ABSTRACT

WANG, BINGQING. Production of Polyhydroxybutyrate (PHB) by *Alcaligenes latus* using Sucrose based Synthetic Media and Sugarbeet Juice. (Under the direction of Dr. Ratna R. Sharma-Shivappa.)

This study focused on exploring the potential of sucrose based media and the suitability of sugarbeet juice as natural media to produce polyhydroxybutyrate (PHB), a kind of biodegradable plastic, which has gained attention over the last 20 years because of its sustainability, degradability and some properties comparable to traditional petro-chemical derived plastics. Three different PHB producing microbial strains, *Ralstonia eutropha* (ATCC 17699), *Alcaligenes latus* (ATCC 29712) and *Alcaligenes latus* (ATCC 29712) were investigated in this research to identify the optimal strain for PHB production based on their growth kinetics when cultured in sucrose-based media. Based on the growth measurement data (OD, maximum growth rate based on dry cell weight (μ_m) and doubling time (T_d)), *A. latus* (ATCC 29714) was found to have the highest μ_m ($0.38 \pm 0.01\ h^{-1}$) and significantly ($P \leq 0.05$) lower T_d (1.80 ± 0.05 h) and was thus selected to perform PHB fermentations. Two-stage batch fermentations with 3 time points (14 h, 16 h or 18 h) for introducing nitrogen limited media and fed-batch fermentations with similar time points (the same as two-stage) for initiating feeding of nitrogen limited media, were conducted using synthetic media with sucrose as the only carbon source. Two-stage batch fermentation with introduction of nitrogen limitation at 16 h with the second stage ending at 26 h post induction of nitrogen limitation was found to be the optimal fermentation mode for PHB production and was thus further investigated for fermentation of sugarbeet juice. The fermentation process with synthetic media resulted

in a dry cell weight (DCW) of 7.88 ± 0.10 g/l, PHB yield coefficient relative to cell dry weight ($Y_{p/x}$) 0.47 ± 0.12, productivity 0.13 ± 0.04 g/l · h and PHB content 48.42 ± 7.06%. And the PHB yield based on sugar consumed was 0.71 ± 0.14. The values for PHB content and the maximum productivity from 2 stage batch fermentation using sugarbeet juice adjust for sugar and nutrient content with partial were 38.66 ± 7.28% and 0.22 g/l.h, respectively, which were both significantly higher ($P \leq 0.05$) than those obtained by other two strategies (no nutrient addition and complete nutrient addition fermentation). This indicates that a sugarbeet based media is suitable for production of PHB. It was also observed that fermentation of sugarbeet juice with partial nutrient addition resulted in a $Y_{p/x}$ value of 0.39 ± 0.07 g PHB/g biomass and PHB concentration of 4.01 ± 0.95 g/l. However, a more comprehensive economic analysis and scale-up studies still need to be done to develop an efficient process for industrialized production. Additionally other accessible and cheaper feedstocks such as sugarbeet molasses and pulps need to be explored in depth, as do production of PHB derivatives like PHBV, P(3HB-4HB) with sugarbeet based media, to obtain more competitive end products with a more economical pathway.

Production of Polyhydroxybutyrate (PHB) by Alcaligenes latus using Sucrose based Synthetic Media and Sugarbeet Juice

by
Bingqing Wang

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APPROVED BY:

_____	_____
Ratna R. Sharma-Shivappa Committee Chair	Jonathan W. Olson
_____	
Saad A. Khan	

DEDICATION

This is for my beloved parents and friends, their endless love and everlasting support encouraging me to always go forward without fear and regret.

BIOGRAPHY

Bingqing Wang, who was born in July 1986, is currently a master student in the Department of Biological and Agricultural Engineering at North Carolina State University. This is the 25th year since he came to this lively world, along with his girlish given name. "Bingqing" in Chinese is always used to describe a naive and virtuous girl. That is why he was usually taken for a girl among people without seeing him. This particularity makes him easy to be remembered by others, while giving him a kind and sincere heart. During the first 18 years of his life, he spent his childhood and school time in Shijiazhuang, the capital city of Hebei province, a newly-emerging industrialized city near Beijing that is well known for its light manufacturing sector, textile and important geographical position as transport hub. However, the high rate of development over the past 30 years brought a lot of environmental issues including air and water pollution, and disposal of excess municipal wastes, making it, as reported, to be one of the top polluted cities in China. Growing up in this city, I`ve gained a strong desire, since my childhood, to investigate the root of the pollution and conquer it by using mild and sustainable ways. Carrying this dream, Bingqing went to Nanjing Agricultural University (NJAU) for undergraduate study and majored in biological engineering. There, he was introduced to a kind of invisible amazing creature called microorganism. Soon after that, he learnt another type of magic tool named fermentation, which can realize conversion of matter with microbes as vector. He gradually showed more and more interest in this field and through hard course work and lab work he gained lots of professional knowledge on micro-organism and sufficient hands-on experiences in the lab. Other than that, he also

positively involved himself in college students management work and volunteer activities towards collaboration agencies such as municipal school for the deaf and Jinghai Temple. After graduation from NJAU in 2009, he travelled from China to United States, pursuing his master degree at North Carolina State University (NCSU). He worked with Dr. Ratna Sharma-Shivappa in Department of Biological and Agricultural Engineering on project of biodegradable plastics. Here he learned and explored how to convert agricultural products to value-added chemicals, which will reduce the plastic garbage discharged into the environment, continually moving forward on the road to chase his dream.

