional food (Fig 1.1) and it also holds the pride as the biggest functional food product in the world. It is estimated that there is a 7% growth in the probiotic market annually, with Asian and European region as the main consumers, which further strengthened with a forecast of 48 billion dollars in the next five years (Foligné *et al.,*, 2013). To cater the increasing demand for probiotic based functional food product, research efforts to develop probiotic food product should be intensified and must be made industrially feasible for commercial applications.

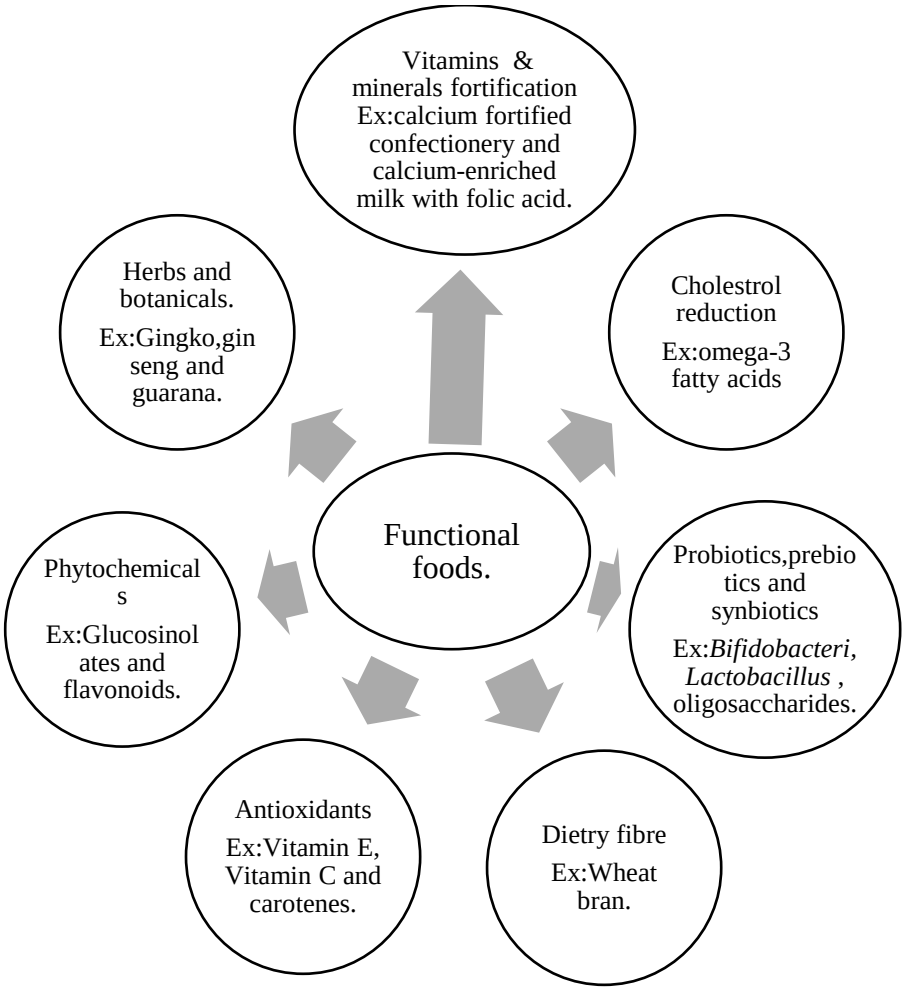

Figure 1.1 Clusters of Functional Food adapted from (Jones and Jew, 2007)

The word “probiotic” originates from Greek which means “for life” and nowadays, probiotic is defined as ‘live microorganism, which when administered in adequate amounts confer a health benefit on the host’ (Kingsley C. Anukam 2007). They mostly belong to the group of lactic acid producing microorganism such as the *Lactobacillus* and *Bifidobacterium*, but not limited to this category only. *Lactobacilli* are an important constituent of a healthy human body microbiome as they are found in the oral cavity, ileum, colon (10^4-10^8 cfu/g and a significant portion of the vaginal microorganism (Borriello *et al.,* 2003).

Lactobacillus casei is the microorganism of interest in this study for high cell mass cultivation. *Lactobacillus casei* strains pose an outstanding genetic variability which allows them to colonize diverse ecological niches, such as the human gastrointestinal tract and eventually obtain wide range of commercial applications (Aguirre-Ezkauriatza *et al.,* 2010). Besides this, the successful commercial application of *L. casei* as probiotics was due to the evident from various clinical studies which proved favorable results for *L. casei*, such as antimicrobial activity, anti-diarrheal activity, anti-mutagenic activity, cholesterol lowering activities, immune system enhancement and lowering of blood glucose level too (Aguirre-Ezkauriatza *et al.,* 2010).

Besides probiotic potential, *L. casei* also shines as a significant microorganism in the fermentation field due to its ability to produce high amount of lactic acid. Fermentation is a beneficial and cost-effective method of producing this compound and its derivatives such as the poly-lactic acid (PLA) (Thakur *et al.,* 2018). Lactic acid and PLA are applied in various industries such as leather, food and polymer industry for leather treatments, food preservation and application for medical (Schiraldi *et al.,* 2003). Therefore, most fermentation and bioprocess engineering research associated with *L. casei* focus on the lactic acid production, rather than the high cell mass for probiotic application (Aguirre-Ezkauriatza *et al.,* 2010).

