OPTIMIZATION OF MEDIUM AND FERMENTATION PARAMETERS FOR HIGH CELL DENSITY CULTIVATION OF *Azotobacter vinelandii*

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To my beloved mother and father

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ABSTRACT

Azotobacter vinelandii is a free-living N-fixing bacterium capable of converting atmospheric nitrogen into ammonia, a nitrogen source easily assimilated by plants. Although numerous researches have been done on the genetics and metabolism of *A. vinelandii*, little information on cell mass production for biofertilizer applications is available. Therefore, the objective of this research is to develop an industrial culture medium and a cultivation strategy for the mass production of *A. vinelandii* in a semi-industrial scale. Based on previous works, several media formulations were tested for their cell growth potential. The best medium yielded a cell mass of only 3.94 g L^{-1} in shake flask cultures and was optimized using both a classical and statistical approach, achieving a maximum cell mass of 7.71 g L^{-1} and 8.82 g L^{-1}, respectively. The cell yield on glucose of the classically optimized medium was approximately 35.5% higher than the statistically optimized medium and was thus used in subsequent bioreactor experiments. Batch cultivations in 16-L stirred tank bioreactors with and without pH control yielded cell mass concentrations of 7.52 g L^{-1} and 15.86 g L^{-1} respectively. A series of fed-batch cultivations was carried out to determine the factors limiting cell growth. A combination of a constant feeding strategy coupled with pH and dissolved oxygen control with additional pure oxygen sparging was found to yield the highest cell mass concentration of 40.65 g L^{-1} in 16-L bioreactor cultivations. The cultivation in a 150-L stirred tank bioreactor revealed that oxygen is one of the most critical factors affecting cell mass production of the highly aerobic *A. vinelandii*. The decreased oxygen transfer rate limited cell growth but increased alginate production. The maximum cell mass obtained in a fed-batch culture of *Azotobacter vinelandii* in a 150-L stirred tank bioreactor was 28.35 g L^{-1} while the maximum alginate concentration was 18.60 g L^{-1}.

ABSTRAK

Azotobacter vinelandii merupakan sejenis bakteria pengikat-N yang boleh menukarkan nitrogen di atmosfera kepada ammonia yang boleh diserap oleh tumbuh-tumbuhan.Walaupun banyak kajian telah dijalankan ke atas genetik dan metabolisme bakteria ini, namun maklumat tentang strategi pengkulturan ketumpatan sel tinggi untuk pengaplikasian bio-baja masih kekurangan. Oleh itu, objektif utama kajian ini adalah untuk menghasilkan mediakultur dan strategi yang sesuai untuk pengkulturan ketumpatan sel tinggi *A. vinelandii*. Berdasarkan kajian yang lepas, beberapa mediakultur telah dipilih untuk diuji kesesuaiannya untuk pengkulturan *A. vinelandii*. Media terbaik memberi ketumpatan sel sebanyak 3.94 g L^{-1} dalam pengkulturan di dalam kelalang kon. Pengoptimuman komposisi media dijalankan atas media ini menggunakan kaedah klasik dan statistik.Ketumpatan sel yang diperoleh dari media optimum klasik dan statistik setiap satunya adalah 7.71 g L^{-1} dan 8.82 g L^{-1}. Media optimum klasik yang menunjukkan hasil sel yang lebih tinggi (35.5%) dipilih untuk pengkulturan seterusnya dalam bioreaktor 16-L. Pengkulturan berkelompok di dalam bioreaktor 16-L dengan dan tanpa kawalan pH masing-masing menghasilkan 7.52 g L^{-1} dan 15.86 g L^{-1} sel. Satu siri pengkulturan suap kelompok dijalankan untuk menentukan faktor-faktor yang mempengaruhi pertumbuhan sel. Kombinasi pengkulturan suap kelompok dengan aliran nutrien malar, kawalan pH dan oksigen dengan aliran gas oksigen tulen menghasilkan ketumpatan sel tertinggi, 40.65 g L^{-1} dalam bioreaktor 16-L. Pengkulturan *A. vinelandii* dalam bioreaktor 150-L menghasilkan ketumpatan sel sebanyak 28.35 g L^{-1} dan alginate adalah 18.60 g L^{-1}. Ini menunjukkan bahawa oksigen merupakan salah satu faktor utama yang mempengaruhi pertumbuhan sel di mana penurunan kadar pemindahan oksigen meningkatkan penghasilan alginat berbanding dengan penghasilan sel.

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LIST OF ABBREVIATIONS

Abbreviations

A.	-	*Azotobacter*
BNF	-	Biological Nitrogen Fixation
CDW	-	Cell dry weight
DO	-	Dissolved Oxygen
FAO	-	Food and Agriculture Organization
OD	-	Optical density
OD_{540}	-	Optical density at 540 nm
OD_{600}	-	Optical density at 600 nm
sp.	-	Species
USD	-	US Dollar