Bingqing is an industrious, easy-going and active person. His chinese zodiac is tiger and constellation is Leo, which gives him a brave heart, endless passion to explore new things and strong desire for adventure. In his spare time, he gets involved in a broad range of interests including watching sports game, playing basketball, reading, watching movies, travelling and most important, spending time with beloved families and friends. He likes to be active all the time and by managing time suitably makes each day meaningful. Bingqing always believes the precious experiences in NC State give him more confidence and motivation to keep moving towards his dream. He is ready to start a new journey.

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On the road of completing this thesis for my master`s degree, lots of people kindly provided great assistance. The first person I want express my supreme thanks and respects to is my advisor, beloved Dr. Ratna Sharma-Shivappa. She is a knowledgeable, patient, wise and kind-hearted person, not only taught me how to design, conduct and present my research, but also imparted me how to think, how to get adapted to a new culture and how to overcome the setbacks come all over the life. During these two years, I was once knocked by some sudden issues and lost the motivation and goal of life, it was her, who offered me timely guidance, her careful and valuable instructions will be always imprinted in my heart, going with me throughout rest of my life. I also appreciate her for generously supporting me to join in several academic conferences and giving me freedom to choose the courses and conduct experiments obeying my own thought.

The next person I gratefully acknowledge is Dr. Tungate from Solar Center, I suffered the funding shortage when I just came to Raleigh. She nicely offered me a part time job from sugarbeet project to help me extricate oneself from a plight and I really enjoy a good time working with her. She is kind, knowledgeable and vigorous person and gave me some inspirations on my research. And I also enjoyed the sugarbeets she offered during my research.

I also want to show my hearty gratitude to my other committee members: Dr. Jonathan W. Olson from Department of Microbiology and Dr. Saad A. Khan from Department of Chemical Engineering. Dr. Olson is a well-read man especially on microbiology and gave me some critical advices on micro-organism and fermentation

design to make my research design more professional and feasible. Dr. Khan is an amiable, generous and Knowledge-rich scholar, who kindly provide technology and equipment support on plastic characterization. I also want to thank Dr. Dan Willits and Dr. Mari Chinn in BAE, for their unselfish guidance and encouragement, which greatly helped me to conduct my experiments smoothly.

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In addition, I would like thank all my dear friends, here in USA and back in China, without their encouragement and advice, I wouldn`t have gained the power to shake off the bonds and keep moving forward.

At last, thank you, my beloved parents, you always stand by my side and give me good-to-excellent care. Because of you, I will keep fighting for brilliant future.

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Introduction and Literature Review

1.1 Plastics

Polymeric materials like rubber, resin, ployester etc. have been widely accepted because of their ease of their processability and amenability in providing a large variety of cost-effective products that help to enhance comfort and quality of life in modern industrial society (Khare and Deshmukh, 2006). The varied types of polymers have brought wide applications (Belenkiy, 2006), such as commodity products (bags, electrical insulation, siding, roofing membranes, packaging, bottles, buckets, cabinets for TVs and computers, parts for washing machines, refrigerators and vacuum cleaners, etc.) and specialty products (made with conducting, photosensitive, liquid crystalline and bio-polymers for agricultural, aerospace, and electronics applications). Therefore, they play an important role in mankind`s daily life. The total amount of plastics in Municipal Solid Waste (MSW) stream—about 30 million tons—represented 12.0% of total MSW stream generated in 2008 (EPA, 2008).

There are two main types of plastics which are widely used today (Khare and Deshmukh, 2006): petroleum-based plastics, which belong to thermosets, and biodegradable plastics, which belong to thermoplastics.

1.2 Petroleum-derived plastics

Petroleum based plastics are almost exclusively made from a nonrenewable resource (petroleum), which is also the main source of energy for today`s world, which mainly

include polypropylene (PP), polyethylene (PE) and polystyrene (PS) (Yu, 2000). In western world, a large amount of fossil carbon was used by chemical industries and a part of this is used for production of plastics and polymers every year (Eggersdorfer et al., 1992). The current nationwide dependence on fossil fuels for manufacturing plastic is around 270 million metric tonnes per year (Khardenavis et al., 2007).Therefore if petroleum keeps being consumed at this speed for a various applications, it will be almost complete dried out in the next 60 - 80 years (Khare and Deshmukh, 2006).