The production of lactic acid unfortunately inhibits cell growth which makes it challenging to produce dense probiotic biomass of *L. casei*. To cultivate high cell mass of probiotic biomass, the media components play an important role, as each type of nutrient produce different biochemical reaction in the cell which in turn reflects in terms of growth and the metabolites secretion of the particular cell. Different growth media has been used for this bacteria such as the MRS, Eliker's, skim milk and whey media, but all of them have their own limitations in terms of cost efficiency, ability for large scale cultivation and harvesting method (Ha *et al.,* 2003). Hence, those limitations will be taken into account in this study to design an appropriate medium for the high density cultivation of probiotic biomass.

One factor at a time (OFAT) method and statistical analysis using Response Surface Methodology (RSM) will be used to investigate effect of each media component of on the growth and lactic acid production of *L. casei*. OFAT method will be able to describe the effect of individual factors while RSM method improves the media design by optimizing process settings, troubleshoot process problems, and weak points and will result in a media which is robust against external factors (Liong and Shah, 2005). The strategy to use both OFAT and RSM for optimization process is still preferred and reported to be used in previous published works (Dong, 2014).

Moreover, the optimized medium obtained from shake flask level will be used in a 16L bioreactor cultivation to further study and optimize the bioreactor process conditions. Scaling up from shake flask to bioreactor is not just simply a matter of increasing culture and vessel volume, but also dominated by a number of engineering considerations (Hewitt and Nienow, 2010). Hence, bioprocessing parameters such pH and aeration rate were studied in the 16-L bioreactor. An optimum media and bioreactor process conditions will be derived to cultivate high cell mass of *L. casei* with low lactic acid concentration in an industrially feasible scale at the end of this study. Up to date, no report was shown for the effect of pH and aeration of for the biomass production of *L. casei* in 16L bioreactor scale.

1.2 Problem Statement

Probiotics are useful to treat and prevent the occurrence of chronic diseases. The demand for *L. casei* biomass as a probiotic product has increased due to the health benefit that it can give to the host. Developing a fermentation process for biomass production from laboratory scale to a commercial one involves many challenges such as examining the factors affecting the scale-up process.

There were many *Lactobacillus* sp related studies conducted for the production of lactic acid or other bacteriocins; however, there is a limitation on the number of studies describing the *Lactobacillus* sp biomass production for commercial applications (Hwang *et al.*, 2012). The de Man, Rogosa, and Sharp (MRS) medium is

the standard medium used to grow LAB but it is costly, and the fermentation field never stop finding an alternative low-cost medium (Ayad *et al.,* 2020). Besides this, a major engineering challenge to obtain high bacterial cell mass is the secretion of lactic acid by the bacteria themselves, which eventually inhibits their growth.

This problem needs to be resolved by identifying an appropriate medium and cultivation conditions which results in less lactic acid and high cell mass. The experimental design which achieves high cell mass and low lactic acid should be applicable in industrial scale too, not just the laboratory level which is not practical for industrial applications. So, designing an experimental design which fits industrial expectation is also another problem faced here.

1.3 Objectives

i. To optimize medium composition which support cell mass production of *L. casei* for probiotic applications using One Factor At a Time method and Response Surface Methodology.

ii. To investigate the effect of controlled and uncontrolled pH condition in 16-L bioreactor towards high biomass production.

iii. To investigate the effect of different aeration condition on the biomass production of *L. casei* in a 16-L semi industrial scale for cell mass production.

1.4 Scope of study

i. Optimization of medium composition for cell mass production of *L. casei* by using One Factor At a Time method and Response Surface Methodology at shake flask level cultivation.

ii. Batch cultivation strategy under controlled and uncontrolled pH conditions in the 16L bioreactor cultivation. The pH was controlled at pH6.5. The temperature was maintained at 37°C, aeration at 0.2vvm, agitation at 200rpm. 10% inoculum was used to initiate the cultivation and the bioreactor was operated at 8L of working volume.

iii. Batch cultivation strategy with different aeration condition such as 0.2vvm microaerophilic aeration, 0.2vvm CO_2 and 0 aeration in 16L bioreactor cultivation. The initial pH was pH 6.5, temperature maintained at 37°C, agitation at 200rpm. 10% inoculum and with 8L working volume of the bioreactor.

1.5 Research Significance

The increasing demand for food products containing probiotic microorganisms arises a need to establish effective fermentation techniques for probiotic cell mass production. Cell mass is utmost important aspect of a probiotic product because its efficiency is directly proportional to number of viable probiotic cells in the product. Therefore, at the end of the study, an optimum media to produce high cell mass of *L. casei* biomass will be able to be produced in an industrially feasible way. The bioprocessing parameters such as the cultivation media composition, optimum pH condition and optimum aeration condition specifically for *L. casei* biomass production will be found in the end of this work. This will allow new probiotic product development and application of *L. casei* as probiotic in food and pharma industry.

It should be noted that there are many medium optimization and bioreactor cultivation studies conducted for *Lactobacillus* sp, but most are focused on the lactic acid, exopolysaccharide and bacteriocin production. The studies related to *L. casei* cell mass production in bioreactor scale is limited. Hence, this research was done in 16-L bioreactor scale to study the effect of pH and different aeration condition as this could fit the industrial scale cultivation better compared to the shake flask level.