Chemicals

$(NH_4)_2SO_4$	-	Ammonium sulfate
C	-	Carbon

$CaC1_2$	-	Calcium chloride (anhydrous)
$CaCl_2.2H_2O$	-	Calcium chloride dihydrate
$CaSO_4$	-	Calcium sulfate
CoA	-	Coenzyme A
$CoCl_2$	-	Cobalt (II) chloride
$CuSO_4.5H_2O$	-	Copper (II) sulfate pentahydrate
Cyt	-	Cytochrome
DHAP	-	Dihydroxyacetone phosphate
DNS	-	3, 5-dinitro-salicylic acid
$FeCl_3$	-	Iron (III) chloride (anhydrous)
$FeS0_4.7H_2O$	-	Iron (II) sulfate heptahydrate
$FeSO_4$	-	Iron (II) sulfate (anhydrous)
GDP	-	Guanosine diphosphate
H^+	-	Proton
H^+/e	-	Proton to electron ratio
H_2	-	Dihydrogen
H_3BO_3	-	Boric acid
HCl	-	Hydrochloric acid
K_2HPO_4	-	Dipotassium hydrogen phosphate
KH_2PO_4	-	Potassium dihydrogen phosphate
$MgS0_4 .7H_2O$	-	Magnesium sulfate heptahydrate
$MnCl_2.4H_2O$	-	Manganese (II) chloride tetrahydrate
$MnSO_4.4H_2O$	-	Manganese (II)sulfate heptahydrate
MOPS	-	3-(N-morpholino)-propanesulfonic acid
MSG	-	Monosodium glutamate
N	-	Nitrogen
N_2	-	Dinitrogen
Na	-	Sodium
Na_2HPO_4	-	Disodium hydrogen phosphate
$Na_2MoO_4.2H_20$	-	Sodium molybdate dihydrate
NaCl	-	Sodium chloride
NADH	-	Reduced nicotinamide adenine dinucleotide
NADPH	-	Reduced nicotinamide adenine diphosphate
NaOH	-	Sodium hydroxide
NDH I	-	NADH dehydrogenase I
NDH II	-	NADH dehydrogenase II

NH_3	-	Ammonia
$NH_4CH_3CO_2$	-	Ammonium acetate
NO	-	Nitrogen oxide
N_2O	-	Nitrous oxide
O_2	-	Oxygen
P	-	Phosphate
PHA	-	Polyhydroxyalkanoate
PHB	-	Poly-β-hydroxybutyrate
P_i	-	Inorganic phosphate group
TCA	-	Tricarboxylic acid
$ZnSO_4.7H_2O$	-	Zinc sulfate heptahydrate

LIST OF SYMBOLS

Symbols

%	-	Percent
>	-	Greater than
μ	-	Specific growth rate (h^{-1})
μ_{max}	-	Maximum specific growth rate (h^{-1})
F	-	Feed rate ($g\ L^{-1}h^{-1}$)
K_S	-	Substrate utilization constant
m	-	Maintenance coefficient (mol substrate g^{-1} cell L^{-1})
q_p	-	Specific rate of product formation (mg product g^{-1} biomass h^{-1})
S	-	Substrate concentration ($g\ L^{-1}$)
S_o	-	Feed substrate concentration (g substrate L^{-1})
t	-	Time interval (h)
t_0	-	Initial time (h)
V	-	Volume of reactor liquid (L)
v/v	-	Volume per volume
vvm	-	Volume per volume per minute
X	-	Biomass concentration ($g\ L^{-1}$)
x_0	-	Original biomass concentration ($g\ L^{-1}$)

x_t	-	Biomass concentration after the time interval (g L^{-1})
$Y_{P/X}$	-	Alginate productivity (g alginate g^{-1} biomass)
$Y_{X/S}$	-	Substrate yield coefficient (g biomass g^{-1} substrate)
α	-	Alpha
β	-	Beta

Units

°C	-	Degrees Celsius
µl	-	Micro liter
µm	-	Micrometer
µM	-	Micromole
g	-	Gram
h	-	Hour
kg	-	Kilogram
L	-	Liter
M	-	Molarity
min	-	Minute
ml	-	Milliliter
rpm	-	Revolutions per minutes

CHAPTER I

INTRODUCTION

1.1. Background

Food security is one of the most important global issues affecting the world today. In 2007, the Food and Agriculture Organization of the United Nations, (FAO) estimated that the number of chronically hungry people in the world rise by 75 million to reach a total of 923 million. Current food insecurity will be further aggravated by the increasing world population. The FAO estimates that the global population will reach approximately 8 billion in 2025 and around 9 billion in 2050 before stabilizing at slightly more than 10 billion after 2100. Even before the world population stabilizes, world food production must increase by more than 75 percent to feed the entire world population by 2025 (FAO). Since little new land is suitable for crop production, the output per unit area must increase to meet increasing food demand (Mosier et al., 2004; Bumb and Baanante, 1996).

Efforts to increase the productivity of crops have seen a tremendous increase in fertilizer usage over the years. FAO estimates the world fertilizer consumption to grow annually at about 1.7 percent from 2007/2008 to 2011/12. This growth is equivalent to an increment of about 15 million tonnes and is expected to reach a total demand of 180 million tonnes by 2030. Although chemical fertilizers are able to supply crops with the necessary nutrients for greater yield and productivity, their excessive use may lead to numerous health hazards and detrimental effects to the environment. For instance, the excessive use of nitrogen, N fertilizers may lead various implications such as nitrate leaching which could result in the pollution of water systems (Fisher and Newton, 2002), emission of greenhouse gases such as nitrogen oxides (NO, N_2O) and volatilization of ammonia (Roy *et al*., 2006; Mosier *et al*., 2004; Hernandez, 2002).

Since nitrogen is one of the most vital minerals required by plants, a viable alternative is to supply this element through biological nitrogen fixation, BNF. BNF is a natural process whereby diazotrophs such as *Acetobacter*, *Azoarcus*, *Azotobacter*, *Azospirillum*, Cyanobacteria, *Glucoacetobacter*, *Pseudomonas* and *Rhizobium* reduce stable molecular nitrogen from the atmosphere to ammonia which can then be readily available to plants (Newton, 2007; Roy *et al*, 2006).

$$N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$$

Azotobacters are also known to produce a wide range of growth promoting nutrients to plants apart from fixing nitrogen. This not only supplies the necessary nutrients to plants but also helps rejuvenate the soil (Roy *et al*., 2006). Hence, microbial inoculants or biofertilizers containing diazotrophs like *Azotobacter vinelandii* are seen as viable replacements for detrimental mineral nitrogen fertilizers.