Besides the problem of relying on diminishingly non-renewable energy sources for production of plastic, the problem of environmental pollution caused by indiscriminate dumping of non-biodegradable plastic waste has also assumed global proportions. Plastics accumulate easily in landfills, oceans and even bodies of animals, defying any kinds of attempt at disposal – be it through recycling, burning, or land-filling. In 2006, 12% of the weight of landfills in USA were plastic materials (Khare and Deshmukh, 2006). Recycling petroleum-derived plastic has been tried, but it is usually associated with human health issues, resulting from exposure to and inhalation of toxic fumes, especially gas and other residues released during the process.

Because of the inert nature of petroleum-derived plastics, how solid wastes to be disposed has been a new concern of waste management (Choi et al., 1997). Therefore, problems with solid waste management and further environmental impact called for the development of biodegradable plastics, which is possessed of comparable physical and chemical properties paralleled with conventional synthetic plastics (Patwardhan et al., 2004). Also, in part, due to growing legislation in the US and Europe aimed at barring the

use of non-degradable plastics in a variety of consumer products, the development and production of degradable plastics is rapidly expanding (Leaversuch, 1987).

Approximately 93% of fossil resources consumed in the world are for energy production, while only 7% are used by industries for the production of a variety of organic chemicals, including solvents and plastics (Eggersdorfer, 1992). Replacing a part of synthetic plastics with biodegradable polymers produced from renewable resources is likely to have only a small impact on the overall consumption of fossil fuel. However, demand for biodegradable plastic in the US is expected to rise more than 15%/year to 200 million tons valued at $845 million in 2012 (PFFC, 2008). The more widespread application of biodegradable plastics could, thus, significantly contribute to solving problems caused by waste disposal and environmental pollution.

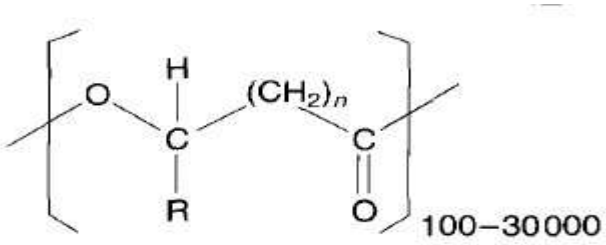
1.3 Biodegradable plastics

It has been proposed that an environmentally acceptable degradable plastic or polymer is the one which are degraded through photo-degradation, oxidation, hydrolysis and/or biodegradation without discharging harmful residue to the environment (Swift, 1993). Biodegradable plastics are either partly or fully degraded either by non-enzymatic hydrolysis or by organized enzymes presented possessed by some microorganisms (Poirier, 1995). Some types of the biodegradable plastics being developed include polyhydroxyalkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides, and copolymers and blends of starch and polypropylene (Lee, 1996). Blends of starch and polypropylene can be regarded only as semi-biodegradable while PHA is 100%

biodegradable (Patwardhan et al., 2004). One of most widely used biodegradable plastics is polylactic acid (PLA). Compared to polyhydroxybutyrate (PHB) and petroleum-derived plastics, it is cheaper (Datta et al., 1995), but suffers from defects like slow crystallization, poor heat resistance and mechanical brittleness, which have hindered further application of PLA (Yokohara and Yamaguchi, 2008) . In addition, although lactic acid can currently be produced by industrial scale fermentation, it still needs chemical polymerization to get PLA, which makes the production process more complicated and time-consuming (Datta et al., 1995). Hence, further exploration of other biodegradable plastics like PHB is warranted.

1.4 A potential biodegradable plastic---Polyhydroxybutyrate (PHB)

PHB belongs to the class of biodegradable plastics, Polyhydroxyalkanoates (PHAs) . The general structure of PHAs is showed in Figure 1.1.



n = 1	R = hydrogen	Poly(3-hydroxypropionate)
	R = methyl	Poly(3-hydroxybutyrate)
	R = ethyl	Poly(3-hydroxyvalerate)
	R = propyl	Poly(3-hydroxyhexanoate)
	R = pentyl	Poly(3-hydroxyoctanoate)
	R = nonyl	Poly(3-hydroxydodecanoate)
n = 2	R = hydrogen	Poly(4-hydroxybutyrate)
	R = methyl	Poly(4-hydroxyvalerate)
n = 3	R =hydrogen	Poly(5-hydroxyvalerate)
	R = methyl	Poly(5-hydroxyhexanoate)
n = 4	R = hexyl	Poly(6-hydroxydodecanoate)

Figure 1.1 General structure of Polyhydroxyalkanoates(PHAs) and some representative members (Steinbuchel, 1991)

PHB is a kind of polyesters synthesized by various microorganisms as energy reserve materials under unfavorable conditions, i.e. limitation of some essential nutrients or excess availability of carbon source (Lee, 1996). They play the same role in bacteria as fat in humans or starch in plants.

Properties of pure PHB are comparable to commonly used bulk plastics, e.g. polypropylene. It is an unique natural biopolymer, which combines three exceptional features (Hrabak, 1992): (i) thermoplastic processability; (ii) 100% resistance to water and moisture; and (iii) 100% biodegradability. It could therefore be used for applications similar to those of common plastics and would fit well into new waste-management strategies.