Apart from that, the increased biomass may facilitate the recovery process and reduce production cost. The benefit of higher amounts of biomass may shorten the fermentation time, reduce wastewater volume, and accelerate downstream processes. Moreover, with the new strains that are being continuously found, there is always a need to carry out optimization experiments.

REFERENCES

Aguirre-Ezkauriatza, E., Aguilar-Yáñez, J., Ramírez-Medrano, A. and Alvarez, M. (2010) 'Production of probiotic biomass (*Lactobacillus casei*) in goat milk whey: Comparison of batch, continuous and fed-batch cultures', *Bioresource Technology*, 101(8), pp. 2837-2844.

Ai, Z., Lv, X., Huang, S., Liu, G., Sun, X., Chen, H., Sun, J. and Feng, Z. (2017) 'The effect of controlled and uncontrolled pH cultures on the growth of *Lactobacillus delbrueckii subsp. Bulgaricus', LWT*, 77, pp. 269-275.

Anderson, R. and Jayaraman, K. (2003) 'Influence of carbon and nitrogen sources on the growth and sporulation of *Bacillus thuringiensis var Galleriae* for biopesticide production', *Chemical and biochemical engineering quarterly*, 17(3), pp. 225-232.

Arasaratnam, V., Senthuran, A. and Balasubramaniam, K. (1996) 'Supplementation of whey with glucose and different nitrogen sources for lactic acid production by *Lactobacillus delbrueckii', Enzyme and Microbial Technology*, 19(7), pp. 482-486.

Archambault, J., Williams, R. D., Bédard, C., and Chavarie, C. (1996) 'Production of sanguinarine by elicited plant cell culture in shake flask suspension cultures', *Journal of biotechnology*, *46*(2), pp. 95-105.

Abbasiliasi, S., Tan, J. S., Ibrahim, T. A. T., Bashokouh, F., Ramakrishnan, N. R., Mustafa, S., and Ariff, A. B. (2017) 'Fermentation factors influencing the production of bacteriocins by lactic acid bacteria: a review'. *Rsc Advances*, *7*(47), pp. 29395-29420.

Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gómez-Llorente, C. and Gil, A. (2012) 'Probiotic mechanisms of action, *Annals of Nutrition and Metabolism',* 61(2), pp. 160-174.

Broadbent, J. R., Larsen, R. L., Deibel, V. and Steele, J. L. (2010) 'Physiological and transcriptional response of *Lactobacillus casei* ATCC 334 to acid stress, *Journal of bacteriology',* 192(9), pp. 2445-2458.

Borriello, S., Hammes, W., Holzapfel, W., Marteau, P., Schrezenmeir, J., Vaara, M. and Valtonen, V. (2003) 'Safety of probiotics that contain lactobacilli or bifidobacteria, *Clinical Infectious Diseases',* 36(6), pp. 775-780.

Box, G. and Wilson, K. (1951) Royal Statistical Society–Series B, *Statistical Methodology,* 13, pp. 1.

Boze, H., Moulin, G. and Galzy, P. (1995) 'Production of microbial biomass', *Biotechnology Set, Second Edition*, pp. 165-220.

Brinques, G. B., do Carmo Peralba, M. and Ayub, M. A. Z. (2010) 'Optimization of probiotic and lactic acid production by *Lactobacillus plantarum* in submerged bioreactor systems', *Journal of industrial microbiology & biotechnology,* 37(2), pp. 205-212.

Cerning, J., Renard, C., Thibault, J., Bouillanne, C., Landon, M., Desmazeaud, M. and Topisirovic, L. (1994) 'Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer', *Applied and Environmental Microbiology,* 60(11), pp. 3914-3919.

Chaisu, K., Charles, A. L., Guu, Y.-K., Yen, T.-B. and Chiu, C.-H. (2014) 'Optimization Lactic Acid Production from Molasses Renewable Raw Material through Response Surface Methodology with *Lactobacillus casei* M-15', *APCBEE procedia*, 8, pp. 194^{-1}98.

Chang, C. and Liew, S. L. (2013) 'Growth medium optimization for biomass production of a probiotic bacterium, *Lactobacillus rhamnosus* ATCC 7469, *Journal of Food Biochemistry*, 37(5), pp. 536-543.

Chauhan, K., Trivedi, U. and Patel, K. C. (2007) 'Statistical screening of medium components by Plackett–Burman design for lactic acid production by *Lactobacillus* sp. KCP01 using date juice', *Bioresource Technology*, 98(1), pp. 98-103.

Coghetto, C. C., Vasconcelos, C. B., Brinques, G. B. and Ayub, M. A. Z. (2016) '*Lactobacillus plantarum* BL011 cultivation in industrial isolated soybean protein acid residue, *Brazilian journal of microbiology',* 47(4), pp. 941-948.

Collado, M. C., Isolauri, E., Salminen, S. and Sanz, Y. (2009) 'The impact of probiotic on gut health', *Current drug metabolism,* 10(1), pp. 68-78.

Czitrom, V. (1999) 'One-factor-at-a-time versus designed experiments', *The American Statistician,* 53(2), pp. 126-131.