1.2. Problem Statement

Although there is much data on *Azotobacter vinelandii*, most researches are either focused on the genetics and metabolism of the bacteria or alginate production. There is however still little information regarding high cell density cultivation strategies for *Azotobacter vinelandii*. On the contrary, most cultivation media are designed to favor the production of alginate over biomass. Therefore, there is a need to develop an industrial medium and high cell density cultivation strategy to maximize cell mass production while minimizing alginate formation. Conditions for optimal alginate production are known to limit cell growth.

1.3. Objective

The main objective of the present work is to develop an industrial medium and cultivation strategy to maximize the biomass production by *Azotobacter vinelandii* NRRL B-14641 with minimal alginate production.

1.4. Scope

a) Media optimization for high cell mass production of *Azotobacter vinelandii* using classical approach.
b) Media optimization for high cell mass production of *Azotobacter vinelandii* using statistical approach.
c) Comparison between classical media optimization approach and statistical media optimization approach.

d) Batch cultivation of *Azotobacter vinelandii* in a 16-L pilot scale stirred tank bioreactor for high cell mass production.

e) Fed-batch cultivation of *Azotobacter vinelandii* in a 16-L pilot scale stirred tank bioreactor for high cell mass production.

f) Scaling-up of cultivation process for *Azotobacter vinelandii* to a 150-L pilot scale stirred tank bioreactor for high cell mass production.

REFERENCES

Aldor I.S. and Keasling J.D. (2003).Process design for microbial plastic factories metabolic engineering of polyhydroxyalkanoates.*Current Opinion in Biotechnology*, 14, 475-483.

Alexander M.A., Chapman T.W. and Jeffries T.W. (1988).Xylose metabolism by *Candida shehatae* in continuous culture.*Applied, Microbiology and Biotechnology*, 28, 478 – 486.

Anderson A.J. and Dawes E.A. (1990). Occurrence, metabolism, metabolic rate, and industrial uses of bacterial polyhydroxyalkanoates.*Microbiological Reviews*,54, 450-472.

Annison G. and Couperwhite I. (1986). Influence of calcium on alginate production and composition in continuous cultures of *Azotobacter vinelandii*.*Applied, Microbiology and Biotechnology*,25, 55 – 61.

Anonymous.(2005).*Current World Fertilizer Trends and Outlook to 2009/10*.Rome: FAO.

Anonymous, (2008), *Current World Fertilizer Trends and Outlook to 2011/12*, Rome:FAO.

Anonymous, (2008), *The State of Food Insecurity in the World 2008*.Rome: Food and Agriculture Organization, FAO.

Anthony J., (2003), *Design of Experiments for Engineers and Scientists*, Oxford, UK: Elsevier Science & Technology Books.

Anthony P., Davey M.R., Power J.B., Washington C. and Lowe K.C. (1994).Synergistic enhancement of protoplast growth by oxygenated perfluorocarbon and Pluronic F-68.*Plant Cell Reports*,13, 251 – 255.

Aquilanti L., Favilli F., Clementi F., (2004), Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples, *Soil Biology & Biochemistry***36**, 1475–1483.

Azcon R. and Barea J.M. (1975). Synthesis of auxins, gibberellins and cytokinins by *Azotobacter vinelandii* and *Azotobacter beijerinckii* related to effects produced on tomato plants.*Plant and Soil*, 43, 609 – 619.

Bae S. and Shoda M. (2004).Bacterial cellulose production by fed-batch fermentation in molasses medium.*Biotechnology Progress*, 20, 1366 – 1371.

Bakulin M.K., Grudsyna A.S. and Pletneva A.Y. (2007).Biological fixation of nitrogen and growth of bacteria of the genus *Azotobacter* in liquid media in the presence of perfluorocarbons.*Applied Biochemistry and Microbiology*, 43, 399 – 402.

Barrera A.D., Pena C. and Galindo E. (2007). The oxygen transfer rate influences the molecular mass of alginate roduced by *Azotobacter vinelandii*.*Applied Microbial and Cell Physiology*, 76, 903-910.

Becking J.H. (2006), *The Family Azotobacteraceae*. In: Dworkin M., Schleifer Falkow S., Rosenberg E., Schleifer K.-H. and Stackebrandt E. *The Prokaryotes*, Vol. 6, 3rd edition.(pp. 759 – 783). Singapore: Springer Science + Business Media.

Bormann E.J., Leibner M. and Beer B. (1998). Growth-associated production of poly(hydroxybutyric acid) by *Azotobacter beijerinckii* from organic nitrogen substrates.*Applied, Microbiology and Biotechnology,* 49, 84-88.

BRENDA, the enzyme information system in 2011, Scheer M., Grote A., Chang A., Schomburg I., Munaretto C., Rother M., Söhngen C., Stelzer M., Thiele J. and Schomburg D. (2011), *Nucleic Acids Research.* 39.

Brivonese A.C. and Sutherland I.W. (1989).Polymer production by a mucoid strain of *Azotobacter vinelandii* in batch culture.*Applied Microbiology and Biotechnology,* 30, 97 – 102.

Brown M.E., Burlingham S.K. and Jackson R.M. (1962).Studies on *Azotobacter* species in soil.*Plant and Soil,* 17, 309 – 319.

Buhler T., Sann R., Monter U., Dingier C., Kuhla J. and Oelze J. (1987).Control of dinitrogen fixation in ammonium-assimilating cultures of *Azotobacter vinelandii*.*Archives of Microbiology,* 148, 247- 251.

Bumb B.C. and Baanante C.A., (1996). The role of fertilizer in sustaining food security and protecting the environment to 2020, Washington, D.C., USA:International Food Policy Research Institute.

Burma D.P. and Burris R.H. (1957).Metabolism of nitrogen by cell-free preparations from *Azotobacter vinelandii*.*Journal of Biological Chemistry,* 225, 723-733.