PHB also has some addition advantages which favor interest in this material. Firstly, the 3-hydroxybutyric acid monomer is a chiral molecule. The monomer units of PHB can be used as basic molecules for the chemical production of complex chiral pharmaceutical or agrochemical agents, which is an area of growing importance due to worldwide governmental regulations on pharmaceutical production, as reported by Seebach et al., 1987. Another very important property is that PHB is biocompatible, which means that it can be implanted in the body and will not cause inflammations. The rate of degradation of PHB is very slow inside the body, usually lasts over a period of months to years, which is not the case for example for PLA. Therefore, PHB as a kind of emerging materials for slow release of pharmaceuticals has also been investigated (Korsatko et al., 1983; Lafferty et al, 1988).

Relative to physical properties of PHB, Yezza et al. (2007) reported the average molecular weight of PHB produced from maple sap and pure sucrose media were $300 \pm 66 \times 10^3$ and $313 \pm 104 \times 10^3$ g/mol, respectively. The melting temperature and enthalpy of fusion of PHB produced from maple sap (177.0 ℃ and 77.2 J/g, respectively) perfectly matched results obtained from sucrose (176.6 ℃ and 81.0 J/g, respectively). The PHB recovered from maple sap expressed a quick thermal degradation between 288.0 and 319.4 ℃ with a peak at 308.8 ℃. For that in sucrose-based media, it showed a rapid degradation between 286.5 and 317.7 ℃ with a peak at 307.9 ℃. The results of research conducted by Pereira et al. (2008) showed that PHB produced from sucrose-based media had an average molecular weight of 790×10^3 g/mol, the corresponding melting temperature and enthalpy of fusion was 169.9 ℃ and 63.4 J/g respectively.

Although PHB has several desirable properties, the price of commercial grade PHB—around 15 times higher than comparable synthetic plastics—greatly limits its further applications. For example, Biopol®, a kind of copolymer of β-hydroxybutyric acid and β-hydroxyvaleric acid produced by *Ralstonia eutropha*, sells for about 8-10 times the price of synthetic plastics (Mudliar et al., 2007). Wider use of PHB requires economic feasibility, hence, optimized fermentation strategies, low-cost media and more efficient downstream recovery methods are needed (Chisti, 1998; Tamer et al., 1998). In PHB production, about 40% of the total production cost is brought by raw material (Choi et al., 1999). Thus, the use of a cheaper carbon source is essential in order to reduce the high production cost of PHB.

1.5 Production of PHB

PHB was the first member in the family of PHAs to be found by Lemoigne (1926) as a type of composition of bacterium *Bacillus megaterium*. After that PHB has been detected to exist in various taxonomically different strains. Many kinds of microbes such as *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium*, *Methylotroph* are able to generate PHB up to 30 – 80% of their dry cell weight (Lafferty et al., 1988). Although production of PHB can be realized through chemical synthesis utilizing β-butyrate or β-hydroxybutyric acid as monomers, the difficulty in controlling process conditions and high production cost make this approach economically unfeasible (Wang et al. 2001). Compared to chemical synthesis, biosynthesis is advantageous by virtue of its simple and

mild conditions during production (Li, 2007). Therefore, fermentation production of PHB using specific microorganisms is the most appropriate path.

Over the years, research has been done on the production of PHB in plants. By transferring the capability for PHB synthesis from bacteria to higher plants, transgenic *Arabidopsis* plants can express all three enzymes (β -ketothiolase, acetoacetyl-CoA reductase and PHB synthase) for PHB synthesis in the plastid (Benfey et al., 1990; Poirier et al., 1992; Nawrath et al., 1994). However, plant cells can obtain low yields [<10% (w/w) of dry sugarbeet] of polymer. Higher levels of polymer inhibit growth and development of plants (Verlinden et al., 2007). On the contrary, PHAs can be accumulated up to 90% (w/w) of the dry cell mass inside bacteria (Verlinden et al., 2007). Therefore, this research focuses on fermentation production of PHB from inexpensive substrate using a specific kind of microorganism.

1.5.1 PHB producing microorganisms

The microorganisms widely reported to produce PHB include *Ralstonia eutroph*a (Kim et al., 1994; Ryu et al., 1997; Yu et al., 2006), *Alcaligenes latus* (Hrabak, 1992; Yamane et al., 1996; Wang et al., 1997; Grothe et al., 1999), *Azotobacter vinelandii* (Page and Comish, 1993), several strains of *methylotrophs* (Kim et al., 1996), and recombinant *Escherichia coli* (Fidler and Dennis, 1992; Wang and Lee, 1997) among the others. Generally speaking, among the various microbes studied, microbial production of PHB has been mainly conducted by the bacterium *R.eutropha*, and then, *A.latus* (Grothe et al., 1999).