Dailin, D. J., Elsayed, E. A., Othman, N., Malek, R., RamLi, S., Sarmidi, M. R., Aziz, R., Wadaan, M. A. and El Enshasy, H. A. (2014) 'Development of cultivation medium for high yield kefiran production by *Lactobacillus kefiranofaciens'*, *Int. J. Pharm. Pharm. Sci*, 7, pp. 159-163.

Dash, P. K. M., Sonali Swain, Manas Ranjan Thatoi, Hrudayanath (2017) 'Optimization of bioethanol production from saccharified sweet potato root flour by co-fermentation of *Saccharomyces cerevisiae* and *Pichia* sp. using OFAT and response surface methodologies', *Acta Biologica Szegediensis,* 6.

Dave, M., Higgins, P. D., Middha, S. and Rioux, K. P. (2012) 'The human gut microbiome: current knowledge, challenges, and future directions, *Translational Research',* 160(4), pp. 246-257.

De Lima, C. J., Coelho, L. F. and Contiero, J. (2010) 'The use of response surface methodology in optimization of lactic acid production: focus on medium supplementation, temperature and pH control', *Food Technology and Biotechnology,* 48(2), pp. 175-181.

Dong, Z., Lei Zhang, Juan Wang, Miao Du, Guocheng Chen, Jian Li, Huazhong (2014) 'Optimisation for high cell mass cultivation of *Lactobacillus salivarius* BBE 09-18 with response surface methodology', *International Dairy Journal,* 34(2), pp. 230-236.

Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. and Relman, D. A. (2005) 'Diversity of the human intestinal microbial flora', *Science,* 308(5728), pp. 1635-1638.

El Enshasy, H., Malik, K., Malek, R. A., Othman, N. Z., Elsayed, E. A. and Wadaan, M. (2015) Anaerobic probiotics: the key microbes for human health, In Anaerobes in Biotechnology, pp. 397-431, Springer, Cham.

Elsayed, E. A., Othman, N. Z., Malek, R., Tang, T. and El Enshasy, H. (2014) 'Improvement of cell mass production of *Lactobacillus delbrueckii sp. bulgaricus WICC-B-02*: A newly isolated probiotic strain from mother's milk', *Journal of Applied Pharmaceutical Science,* 4(11), pp. 008-014.

Erickson, D. R. (2015) *Practical handbook of soybean processing and utilization.* Elsevier.

Felis, G. E. and Dellaglio, F. (2007) 'Taxonomy of lactobacilli and bifidobacteria', *Current issues in intestinal microbiology,* 8(2), pp. 44.

Foligné, B., Daniel, C. and Pot, B. (2013) ‘Probiotics from research to market: the possibilities, risks and challenges’, *Current opinion in microbiology*, 16(3), pp. 284-292.

Frey, D. D., Engelhardt, F. and Greitzer, E. M. (2003) 'A role for "one-factor-at-a-time" experimentation in parameter design', *Research in Engineering Design*, 14(2), pp. 65-74.

Fuller, R. (1992) History and development of probiotics, *Probiotics*: Springer Netherlands, pp. 1-8.

Galdeano, C. M. and Perdigon, G. (2006) 'The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity', *Clinical and Vaccine Immunology*, 13(2), pp. 219-226.

Gänzle, M. and Follador, R. (2012) 'Metabolism of oligosaccharides and starch in lactobacilli: a review', *Frontiers in microbiology*, 3, pp. 340.

Gerdes, S. and Overbeek, R. 'Embden-Meyerhof glycolytic pathway and Gluconeogenesis'.

Ge, X.-Y., Xu, Y., Chen, X. and Zhang, L.-Y. (2015) 'Regulation of metabolic flux in *Lactobacillus casei* for lactic acid production by overexpressed ldhL gene with two-stage oxygen supply strategy', *J Microbiol Biotechnol*, 25(1), pp. 81-88.

Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M. and Nelson, K. E. (2006) Metagenomic analysis of the human distal gut microbiome, *science*, 312(5778), pp. 1355-1359.

Giovanni, M. (1983) 'Response surface methodology and product optimization', *Food technology*.

Gold, R. S., Meagher, M., Hutkins, R. and Conway, T. (1992) Ethanol tolerance and carbohydrate metabolism in lactobacilli, *Journal of Industrial Microbiology & Biotechnology*, 10(1), pp. 45-54.

Goldsmith, P. D. (2008) ‘Economics of soybean production, marketing, and utilization’, *Soybeans*: Elsevier, pp. 117-150.

Gomes, A. M. and Malcata, F. X. (1999) ‘*Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics’, *Trends in Food Science & Technology*, 10(4), pp. 139-157.

Granato, D., Branco, G. F., Nazzaro, F., Cruz, A. G. and Faria, J. A. (2010) 'Functional foods and nondairy probiotic food development: trends, concepts, and products', *Comprehensive reviews in food science and food safety,* 9(3), pp. 292-302.

Guyot, J. P., & Morlon-Guyot, J. (2001) 'Effect of different cultivation conditions on *Lactobacillus manihotivorans* OND32T, an amylolytic lactobacillus isolated from sour starch cassava fermentation', *International journal of food microbiology, 67*(3), pp. 217-225.