Cascaval D., Galaction A.-I., Folescu E. and Turnea M. (2006).Comparative study on the effects of n-dodecane addition on oxygen transfer in stirred bioreactors for simulated, bacterial and yeasts broths.*Biochemical Engineering Journal,* 31, 56 – 66.

Chang C.-Y., Lee C.-L. and Pan T.-M. (2006). Statistical optimization of medium components for the production of *Antrodia cinnamomea* AC0623 in submerged cultures.*Applied Microbiology and Biotechnology,* 72, 654 – 661.

Chauhan, Kishor, Trivedi U. and Patel K.C. (2006).Application of response surface methodology for optimization of lactic acid production using date juice.*Journal of Microbiology and Biotechnology*, 16, 1410 – 1415.

Chen B.-Y., You J.-W., Hsieh Y.-T.and Chang J.-S (2008).Feasibility study of exponential feeding in fed-batch cultures for phenol degradation using *Cupriavidus taiwanensis*.*Biochemical Engineering Journal*, 41, 175-180.

Chen G.-Q.and Page W.J. (1997).Production of Poly-β-hydroxybutyrate by *Azotobacter vinelandii* in a two-stage fermentation process.*Biotechnology Techniques*,11, 347-350.

Chen W.-P., Chen J.-Y., Chang S.-C.and Su C.-L. (1985). Bacterial alginate produced by a mutant of *Azotobacter vinelandii*.*Applied and Environmental Microbiology*, 49, 543 – 546.

Clementi F., Moresi M. and Parente E. (1999).Alginate from *Azotobacter vinelandi, Methods in Biotechnology*, 10, 23 – 42.

Dalton D.A. and Kramer S. (2007).*Nitrogen-fixing bacteria in non-legumes*, In: Gnanamanickam S.S. *Plant-Associated Bacteria* (pp. 105 – 130). The Netherlands: Springer.

De La Vega M.G. and Cejudo F.T., Paneque A. (1991).Production of Exocellular Polysaccharide by *Azotobacter chroococcum*.*Applied, Biochemistry and Biotechnology*, 30, 273-284.

Dhanasekar R., Viruthagiri T. and Sabarathinam P.L. (2003).Poly(3-hydroxy butyrate) synthesis from a mutant strain *Azotobacter vinelandii* utilizing glucose in a batch reactor.*Biochemical Engineering Journal*, 16, 1 – 8.

Diaz-Barrera, Pena C. and Galindo E. (2007). The oxygen transfer rate influences the molecular mass of the alginate produced by *Azotobacter vinelandii*.*Microbiology and Biotechnology*, 76, 903–910.

Dixon R.A. and Cannon F.C. (1976).*Recent advances in the genetics of nitrogen fixation,* In:Nutman P.S. *Symbiotic nitrogen fixation in plants* (3 – 24).Great Britain: Cambridge University Press.

Dommelen A.V. and Vanderleyden J. (2007).*Associative nitrogen fixation,* In: Bothe H., Ferguson S.J. and Newton W.E. *Biology of the Nitrogen Cycle* (pp. 179 – 192). The Netherlands: Elsevier.

El-Enshasy, H.A. (1998).*Optimization of glucose oxidase production and excretion by recombinantAspergillus niger*, Ph.D.thesis, TU-Brauschweig, Germany.

Ferreira S.L.C, Bruns R.E., Ferreira H.S., Matos G.D., David J.M., Brand G.C., da Silva E.G.P., Portugal L.A., dos Reis P.S., Souza A.S. and dos Santos W.N.L. (2007). Box-Behnken design: An alternative for the optimization of analytical methods, *Analytica Chimica Acta,* 597, 179 – 186.

Ferreira S.L.C, dos Santos W.N.L., Quitella C.M., Neto B.B. and Bosque-Sendra J.M. (2004). Doehlert matrix: a chemometric tool for analytical chemistry—review, *Talanta,* 63, 1061 -1067.

Fisher K. and Newton W.E. (2002).*Nitrogen fixation – A general overview*. In:Leigh J.F.*Nitrogen Fixation at the Millenium* (pp. 1 – 34).Amsterdam, The Netherlands: Elsevier.

Galindo F., Pena C., Nunez C., Segura D. and Espin G. (2007).Molecular and bioengineering strategies to improve alginate and polyhydroxyalkanoate production by *Azotobacter vinelandii*, *Microbial Cell Factories,* 6, 1-16.

Ganuza E., Anderson A. J. and Rattleledge C. (2008).High cell density cultivation of *Schizochytrium sp*. in an ammonium/pH-auxostat fed-batch system.*Biotechnology Letters*, 30, 1559-1564.

Garcia-Arrazola R., Sun C.S., Chan G., Buchanan I., Doyle B., Titchener-Hooker N. and Baganz F. (2005).Evaluation of a pH-stat feeding strategy on the

production and recovery of Fab' fragments from *E. coli*.*Biochemical engineering Journal,* 23, 221 – 230.

Garcia-Ochoa F. and Gomez E. (2009). Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview.*Biotechnology Advances,* 27, 153 – 176.

George S.E., Costenbader C.J. and Melton T. (1985). Diauxic Growth in *Azotobacter vinelandii*.*Journal of Bacteriology*, 164, 866-871.

Giridhar R. and Srivastava A.K. (2000).Fed-batch sorbose fermentation using pulse and multiple feeding strategies for productivity improvement.*Biotechnology & Bioprocess Engineering*, 5, 340-344.

Gorin P.A.J. and Spencer J.F.T. (1966).Exocellular alginic acid from *Azotobacter vinelandii*, *Canadian Journal of Chemistry*, 44, 993 – 998.

Gothwal R..K., Nigam V.K., Mohan M.K., Sasmal D. and Ghosh P. (2008). Screening of Nitrogen Fixers from Rhizospheric Bacterial Isolates Associated with Important Desert Plants.*Applied Ecology and Environmental Research,* 6, 101-109.