1.5.1.1 PHB production in *Ralstonia eutropha*

R.eutropha has drawn most research interest by virtue of its ability to produce large amount of PHB using simple carbon sources such as glucose, fructose and acetic acid (Ramsay et al., 1991; Braunegg et al., 1995; Koyama and Doi, 1995). It can produce PHB in the presence of a carbon source, but in the meantime an essential growth nutrient should be missing (Suzuki et al., 1986). Thus, a two-step process is necessary, which involves an initial cell growth stage followed by a PHB production step. The glucose concentration was sustained at 10–20 g/l during fed-batch cultivation, final cell mass, PHB concentration and PHB content was obtained as 164 g/l, 121 g/l, and 76%, respectively by *R. eutropha* within 50 h cultivation, giving the highest PHB productivity of 2.42 g/(L·h) (Kim et al., 1994). Although some mutants of *R.eutropha* such as ATCC 17697 have been reported to use sucrose to produce PHB (El-Sayed et al., 2009; Abdelhad et al., 2009; Tanamool et al., 2009), little research has been done to prove that mutants of *R.eutropha* can produce PHB from sucrose as efficiently as with glucose or fructose.

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1.5.1.2 PHB production in *Acaligenes latus*

A.latus is a kind of non-sporulating, gram-negative, obligate aerobe bacteria. The shape bacterial cells are more like short rods or coccoids, which are around 1.2–2.4 mm in diameter and 1.6 –2.4 mm in length. The cells exist singly, in pairs, or in short chains (Palleroni et al., 1978; Holt et al., 1994). The bacterium is sluggishly motile using 5–10 peritrichous flagella (Palleroni et al., 1978; Holt et al., 1994). Optimal growth

temperature is around 35 ℃. Growth takes place within the pH range of 6.0–7.5. Colonies are opaque, yellowish to grayish pink, and round.

A.latus, a kind of growth-associated PHB producer, can accumulate PHB up to 80% of dry cell weight without limitation of any nutrient (Braunegg et al., 1985). Thus, the single-stage fermentation could potentially realize the desired PHB production levels (Hanggi, 1990; Hrabak, 1992). Recent research reported that by culturing in nitrogen limitation medium, higher PHB synthesis rate and content could be achieved (Wang et al., 1997; El-Sayed et al., 2009). Furthermore, *A. latus* can be cultured readily on sucrose, which is less expensive than glucose that is typically used in *R.eutropha* fermentations, thus providing an opportunity to reduce the costs of PHB production greatly. Wang et al. (1997) enhanced PHB content (% of dry cell weight) in *A. latus* from 50% to 87% in fed-batch cultivation by induction nitrogen limitation. Strains of *A. latus* can also be used to produce PHB from molasses or sugar syrup. Batch culture of *A. latus* ATCC 29713 in regular nitrogen-rich media was reported to produce PHB up to 63% of dry cell mass after 93 h of cultivation (Grothe et al., 1999). The average biomass yield coefficient on sucrose was about 0.4 kg/kg. Consequently, *A.latus* offers the double advantages at increasing PHB productivity and reducing raw material cost that thus allowing significant savings in production cost. Additionally *A. latus* cells can be easily lysed to enhance recovery of PHB (Byrom, 1990). In spite of the advantages offered by *A. latus* are appealing, there has been limited research on PHB production by this microorganism due to the media cost and yield, besides industrial PHB production with *A. latus* by Chemie Linz GmbH, Austria (Hrabak, 1992).

1.5.1.3 PHB production in other microorganisms

It has been demonstrated that a very high concentration (149 g/l) of PHB could be obtained by fully automatic fed-batch culture of *Pseudogenes* oleovorans using methanol as a carbon source. However, it took 170 h to reach this concentration, resulting in low productivity of 0.88 g/ (l· h) (Suzuki et al., 1986). Production of PHA by fed-batch and continuous cultivation of *P. oleovorans* resulted in high concentration of PHA and high productivity. With optimized culture conditions, the cell concentration and productivity at steady state could be 11.6 g/l and 0.58 g/ (l· h), respectively, under a continuous mode of operation (Preusting et al., 1993). Production of PHA by recombinant *E. coli* harbouring *R. eutropha* PHA biosynthesis genes was investigated by Kim et al. (1992). The accumulation of PHB in recombinant *E. coli* reached 80–90% of dry cell weight at the end of cultivation. The PHB concentration of higher than 80 g/l with productivity more than 2 g/(l· h) was obtained by pH-stat fed-batch culture of recombinant *E. coli*.