Gupta, S., Abu-Ghannam, N., & Scannell, A. G. (2011) 'Growth and kinetics of *Lactobacillus plantarum* in the fermentation of edible Irish brown seaweeds', *Food and Bioproducts Processing, 89*(4), pp. 346-355.

Ha, M.-Y., Kim, S.-W., Lee, Y.-W., Kim, M.-J. and Kim, S.-J. (2003) 'Kinetics analysis of growth and lactic acid production in pH-controlled batch cultures of *Lactobacillus casei* KH^{-1} using yeast extract/corn steep liquor/glucose medium', *Journal of Bioscience and Bioengineering,* 96(2), pp. 134-140.

Hill, W. J. and Hunter, W. G. (1966) 'A review of response surface methodology: a literature survey', *Technometrics,* 8(4), pp. 571-590.

Holzapfel, W. and Wood, B. J. (2012) *The genera of lactic acid bacteria.* Springer Science & Business Media.

Hujanen, M. and Linko, Y.-Y. (1996) 'Effect of temperature and various nitrogen sources on L (+)-lactic acid production by *Lactobacillus casei*, *Applied microbiology and biotechnology,* 45(3), pp. 307-313.

Hujanen, M., Linko, S., Linko, Y. Y., & Leisola, M. (2001). Optimisation of media and cultivation conditions for L (+)(S)-lactic acid production by Lactobacillus casei NRRL B-441. *Applied Microbiology and Biotechnology*, *56*(1-2), 126-130.

Hwang, C.-F., Chang, J.-H., Houng, J.-Y., Tsai, C.-C., Lin, C.-K. and Tsen, H.-Y. (2012) 'Optimization of medium composition for improving biomass production of *Lactobacillus plantarum Pi06* using the Taguchi array design and the Box-Behnken method', *Biotechnology and bioprocess engineering,* 17(4), pp. 827-834.

Joint, F. (2002) 'WHO working group report on drafting guidelines for the evaluation of probiotics in food', *London, Ontario, Canada,* 30.

Jones, P. J. and Jew, S. (2007) 'Functional food development: concept to reality', *Trends in Food Science & Technology,* 18(7), pp. 387-390.

Kandler, O. (1983) 'Carbohydrate metabolism in lactic acid bacteria', *Antonie van Leeuwenhoek,* 49(3), pp. 209-224.

Kapila, S. and Sinha, P. (2006) 'Antioxidative and hypocholesterolemic effect of *Lactobacillus casei ssp casei* (biodefensive properties of lactobacilli)', *Indian journal of medical sciences,* 60(9), pp. 361.

Kadam, S. R., Patil, S. S., Bastawde, K. B., Khire, J. M. and Gokhale, D. V. (2006) 'Strain improvement of *Lactobacillus delbrueckii NCIM 2365* for lactic acid production', *Process Biochemistry,* 41(1), pp. 120-126.

Karr-Lilienthal, L., Kadzere, C., Grieshop, C. and Fahey Jr, G. (2005) 'Chemical and nutritional properties of soybean carbohydrates as related to nonruminants: A review', *Livestock Production Science,* 97(1), pp. 1-12.

Karr-Lilienthal, L. K., Grieshop, C. M., Merchen, N. R., Mahan, D. C. and Fahey, G. C. (2004) 'Chemical composition and protein quality comparisons of soybeans and soybean meals from five leading soybean-producing countries', *Journal of Agricultural and Food Chemistry,* 52(20), pp. 6193-6199.

Karska-Wysocki, B., Bazo, M. and Smoragiewicz, W. (2010) 'Antibacterial activity of *Lactobacillus acidophilus* and *Lactobacillus casei* against methicillin-resistant *Staphylococcus aureus* (MRSA)', *Microbiological research,* 165(8), pp. 674-686.

Kashket, E. R. (1987) 'Bioenergetics of lactic acid bacteria: cytoplasmic pH and osmotolerance, *FEMS Microbiology Reviews*, *3*(3), pp. 233-244

Kawai, T. O., T Shin, R Ikawa, S Maeda (2016) 'Determination of the Antibacterial Constituents Produced by Lactobacilli against a Periodontal Pathogen: Sodium Lactate and a Low Molecular Weight Substance', *J Prob Health,* 4(1359), pp. 1-7.

Kingsley C. Anukam , P., MHPM and Gregor Reid PhD, MBA, ARM, CCM (2007) 'Probiotics: 100 years (1907-2007) after Elie Metchnikoff's Observation', *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, pp. 466-474.

Kostov, G., Angelov, M., Denkova, Z., Dobrev, I. and Goranov, B. (2011) 'Lactic acid production with *Lactobacillus casei* ssp. rhamnosus NBIMCC 1013: Modeling and optimization of the nutrient medium', *Engineering in Life Sciences,* 11(5), pp. 517-527.

Lambert, R. and Stratford, M. (1999) Weak-acid preservatives: modelling microbial inhibition and response, *Journal of applied microbiology,* 86(1), pp. 157-164.

Lee, J., Rheem, S., Yun, B., Ahn, Y., Joung, J., Lee, S. J., Oh, S., Chun, T., Rheem, I. and Yea, H. S. (2013) Effects of probiotic yoghurt on symptoms and intestinal microbiota in patients with irritable bowel syndrome, *International Journal of Dairy Technology,* 66(2), pp. 243-255.