Goucher C.R. and Kocholaty W. (1954).Effects of various ions on the respiration of *Azotobacter*, *Journal of Biological Chemistry,* 211, 613 – 620.

Goucher C.R., Sarachek A. and Kocholaty W. (1955). A time-course respiratory inactivation associated with *Azotobacter* cells deprived of Mg^{2+}, *Journal of Bacteriology,* 70, 120 – 124.

Gowariker V., Krishnamurthy V.N., Gowariker S., Dhanorkar M., Paranjape K. and Borlaug N. (2009).*The Fertilizer Encyclopedia*. Hoboken, New Jersey: John Wiley and Sons Limited.

Grant C.L. and Pramer D. (1962).Minor element composition in yeast extract.*Journal of Bacteriology,* 84, 869 – 870.

Haddock B.A. and Jones C.W. (1977). Bacterial Respiration, *Bacterial Reviews*, 41, 47-99.

Hardy R.W.F., Holsten R.D., Jackson E.K. and Burns R.C. (1968). The acetylene-ethylene assay for n_2 fixation: laboratory and field evaluation.*Plant Physiology*, 43, 1185-1207.

Haug A. and Larsen B. (1971). Biosynthesis of alginate, *Carbohydrate Research*, 17, 287 – 296.

Hernandez G. (2002).*Chair's Comments: Biological nitrogen fixation and sustainable agriculture, nitrogen fixation: global perspectives*, Wallingford, UK:CAB International.

Ho C.S., Ju L.-K. and Baddour R.F. (1990).Enhancing penicillin fermentations by increased oxygen solubility through the addition of n-hexadecane.*Biotechnology and Bioengineering*, 36, 1110 – 1118.

Hong L. (2002).*Azotobacter vinelandii* nitrogenase: *Multiple substrate-reduction sites and effects of pH on substrate reduction and CO inhibition*, Ph.D. thesis, Virginia Polytechnic Institute and State University, United States.

Horan N.J., Jarman T.R. and Dawes E.A. (1981).Effects of carbon source and inorganic phosphate concentration on the production of alginic acid by a mutant of *Azotobacter vinelandii* and on the enzymes involved in its biosynthesis.*Journal of General Microbiology*, 127, 185-191.

Horan N.J., Jarman T.R. and Dawes E.A. (1983).Studies of some enzymes of alginic acid biosynthesis in *Azotobacter vinelandii* grown in continuous culture.*Journal of General Microbiology*, 129, 2985 – 2990.

Jakobsons A., Zell E.A. and Wilson P.W. (1962).A re-investigation of the calcium requirement if *Azotobacter vinelandii* using purified media. *Archives of Microbiology*, 41, 1 – 10.

Jarman T. R., Deavin L., Slocombe S. and Righelato R.C. (1978).Investigation of the effect of environmental conditions on the rate of exopolysaccharides synthesis in *Azotobacter vinelandii*.*Journal of General Microbiology*, 107, 59 - 64.

Jensen H.L. (1954). The Azotobacteriaceae, *Bacteriological Reviews*, 18, 195 - 214.

Jones H.L., Margaritis A. and Stewart R.J. (2007). The combined effects of oxygen supply strategy, inoculum size and temperature profile on very-high-gravity beer fermentation by *Saccharomyces cerevisiae*.*Journal of the Institute of Brewing*, 113, 168 – 184.

Kennedy C., Rudnick P., MacDonald M.L. and Melton T. (2005).*Genus III Azotobacter Beijerinck 1901, 567*^{AL}, In: Garrity G.M., Brenner D.J., Krieg N.R. and Staley J.T.*Bergey's Manual of Systematic Bacteriology* Volume 2, Part B, 2nd edition (pp.384 – 401)East Lansing, USA: Springer.

Keevil C.W., Hough J.S. and Cole J.A. (1979). Regulation of respiratory and fermentative modes of growth of *Citrobacter freundii* by oxygen, nitrate and glucose, *Journal of General Microbiology*, 113, 83 – 95.

Khanna S. and Srivastava A.K. (2005). Repeated batch cultivation of *Ralstonia eutropha* for poly(β-hydroxybutyrate) production.*Biotechnology Letters*, 27, 1401-1403.

Kim B.S., Lee S.C., Lee S.Y., Chang Y.K. and Chang H.N. (2004). High cell density fed-batch cultivation of *Escherichia coli* using exponential feeding combined with pH-stat.*Bioprocess Biosystem Engineering*, 26, 147-150.

Kim H.M., Paik S.-Y., Ra K.S., Koo K.B., Yun J.W. and Choi J.W. (2006).Enhanced production of exopolysaccharides by fed-batch culture of *Ganoderma resinaceum* DG-6556.*The Journal of Microbiology*, 44, 233 – 242.

Kim S.S., Lee J.S., Cho J.Y. and Kim Y.E. and Hong E.K. (2010).Effects of C/N ratio and trace elements on mycelial growth and exo-polysaccharide production of *Tricholoma matsutake*.*Biotechnology and Bioprocess Engineering*, 15, 293 – 298.

Kim Y.-H., Kng S.W., lee J.H., Chang H.-I., Yun C.-W., Paik H.-D., Kang C.-W. and Kim S.W. (2007).High cell density fermentation of *Saccharomyces cerevisiae* JUL3 in fed-batch culture for the production of β-Glucan.*Journal of Industrial and Engineering Chemistry*, 13, 153 – 158.

Kleiner D. and Kleinschmidt J.A. (1976).Selective inactivation of nitrogenase in *Azotobacter vinelandii* batch cultures.*Journal of Bacteriology*, 128, 117-122.