1.5.1.4 Comparison PHB production between *A.latus* and *R.eutropha*

Some research has been done on the comparison of *A.latus* and *R.eutropha* using sucrose as carbon source to produce PHB. As Tanamool et al. (2009) reported, when using sweet sorghum juice, whose main composition is sucrose, as sole carbon source to produce PHB, for *R. eutropha* the results appeared that the maximum concentrations of dry cell mass and PHB obtained were 0.920 g/l and 0.034 g/l, respectively. The yield and productivity obtained were 0.037 g PHB/g dry cell and 0.0019 g/ (l· h), respectively. In comparison, *A. latus* showed a maximum biomass concentration of 1.73 g/l and the PHB

concentration of 0.68 g/l, which corresponded with a yield of 0.393 g PHB / g dry cell and productivity of 0.0125 g/ (l^{-} h). The comparison implies that *A. latus* behaved better when using sucrose. A similar conclusion was also reached by El-Sayed et al. (2009), who showed that when using sucrose as carbon source, in comparison to *R. eutropha*, *A. latus* gave higher dry cell weight (7.92 g/l vs. 4.02 g/l) and specific growth rate of 0.055h^{-1} on sucrose media. The specific growth rate was comparable to that obtained with *R. eutropha* on glucose medium, 0.053h^{-1}. As reported by Khannna and Strivastava (2005), when using sucrose as carbon source for *A. latus*, the biomass reached 2.179 g/l. Though this biomass was lower than that gained from using glucose, a little higher PHB concentration (0.042 g/l) vs. 0.031 g/l was obtained.

1.5.2 Cultivation methods for PHB production

To enhance PHB productivity, not only reducing culture time, but also increasing cell density and PHB content have to be taken into consideration. High-cell density cultures increase productivity and reduce the cost of downstream processing and wastewater treatment. Fed-batch culture has been the most popular culture system to achieve a high-cell-density and PHB content (Suzuki et al., 1986; Kim et al., 1992; Lee 1994; Kim et al., 1994). However, besides more complicated operation and increased susceptibility to contamination, fed-batch cultivation relies on high cell density accumulation leading to low growth rates before induction, which sometimes prolongs the time needed for production (Shokri et al., 2004).

In comparison, for batch cultivation, bacteria are inoculated into a stirred tank bioreactor and under certain predetermined conditions (temperature, pH, aeration, etc.) go through the various growth phases (lag, exponential, stationary). At the end of fermentation, the microbial cells are collected. After cleaning and sterilization of the fermentor, the fermentor is ready for another batch. A batch culture offers some advantages which make it comparable to Fed-Batch culture. It can be used for different fermentation reactions every day and can be sterilized, thus minimizing risk of infection or strain mutation. However, batch culture also has some disadvantages like high labor cost and extended idle time (Nielsen et al., 1994).

As discussed above, *A.latus* is a growth-associated producer of PHB, due to this specificity of *A.latus*, it has been reported to accumulate PHB up to 80% of dry cell weight under nutrient sufficient conditions (Braunegg et al., 1985). But in recent years, Wang et al. (1997) reported that after 8 h of nitrogen limitation, the cell density of *A.latus*, PHB concentration, and PHB content reached 111.7 g (dry cell weight)/l, 98.7 g/l, and 88%, respectively, resulting in a productivity of 4.94 g of PHB/ (l^{-} h). The highest PHB productivity, 5.13 g/ (l^{-} h), was obtained after 16 h of nitrogen limitation. Increase in the PHB synthesis rate due to nitrogen limitation together with an optimal sucrose feeding rate resulted in unprecedentedly high PHB productivity with high PHB content. This conclusion was also supported by El-Sayed et al. (2009), who indicated that after nitrogen limitation, higher synthesis rate, 0.025 g/ (L^{-} h), compared to nitrogen sufficient, which is 0.016 g/ (L^{-} h), can be gained along with higher PHB content (56.05% vs. 46.18%) . Thus, Two-stage batch, which derived based on fermentation under nitrogen-

limitation condition, was gained more and more attention these days. For two-stage batch, the microorganism will be first cultured in regular nitrogen sufficient media for some time, then old media will be removed, the cell mass will be suspended in the same amount of nitrogen limitation media, after that, it will follow the procedures of regular batch culture to conduct the rest of fermentation.

In conclusion, the different fermentation modes (Batch, Two-stage batch or Fed-Batch, Nitrogen sufficient or Nitrogen limitation) should be applied based on the different substrates and microbes used.

1.5.3 Substrates for PHB production

The key nutritional elements required for PHB production by fermentation are carbon and nitrogen sources (Kim, 2000). Since the most significant factor impacting production cost of PHB is cost of substrate (mainly carbon source) (Kim, 2000), several low cost carbon sources and corresponding fermentation strategies have been investigated to reduce the cost. Such as soybean oil (Akiyama et al., 2003), food-grade starches (Kim, 2000) made from cassava, corn, potato, sweet potato, wheat, whey (a major by-product from the manufacturing of cheese) (Lee et al., 1997; Sheu et al., 2009), and beet molasses (Page, 1989) have been investigated for PHB production. In recent years maple sap (Yezza et al., 2007) and sweet sorghum juice (Tanamool, 2009), which mainly contains sucrose, but also has significant amounts of glucose and fructose, have been used as carbon source to produce PHB.