Li, H., Zhang, S., Lu, J., Liu, L., Uluko, H., Pang, X., Sun, Y., Xue, H., Zhao, L. and Kong, F. (2014) Antifungal activities and effect of *Lactobacillus casei* AST18 on the mycelia morphology and ultrastructure of *Penicillium chrysogenum*, *Food Control,* 43, pp. 57-64.

Li, Z., Ding, S., Li, Z. and Tan, T. (2006) L-Lactic acid production by *Lactobacillus casei* fermentation with corn steep liquor-supplemented acid-hydrolysate of soybean meal, *Biotechnology Journal,* 1(12), pp. 1453^{-1}458.

Lilly, D. M. and Stillwell, R. H. (1965) 'Probiotics: growth-promoting factors produced by microorganisms', *Science,* 147(3659), pp. 747-748.

Liong, M.-T. (2011) *Probiotics: biology, genetics and health aspects.* Springer Science & Business Media.

Liong, M. and Shah, N. E. (2005) 'Optimization of growth of *Lactobacillus casei* ASCC 292 and production of organic acids in the presence of fructooligosaccharide and maltodextrin', *Journal of food science,* 70(2).

Liu, B., Yang, M., Qi, B., Chen, X., Su, Z. and Wan, Y. (2010a) 'Optimizing l-(+)-lactic acid production by thermophile Lactobacillus plantarum As. 1.3 using alternative nitrogen sources with response surface method', *Biochemical Engineering Journal,* 52(2-3), pp. 212-219.

Liu, M., Bayjanov, J. R., Renckens, B., Nauta, A. and Siezen, R. J. (2010b) 'The proteolytic system of lactic acid bacteria revisited: a genomic comparison', *BMC genomics,* 11(1), pp. 36.

Logothetis, N. and Wynn, H. P. (1994) *Quality through design: Experimental design, off-line quality control, and Taguchi's contributions.* Oxford Univ Pr.

Martirosyan, D. M. and Singh, J. (2015) 'A new definition of functional food by FFC: what makes a new definition unique?', *Functional Foods in Health and Disease*, 5(6), pp. 209-223.

Mateju, Vit. (2017). Re: What is the theoretical yield of lactic acid fermentation from sucrose? Retrieved from: https://www.researchgate.net/post/What_is_the_theoretical_yield_of_lactic_acid_fermentation_from_sucrose/587c6fd9615e27c75050a730/citation/download (Accessed: 6 October 2020).

MacLeod, R. A. and Snell, E. E. (1947) 'Some mineral requirements of the lactic acid bacteria', *J. biol. Chem*, 170(1), pp. 351-365

Mende, S., Krzyzanowski, L., Weber, J., Jaros, D. and Rohm, H. (2012) 'Growth and exopolysaccharide yield of Lactobacillus delbrueckii ssp. bulgaricus DSM 20081 in batch and continuous bioreactor experiments at constant pH', *Journal of bioscience and bioengineering*, 113(2), pp. 185^{-1}91.

Mohseni, M. F. and Alireza Shahab Lavasani, J. (2017) 'Effect of Various Parameters of Carbon and Nitrogen Sources and Environmental Conditions on the Growth of *Lactobacillus casei* in the Production of Lactic Acid', *Global Journal of Medical Research*, 16(2).

Monteagudo, J., Rincon, J., Rodriguez, L., Fuertes, J. and Moya, A. (1993) 'Determination of the best nutrient medium for the production of L-lactic acid from beet molasses a statistical approach', *Engineering in Life Sciences*, 13(2), pp. 103-110.

Mondragón-Parada, M. E., Nájera-Martínez, M., Juárez-Ramírez, C., Galíndez-Mayer, J., Ruiz-Ordaz, N. and Cristiani-Urbina, E. (2006) 'Lactic acid bacteria production from whey', *Applied Biochemistry and Biotechnology*, 134(3), pp. 223-232.

Morris, M. D. (1991) 'Factorial sampling plans for preliminary computational experiments', *Technometrics*, 33(2), pp. 161-174.

Mozzi, F., De Giori, G. S., Oliver, G., and De Valdez, G. F. (1994) 'Effect of culture pH on the growth characteristics and polysaccharide production by *Lactobacillus casei*', *Milchwissenschaft*, *49*(12), pp. 667-669.

Mustafa, S. M., Chua, L. S., El-Enshasy, H. A., Abd Majid, F. A., Hanapi, S. Z., and Abdul Malik, R. (2019) 'Effect of temperature and pH on the probiotication of *Punica granatum* juice using *Lactobacillus* species'. *Journal of food biochemistry*, *43*(4), pp. 12805

Nancib, N., Nancib, A., Boudjelal, A., Benslimane, C., Blanchard, F. and Boudrant, J. (2001) 'The effect of supplementation by different nitrogen sources on the production of lactic acid from date juice by *Lactobacillus casei* subsp. *rhamnosus*', *Bioresource Technology*, 78(2), pp. 149-153.

Nannen, Nancy L., and Robert W. Hutkins (1991) 'Intracellular pH effects in lactic acid bacteria', *Journal of Dairy Science* 74(3), pp741-746.

Naveena, B., Altaf, M., Bhadriah, K. and Reddy, G. (2005) 'Selection of medium components by Plackett–Burman design for production of L (+) lactic acid by *Lactobacillus amylophilus* GV6 in SSF using wheat bran', *Bioresource technology*, 96(4), pp. 485-490.