Korz D.J., Rinas U., Hellmuth K., Sanders E.A. and Deckwer W.-D.(1995). Simple fed-batch technique for high cell density cultivation of *Escherichia coli*.*Journal of Biotechnology*, 39, 59 – 65.

Kulaev I.S., Vagabov V.M. and Kulakovskaya T.V. (2004).*The biochemistry of inorganic polyphosphates*,2nd edition, England: John Wiley and Sons Limited.

Lazic Z.R. (2004).*Design of experiments in Chemical Engineering*, Weinheim:Wiley-VCH Verlag GmbH & Co.

Lee J., Lee S.Y., Park S. and Middelberg A.P.J. (1999).Control of fed-batch fermentations.*Biotechnology Advances*, 17, 29-48.

Lees H. and Postgate J.R. (1973). The behavior of *Azotobacter chroococcum* in oxygen and phosphate limited chemostat culture.*Journal of General Microbiology*, 75, 161 – 166.

Li X.-B., Zhao G.-R. and Yuan Y.-J.(2005). A strategy of phosphorus feeding for repeated fed-batch fermentation of penicillin G.*Biochemical Engineering Journal*, 27, 53–58.

Liu J.-K., Lee F.-T., Lin C.-S., Yao X.-T., Davenport J.W. and Wong T.-Y. (1995). Alternative function of the electron transport system in *Azotobacter vinelandii*: removal of excess reductant by the cytochrome d pathway.*Applied and Environmental Microbiology,* 61, 3998–4003.

Liu Q.-N., Liu R.-S., Wang Y.-H., Mi Z.-Y., Li D.-S., Zhong J.J. and Tang Y.J. (2009).Fed-batch fermentation of *Tuber melanosporum* for the hyperproduction of mycelia and bioactive *Tuber* polysaccharides.*Bioresource Technology,* 100, 3644 – 3649.

Long-Shan T., Tsai T.-H.and Wang T.C., (2002). Application of oxygen vectors to *Aspergillus terreus* cultivation.*Journal of Bioscience and Bioengineering*, 94, 453 – 459.

Macedo J.A. (2007).Optimization of medium composition for transglutaminase production by a Brazilian soil *Streptomyces sp*. *Electronic Journal of Biotechnology,* 10, 618 – 626.

Montgomery D.C. (2000), *Design and analysis of experiments*, 5th edition, New York: John Wiley & Sons Inc.

Mosier A.R., Syers J.K. and Freney J.R. (2004).*Nitrogen fertilizer: An essential component of increased food, feed and fiber production, agriculture and the nitrogen cycle*.Washington, DC, USA:Island Press.

Mousdale D.M., Melville J.C. and Fisher M. (1999). Optimization of fermentation processes by quantitative analysis: from analytical biochemistry to chemical engineering. In: El-Mansi E.M.T. and Bryce C.F.A.*Fermentation Microbiology and Biotechnology*(pp. 147 – 178). London: Taylor & Francis.

Navaneeth S., Bhuvanesh S., Bhaskar V., Vijay Kumar P., Kandaswamy S.K.J. and Achary A. (2009).Optimization of medium for the production of subtilisin from *Bacillus subtilis* MTCC 441.*African Journal of Biotechnology,* 8, 6327 – 6331.

Newton W.E. (2007).Physiology, biochemistry, and molecular biology of nitrogen fixation. In: Bothe H., Ferguson S.J. and Newton W.E.*Biology of the Nitrogen Cycle* (pp. , 109 – 130). Amsterdam, The Netherlands: Elsevier.

Nieto K.F. and Frankenberger Jr. W.T. (1989).Biosynthesis of cytokinins by *Azotobacter chroococcum*.*Soil Biology and Biochemistry*, 21, 967 – 972.

Nikel P.I., Pettinari M.J., Mendez B.S. and Galvagno M.A. (2005). Statistical optimization of a culture medium for biomass and poly(3-hydroxybutyrate) production by a recombinant *Escherichia coli* strain using agroindustrial byproducts.*International Microbiology*, 8, 243 – 250.

Noms J.R. and Jensen H.L. (1958).Calcium requirements of *Azotobacter*.*Archives of Microbiology*, 31, 198 – 205.

Ntwampe S.K.O., Williams C.C. and Sheldon M.S., (2010). Water-immiscible dissolved oxygen carriers in combination with Pluronic F 68 in bioreactors.*African Journal of Biotechnology*, 9, 1106 – 1114.

Oelze J. (2000). Respiratory protection of nitrogenase in *Azotobacter* species: is a widely held hypothesis unequivocally supported by experimental evidence?,*FEMS Microbiology Reviews*, 24, 321-333.

Ogino H., Miyamoto K., Yasuda M., Ishimi K. and Ishikawa H. (1999).Growth of organic solvent-tolerant *Pseudomonas aeruginosa* LST-03 in the presence of various organic solvents and production of lipolytic enzyme in the presence of cyclohexane.*Biochemical Engineering Journal*, 4, 1 – 6.

Okabe E., Nokajima M., Marooka H. and Nisizawa K. (1981).Investigation of carbon and phosphorus sources in cultural media of a selected strain of alginate-producing *Azotobacter vinelandii*.*Journal of Fermentation Technology*, 59, 1 – 7.

Page W.J., Tindale A., Chandra M. and Kwon E. (2001).Alginate formation in *Azotobacter vinelandii* UWD during stationary phase and the turnover of poly-β-hydroxybutyrate.*Microbiology,* 147, 483–490.

Pal S., Manna A. and Paul A.K. (1998).Nutritional and cultural conditions for production of poly-3-hydroxybutyric acid by *Azotobacter chroococcum*.*Folia Microbiology*, 43, 177-181.

Parente E., Cruedele M.A., Aquino M. and Clementi F. (1998).Alginate production by *Azotobacter vinelandii* DSM576 in batch fermentation.*Journal of Industrial Microbiology & Biotechnology,* 20, 171–176.