A variety of inorganic and organic carbon sources such as pure sugars, lactic acid (Linko and Vaheri, 1993), alkanoic acids of odd carbon numbers (Fukui et al., 1997), soybean oil (Akiyama et al., 2003),

Sugars such as glucose, fructose and sucrose are the most widely used (Ramsay et al., 1991; Braunegg et al., 1995; Wang et al., 1997; Tanamool et al., 2009) carbon substrates for PHB production due to their low cost, easy availability, and relatively low toxicity (Khanna et al., 2005) compared to other carbon source such as Acetic acid (200 dollars/Kg), Butyric acid (650 dollars/Kg) and Butanol (160 dollars/Kg). Sucrose is a cheaper sugar priced at 4 dollars/Kg while glucose has a market price of about 25 dollars/Kg (USDA, 2010).

Sucrose has been widely reported to produce PHB using *A.latus* (Hrabak, 1992; Yamane et al., 1996; Wang et al., 1997; Grothe et al., 1999). Page (1992) reported that using *Azotobacter vinelandii* UWD cultured in vigorously aerated cultures (50mL per 500mL) , PHA yield was enhanced from 1 g/l (9% of dry weight) in the 2% sucrose control media to 7 g/l (70% dry weight) in the media containing 5% of beet molasses (which also contained about 2% sucrose), and further increased to 20~25 g/l after 35 h Fed-batch fermentation. Liu et al. (1998) reported that the maximum dry cell weight of *Escherichia coli* could be up to 16.7 g/l along with maximum PHB content of 85% (w/w). Results of the studies on beet molasses (Page, 1989; Liu et al., 1998) indicate that sugarbeets can be a suitable feedstock for PHB production, furthermore, the cost of PHA synthesis from beet molasses is one-third of that obtained with glucose (Page, 1992),

however process optimization relative to fermentation strategy to enhance productivity is required for commercial application of this feedstock.

1.5.3.1 Sugarbeet as biomass resource to produce PHB

Sugarbeet is cultivated global, but primarily in warm and temperate climates with sufficient precipitation. The world-wide growing area for sugarbeet is about 9.6 million hectares (OECD, 2007) and the annual production is about 250 million tons (FAO Statistics, 2007). The leading producers of sugarbeets countries in 2007 were France, USA, Germany, Ukraine, Poland, Turkey, China (FAO Statistics, 2007). It is said over 27.3 % of all sugar produced is from sugarbeets (FAO Statistics, 2007).

Sugarbeets get a sucrose content of around 15-20 % which depend on climate, soil type, variety and cultivation methods (Asadi, 2007). The chemical composition, major minerals and α-amino-nitrogen in sugarbeet roots were shown in Tables 1.1 and 1.2. Sucrose is main component of sugarbeet root which also contains nitrogen and minerals like potassium and sodium, which are all required by *A.latus* for growth during PHB production. Thus an inference can be made that sugarbeet juice can be used directly to grow *A.latus* without adding any other nutrients, and is a focus of this study. Utilization of sugarbeet juice without supplements is ideal for reducing the substrate cost especially when compared to using pure sugars such as glucose and sucrose, or starch and starch based feedstock such as sweet potato. Sweet potato priced at 461.36 dollars/ton in North Carolina(USDA, 2008) is more expensive than sugarbeet, which is around 50 dollars/ton (USDA, 2008). Additionally, starch needs pretreatment like liquefaction and

saccharification to first convert polysaccharides to monomeric sugars, which will increase the expense of production.

Table 1.1 Chemical compositions of sugarbeet roots

(23.0% – 24.6 % dry weight)

Component	23.0% – 24.6% dry weight
Crude ash	5.0-8.1
Crude protein	4.7-6.8
Ether extract	0.3-0.6
Crude fibre	4.9-6.3
Sucrose	64.7-70.0

Source: NOVUS, 1996

Table 1.2 Major minerals and α-amino-nitrogen in sugarbeet roots

(23.0% - 24.6 % dry weight)

Component	23.0% – 24.6% dry weight
Sodium (Na)	0.4-0.8
Potassium (K)	5.6-7.2
Phosphorus (P)	1.4-2.2
α-amino-N	0.7-1.1

Source: Überregionaler Sortenvergleich, 1997,1998,1999.

Institute for Sugarbeet Research

Beet molasses, which is a byproduct of the sugarbeet industry, is also a suitable feedstock for PHB production because of its high sucrose content and presence of nitrogen and some minerals (Page, 1989; Liu et al., 1998). The chemical composition, major minerals and contents of major anions in beet molasses in sugarbeet molasses were shown in Tables 1.3, 1.4, 1.5.