Noro, K. and Yang, Z. (1995) 'Purification and identification of antimicrobial substances produced by two *Lactobacillus casei* strains, *International Dairy Journal'*, 5(5), pp. 503-513.

Oh, S., Rheem, S., Sim, J., Kim, S. and Baek, Y. (1995) 'Optimizing conditions for the growth of *Lactobacillus casei* YIT 9018 in tryptone-yeast extract-glucose medium by using response surface methodology', *Applied and environmental microbiology*, 61(11), pp. 3809-3814.

Othman, M., Ariff, A. B., Rios-Solis, L. and Halim, M. (2017a) 'Extractive fermentation of lactic acid in lactic acid bacteria cultivation: A review, *Frontiers in microbiology*, 8, pp. 2285.

Othman, M., Ariff, A. B., Wasoh, H., Kapri, M. R. and Halim, M. (2017b) 'Strategies for improving production performance of probiotic *Pediococcus acidilactici* viable cell by overcoming lactic acid inhibition', *AMB Express*, 7(1), pp. 215.

Parente, E., Brienza, C., Ricciardi, A., & Addario, G. (1997) 'Growth and bacteriocin production by Enterococcus faecium DPC1146 in batch and continuous culture' *Journal of Industrial Microbiology and Biotechnology*, *18*(1), 62-67.

Paulova, L., Chmelik, J., Branska, B., Patakova, P., Drahokoupil, M., and Melzoch, K. (2020) Comparison of Lactic Acid Production by *L. casei* in Batch, Fed-batch and Continuous Cultivation, Testing the use of Feather Hydrolysate as a Complex Nitrogen Source, *Brazilian Archives of Biology and Technology*, *63*.

Payot, T., Chemaly, Z. and Fick, M. (1999) 'Lactic acid production by *Bacillus coagulans*- Kinetic studies and optimization of culture medium for batch and continuous fermentations', *Enzyme and Microbial Technology,* 24(3-4), pp. 191-199.

Polak-Berecka, M., Waśko, A., Kordowska-Wiater, M., Podleśny, M., Targoński, Z. an Kubik-Komar, A. (2010) Optimization of medium composition for enhancing growth of *Lactobacillus rhamnosus* PEN using response surface methodology, *Pol J Microbiol,* 59(2), pp. 113-118.

Rault, A., Bouix, M., & Béal, C. (2009) 'Fermentation pH influences the physiological-state dynamics of *Lactobacillus bulgaricus* CFL1 during pH-controlled culture', *Applied and environmental microbiology*, *75*(13), pp. 4374-4381.

Ricciardi, A., Zotta, T., Ianniello, R. G., Boscaino, F., Matera, A. and Parente, E. (2019) Effect of respiratory growth on the metabolite production and stress robustness of *Lactobacillus casei* N87 cultivated in cheese whey permeate medium, *Frontiers in microbiology*, 10, pp. 851.

Salvetti, E., Torriani, S. and Felis, G. E. (2012) 'The genus *Lactobacillus*: a taxonomic update', *Probiotics and antimicrobial proteins,* 4(4), pp. 217-226.

Sarika, A., Lipton, A. and Aishwarya, M. (2010) Bacteriocin production by a new isolate of *Lactobacillus rhamnosus* GP1 under different culture conditions, *Adv J Food Sci Technol,* 2(5), pp. 291-297.

Schiraldi, C., Adduci, V., Valli, V., Maresca, C., Giuliano, M., Lamberti, M., Cartenì, M. and De Rosa, M. (2003) High cell mass cultivation of probiotics and lactic acid production, *Biotechnology and bioengineering,* 82(2), pp. 213-222.

Schleifer, K. H. and Ludwig, W. (1995) Phylogeny of the genus Lactobacillus and related genera, *Systematic and Applied Microbiology,* 18(4), pp. 461-467.

Sender, R. F., Shai Milo, Ron (2016) 'Revised estimates for the number of human and bacteria cells in the body', *PLoS biology,* 14(8), pp. e1002533.

Sgouras, D., Maragkoudakis, P., Petraki, K., Martinez-Gonzalez, B., Eriotou, E., Michopoulos, S., Kalantzopoulos, G., Tsakalidou, E. and Mentis, A. (2004) In vitro and in vivo inhibition of *Helicobacter pylori* by *Lactobacillus casei* strain Shirota, *Applied and environmental microbiology*, 70(1), pp. 518-526.

Shahravy, A., Tabandeh, F., Bambai, B., Zamanizadeh, H. and Mizani, M. (2012) Optimization of probiotic *Lactobacillus casei* ATCC 334 production using date powder as carbon source, *Chemical Industry and Chemical Engineering Quarterly,* 18(2), pp. 273-282.

Siciliano, R. A., Pannella, G., Lippolis, R., Ricciardi, A., Mazzeo, M. F. and Zotta, T. (2019) 'Impact of aerobic and respirative life-style on *Lactobacillus casei* N87 proteome', *International journal of food microbiology,* 298, pp. 51-62.

Singh, J., Kaushik, N., & Biswas, S. (2014) Bioreactors–technology & design analysis, *The Scitech Journal*, 1, pp. 28-36.