Park M., Kim C., Yang J., Lee H., Shin W. and Kim S., Sa T. (2005).Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea.*Microbiological Research,* 160, 127-133.

Pecina A. and Paneque A. (1994).Studies of some enzymes of alginic acid biosynthesis in mucoid and nonmucoid *Azotobacter chrococcum* strains.*Applied, Biochemistry and Biotechnology,* 49, 51-58.

Pena C., Campos N. and Galindo E. (1997). Changes in alginate molecular mass distributions, broth viscosity and morphology of *Azotobacter vinelandii* cultured in shake flasks.*Applied, Microbiology and Biotechnology*, 48, 510 – 515.

Pena C., Trujilo-Roldan M.A. and Galindo E. (2000). Influence of dissolved oxygen tension and agitation speed on alginate production and its molecular weight in cultures of *Azotobacter vinelandii*.*Enzyme and Microbial Technology,* 27, 390–398.

Perez F., Riba J.P. and Strehaiano P. (1992).Effect of yeast extract concentration on growth of *Schizoccharomyces pombe*.*Biotechnology Letters*, 14, 123 – 126.

Pestov N.A., Kulakovskaya T.V. and Kulaev I.S. (2004).Inorganic polyphosphate in mitochondria of *Saccharomyces cerevisiae* at phosphate limitation and phosphate excess.*FEMS Yeast Research,* 4, 643 – 648.

Post E., Kleiner D. and Oelze J. (1983). Whole cell respiration and nitrogenase activities in *Azotobacter vinelandiii* growing in oxygen controlled continuous culture.*Achieves of Microbiology*, 134, 68-72.

Priego-Jimenez R., Pena C., Ramirez O.T. and Galindo E. (2005). Specific growth rate determines the molecular mass of the alginate produced by *Azotobacter vinelandii*.*Biochemical Engineering Journal,* 25, 187–193.

Quagliano J.C. and Miyazaki, S.S. (1997). Effect of aeration and carbon/nitrogen ratio on the molecular mass of the biodegradable polymer poly-b-hydroxybutyrate obtained from *Azotobacter chroococcum* 6B.*Applied, Microbiology and Biotechnology*, 48, 662-664.

Rajeshwari K.V., Prakash G. and Ghosh P. (1995).Improved process for xanthan production using modified media and intermittent feeding strategy.*Letters in Applied Microbiology,* 21, 173 – 175.

Rech R. and Ayub M.A.Z. (2007).Simplified feeding strategies for fed-batch cultivation of *Kluyveromyces marxianus* in cheese whey.*Process Biochemistry,*42, 873-877.

Remminghorst U. and Rehm B.H.A. (2006).Bacterial alginates from biosynthesis to applications.*Biotechnology Letters,* 28, 1701-1712.

Reyes C., Pena C. and Galindo E. (2003). Reproducing shake flasks performance in stirred fermentors: production of alginates by *Azotobacter vinelandii*.*Journal of Biotechnology,*105, 189 – 198.

Rocha-Valadez J.A., Albiter V., Caro M.A., Serrano-Carreon L. and Galindo E., (2007).A fermentation system designed to independently evaluate mixing

and/or oxygen tension effects in microbial processes: development, application and performance. *Bioprocess and Biosystem Engineering*, 30, 115 – 122.

Roy R.N., Finck A., Blair G.J. and Tandon H.L.S. (2006).*Plant nutrition for food security*, Rome: FAO.

Sabra W. (1999).*Microaerophilic production of alginate by Azotobacter vinelandii*, Ph.D. thesis, TU-Braunschweig, Germany.

Sabra W., Zeng A.-P., Sabry S., Omar S. and Deckwer W.-D.(1999). Effect of phosphate and oxygen concentrations on alginate production and stoichiometry of metabolism of *Azotobacter vinelandii* under microaerobic conditions.*Applied, Microbiology and Biotechnology*, 52, 773-780.

Sabra W., Zeng A.-P., Lunsdorf H. and Deckwer W.-D.(2000). Effect of oxygen on formation and structure of *Azotobacter vinelandii* alginate and its role in protecting nitrogenase.*Applied and Environmental Microbiology*, 66, 4037-4044.

Sabra W., Zeng A.-P.and Deckwer W.-D. (2001). Bacterial alginate: physiology, product quality and process aspects.*Applied, Microbiology and Biotechnology*, 56, 315 – 325.

Sabra W. and Zeng A.-P. (2009). Microbial production of alginates: Physiology and process aspects, *Microbiology Monographs*, 13, 153 – 173.

Sadoff H.L. (1976).Encystment and germination of *Azotobacter vinelandii*.*Bacteriological Reviews*, 39, 516 – 539.

Saha S.P., Patra A. and Pau A.K. (2006).Incorporation of polyethylene glycol in polyhydroxyalkanoic acids accumulated by *Azotobacter chroococcum* MAL-201.*Journal of Industrial Microbiology and Biotechnology*, 33, 377 – 383.

Santek B. andMarc V. (1995). Temperature and dissolved oxygen concentration as parameters of *Azotobacter chroococcum* cultivation for use in biofertilizers.*Biotechnology Letters*, 17, 453-458.

Saribay G.F. (2003).Growth and nitrogen fixation dynamics of *Azotobacter chroococcum* in nitrogen-free medium and OMW containing medium.Master thesis, The Middle East Technical University, Turkey.

Savenkova L., Gercberga Z., Kizhlo Z. and Stegantseva E. (1999).Effect of phosphate supply and aeration on poly-β-hydroxybutyrate production in *Azotobacter chroococcum*.*Process Biochemistry*, 34, 109–114.

Schmidt F.R. (2005). Optimization and scale up of industrial fermentation processes.*Applied, Microbiology and Biotechnology,* 68, 425 – 435.