Singh, V. H., Shafiul Niwas, Ram Srivastava, Akansha Pasupuleti, Mukesh Tripathi, CKM (2017) 'Strategies for fermentation medium optimization: an in-depth review', *Frontiers in microbiology,* 7, pp. 2087.

Stanbury, P. F., Whitaker, Allan Hall, Stephen J (2013) *Principles of fermentation technology*. Elsevier.

Sakamoto, M. and Komagata, K. (1996) 'Aerobic growth of and activities of NADH oxidase and NADH peroxidase in lactic acid bacteria', *Journal of fermentation and bioengineering,* 82(3), pp. 210-216.

Taleghani, H. G., Najafpour, G. D. and Ghoreyshi, A. A. (2016) 'A study on the effect of parameters on lactic acid production from whey', *Polish Journal of Chemical Technology,* 18(1), pp. 58-63.

Tien, M.-T., Girardin, S. E., Regnault, B., Le Bourhis, L., Dillies, M.-A., Coppée, J.-Y., Bourdet-Sicard, R., Sansonetti, P. J. and Pédron, T. (2006) Anti-inflammatory effect of *Lactobacillus casei* on *Shigella*-infected human intestinal epithelial cells, *The Journal of Immunology,* 176(2), pp. 1228-1237.

Tissier, H. (1906) 'The treatment of intestinal infections by the method of transformation of bacterial intestinal flora', *CR Soc. Biol,* 60, pp. 359-361.

Todar, K. (2013) 'Nutrition and growth of bacteria'.

Vázquez, J.A., Mirón, J., González, M.P. (2005) ‘Effects of aeration of growth and on production of bacteriocins and other metabolites in cultures of eight strains of lactic acid bacteria’, *Appl Biochem Biotechnol,* 127, 111–124.

Vergari, F., Tibuzzi, A. and Basile, G. (2010) 'An overview of the functional food market: from marketing issues and commercial players to future demand from life in space', *Bio-Farms for Nutraceuticals*: Springer, pp. 308-321.

Vinay-Lara, E. W., Song Bai, Lina Phrommao, Ekkarat Broadbent, Jeff R.Steele, James L. (2016) '*Lactobacillus casei* as a biocatalyst for biofuel production', *Journal of Industrial Microbiology & Biotechnology,* 43(9), pp. 1205-1213.

Wang, L., Zhou, H., He, R., Xu, W., Mai, K. and He, G. (2016) 'Effects of soybean meal fermentation by *Lactobacillus plantarum* P8 on growth, immune responses, and intestinal morphology in juvenile turbot (*Scophthalmus maximus L.*)', *Aquaculture,* 464, pp. 87-94.

Wang, X., Ding, J., Guo, W. Q., & Ren, N. Q. (2010) 'Scale-up and optimization of biohydrogen production reactor from laboratory-scale to industrial-scale on the basis of computational fluid dynamics simulation', *International journal of hydrogen energy*, *35*(20),pp. 10960-10966.

Webb, M. (1949) 'The Influence of magnesium on cell division: the effect of magnesium on the growth and cell division of various bacterial species in complex media', *Microbiology*, *3*(3), pp. 410-417.

Wu, C. and Hamada, M. (2000) Experiments: planning, design, and parameter optimization. Wiley, New York. Yingying, L., Qunhui, W. and Jianguo, L. 'Effect of aeration condition on lactic acid production from distiller's grain by *Lactobacillus casei*'. *2010 International Conference on Mechanic Automation and Control Engineering*: IEEE, 2109-2112.

Yang, X., Lai, Z., Lai, C., Zhu, M., Li, S., Wang, J., & Wang, X. (2013) 'Efficient production of L-lactic acid by an engineered *Thermoanaerobacterium aotearoense* with broad substrate specificity' *Biotechnology for biofuels*, *6*(1), pp. 124.

Zhang, Z., Jin, B. and Kelly, J. (2007) 'Production of lactic acid and byproducts from waste potato starch by *Rhizopus arrhizus*: role of nitrogen sources', *World Journal of Microbiology and biotechnology,* 23(2), pp. 229-236.

Zhao, H., Xing, B., Zhang, C., Huang, G., Liu, Q., Yi, G., and Zhang, C. (2018) 'Efficient synthesis of nitrogen and oxygen co-doped hierarchical porous carbons derived from soybean meal for high-performance supercapacitors', *Journal of Alloys and Compounds*, *766*, pp. 705-715.

LIST OF PUBLICATIONS

Journal with Impact Factor

1. **J. Eyahmalay**, D.J Dailin, R.A Malek, S.RamLi, V. Siwapiragam, and H. El. Enshashy (2019), Statistical Optimization Approaches for High Cell Biomass Production of *Lactobacillus casei*, *Journal of Scientific and Industrial Research* (Accepted).(Q4).

Non-Indexed Conference Proceedings

1**.** **J. Eyahmalay**, V. Siwapiragam, D. J Dailin, R. A. Malek, S. RamLi and H. El Enshashy (2019), Effect of Different pH and Aeration Conditions on the Cell Mass Production of Probiotic Strain *Lactobacillus casei*, WICC-B26 in Batch Culture in Semi-Industrial Scale Madridge J Food Technol. 2019 http://dx.doi.org/10.18689/2577-4182.a3.003