Seanez G. Pena C. and Galindo E. (2001). High CO_2 affects alginate production and prevents polymer degradation in cultures of *Azotobacter vinelandii*.*Enzyme and Microbial Technology,* 29, 535 – 540.

Segura D. and Espin G. (1998). Mutational inactivation of a gene homologous to *Escherichia coli* ptsp affects poly-β-hydroxybutyrate accumulation and nitrogen fixation in *Azotobacter vinelandii*.*Journal of Bacteriology,* 180, 4790-4798.

Senior P.J., Beech D.A., Ritchie G.A.F. and Dawes E.A. (1972).The role of oxygen limitation in the formation of poly- β-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*.*Biochemistry Journal,* 128, 1193-1201.

Senior P.J. and Dawes E.A. (1973).The regulation of Poly-β-hydroxybutyrate Metabolism in *Azotobacter beijerinckii*.*Biochemistry Journal*, 134, 225-238.

Standbury P.F., Whitaker A. and Hall S.J. (2003).*Principles of Fermentation Technology*.Burlington:Butterworth-Heinemann.

Sun Z., Ramsay J.A. and Guay M. (2006).Automated feeding strategies for high cell density fed-batch cultivation of *Pseudomonas putida* KT 2440.*Applied, Microbiology & Biotechnology,* 71, 423-431.

Tauchert K., Jahn A. and Oelze J. (1990).Control of diauxic growth in *Azotobacter vinelandii* on acetate and glucose. Journal of Bacteriology, 172, 6447 – 6451.

Tsai J.C., Aladegbami S.L. and Vela G.R. (1979).Phosphate-limited culture of *Azotobacter vinelandii*.*Journal of Bacteriology,* 139, 639-64.

Trujillo-Roldan M.A., Moreno S., Espin G. and Galindo E. (2004).The roles of oxygen and alginate-lyase in determining the molecular weight of alginate produced by *Azotobacter vinelandii*.*Applied, Microbiology and Biotechnology,* 63, 742-747.

Truijilo-Roldan M.A., Pena C., Ramirez O.T. and Galindo E. (2001). Effect of oscillating dissolved oxygen tension on the production of alginate by *Azotobacter vinelandii*.*Biotechnology Progress,* 17, 1042 – 1048.

Vargas-Garcia M.C., Lopez M.J., Elorrieta, Suarez F. and Moreno J. (2001). Influence of nutritional and environmental factors on polysaccharide production by *Azotobacter vinelandii* cultured on 4-hydroxybenzoic acid.*Journal of Industrial Microbiology and Biotechnology*, 27, 5 – 10.

Vermani M.V., Kelkar S.M. and Kamat M.Y. (1995). Production and optimization of certain growth parameters for an exopolysaccharide from *Azotobacter vinelandii* MTCC 2460 isolated from a plant rhizosphere.*Journal of Fermentation and Bioengineering,* 80, 599 – 602.

Villen R.A., Borzani W. and Netto A.S. (2009). Influence of the accumulation of phosphate and magnesium ions in the yeast cells on the ethanol productivity in batch ethanol fermentation.*Brazilian Archives of Biology and Technology*, 52, 153 – 155.

Wang J. (2000). Enhancement of citric acid production by *Aspergillus niger* using n-dodecane as an oxygen-vector.*Process Biochemistry,* 35, 1079 – 1083.

Ward A.C., Rowley B.I. and Dawes E.A. (1977).Effect of oxygen and nitrogen limitation on poly-β-hydroxybutyrate biosynthesis in ammonium-grown *Azotobacter beijerinckii*.*Journal of General Microbiology,* 102, 61-68.

Wong H.H., Kim Y.C., Lee S.Y. and Chang H.N.(1998).Effect of post-induction nutrient feeding strategies on the production of bioadhesive protein in *Escherichia coli*.*Biotechnology and Bioengineering,* 60, 271-276.

Wong T.Y. (1993). Effects of calcium on sugar transport in *Azotobacter vinelandii*.*Applied and Environmental Microbiology,* 59, 89 – 92.

Wu Z., Du G. and Chen J. (2003).Effects of dissolved oxygen concentration and do-stat feeding strategy on coq_{10} production with *Rhizobium radiobacter*.*World Journal of Microbiology & Biotechnology,* 19, 925-928.

Yano T., Endo T., Tuji T. and Nishizawa Y. (1991).Fed-batch culture with a modified DO-stat method.*Journal of Fermentation and Bioengineering,* 71, 35 – 38.

Yeom S.H., Dangulis A.J. and Nielsen D.R. (2010).Estimating the cellular maintenance coefficient and its use in the design of two-phase partitioning bioscrubbers.*Bioprocess and Biosystem Engineering,* 33, 731 – 739.

Yee L. and Blanch H.W. (1993).Recombinant trypsin production in high cell density fed-batch cultures in *Escherichia coli*.*Biotechnology and Bioengineering,* 41, 781-790.

Yoo I.-K., Seong G.H., Chang H.N. and Park J.K. (1996). Encapsulation of *Lactobacillus casei* cells in liquid-core alginate capsules for lactic acid production.*Enzyme and Microbial Technology,* 19, 426 – 433.

Zainal Alam M.N.H. (2005).Scale-up strategies in stirred and aerated bioreactor, Masters Thesis, Universiti Teknologi Malaysia, Malaysia.

Zanzig J. and Scholz C. (2003), Effects of poly(ethylene glycol) on the production of poly(β-hydroxybutyrate) by *Azotobacter vinelandii* UWD.*Journal of Polymers and the Environment*, 11, 145-154.

Zeiger L. and Grammel H. (2010).Model-based high cell density cultivation of *Rhodospirillum rubrum* under respiratory dark conditions.*Biotechnology and Bioengineering*, 105, 729-739.