Table 1.3 Chemical compositions of sugarbeet molasses

(73.0% – 79.0 % dry weight)

Component	**73.0% – 79.0 % dry weight**
Crude ash	6.6-10.0
Crude protein	6.6-11.1
Ether extract	0.0-0.3
Crude fibre	0.0-0.3
Sucrose	43.0-50.5

Source: NOVUS, 1996

Table 1.4 Major minerals in sugarbeet molasses

(73.0% - 79.0% dry weight)

Component	**73.0% – 79.0%** **dry weight**
Sodium (Na)	0.6-1.9
Potassium (K)	3.2-4.7
Phosphorus (P)	0.02-0.06
Calcium (Ca)	0.1-0.5
Magnesium (Mg)	0.01-0.3

Source: NOVUS, 1996

Table 1.5 Contents of major anions in beet molasses

(73.0% - 79.0% dry weight)

Component	**73.0% - 79.0 %** **dry weight**
Chloride	1.0-3.0
Sulfate	0.6-2.0
Phosphorus	0.1-0.06
Nitrate	0.3-0.8
Nitrite	3.0-170mg/kg

Source: Van der Poel et al., 1998

1.6 PHB extraction and recovery

PHB is an intracellular product, which appears as inclusion bodies inside the cell (Kapritchkoff et al., 2006). Therefore, if highly purified PHB is expected, cell lysis should be done to break the cell and release the PHB granules. In another words, extraction of polymer from the biomass is a vital stage of the process (Anderson et al., 1990). And biopolymer recovery and purification steps are important contributions to the production cost (Kapritchkoff et al., 2006). A number of solvents have been studied for PHB extraction, with the aim of finding a relatively cheaper method while achieving higher rate of recovery.

Other solvents have also been reported for recovery of PHB. They include methylene chloride, 1,2-Dichloroethane, 1,1,2,2-etrachloroethane, trichloroethylene (halogenated compound), ethyl acetate (esters), 2,2,2-trifluoro ethanol (alcohols), dimethylformamide (nitrogen compound), dimethylsulphoxide (sulfur compounds) (Braunegg et al., 1978; Choi and Lee 1997; Terada and Marchessaut 1999). Besides these solvents, PHB extraction was also widely reported using solvents like dichloroethane, dichloromethane and chloroform (Hahn et al., 1994). Chloroform is the solvent most commonly used for recovery of PHB (Hahn et al., 1994; Hahn et al., 1995; Lee et al., 1999).

Chloroform and sodium hypochlorite solution are well known solvents for PHB extraction due to advantages of high solubility and easy recovery of PHB (Hahn et al., 1994). Hahn et al. (1994) used chloroform and sodium hypochlorite to extract PHB from dry cell powder. PHB recovers from the chloroform phase by non-solvent (mixture of methanol and water 7:3 v/v) precipitation and filtration, under optimal conditions, the

recovery was up to 91% and the purity of recovered PHB was higher than 97%. Shawaphun and Manangan (2009) studied the extraction of PHA produced by *A. latus* and found that $CHCl_3$ and CH_2Cl_2 gave relatively higher rates of PHA recovery (83.93% and 86.80%) compared to other "non solvents" and protic solvents like toluene and hexane.

1.7 PHB quantification and characterization

As reported, Gas Chromatography (GC) and Fourier Tansform Infrared Spectroscopy (FT-IR) are used for PHB identification and qualification (Hahn et al., 1994; Hong et al., 1999; Yu et al., 1998; Kim, 2000; Yezza et al., 2007; Hemmat et al., 2009). FTIR is sensitive to local molecular environment; therefore, it has been widely applied to reveal the conformational changes of macromolecules during melting and crystallization(Bayar and Severcan, 2005) . After GC analysis, the presence and amount of 3-hydroxybutyric acid were determined. Gel Permeation Chromatography (GPC), Differential Scanning Calorimeter (DSC), Rheology, Transmission electron microscopy (TEM) are applied to do the PHB characterization (Yu et al., 1998; Yezza et al., 2007; Pereira et al., 2008), GPC for molecular weight measurement, DSC and Rheology for melting temperature measurement and TEM for inclusion body observation.

Chapter 2

Production of Polyhydroxybutyrate (PHB) by *Alcaligenes latus* using Two-Stage Batch and Fed-batch Fermentation strategies

Abstract

This research focused on investigating the suitability of sucrose as carbon source to produce polyhydroxybutyrate (PHB), a promising biodegradable plastic, by two-stage batch and fed-batch fermentation with *Alcaligenes latus* ATCC 29714. The study included selection of strain, two-stage batch fermentations using different time points for switching to nitrogen limited media (14 h, 16 h or 18 h) and fed-batch fermentations using varied time points (the same as two-stage) for introducing nitrogen limited media. Based on the results, the best strain to produce PHB using sucrose as carbon source was *A.latus* ATCC 29714 with maximum specific growth rate of 0.38 ± 0.01 h^{-1} and doubling time of 1.80 ± 0.05 h. Inducing nitrogen limitation at 16 h and ending second stage at 26 h gave optimal performance for PHB production, resulting in significantly higher ($P \leq 0.05$) PHB concentration of 3.60 ± 0.06 g/l and PHB content as 46.65 ± 12.24% of cell dry weight.

Keywords: Biodegradable plastics, Polyhydroxyalkanoate (PHA), *Ralstonia eutropha*, Sucrose fermentation, Nitrogen limitation

Abbreviation and Nomenclature

DCW dry cell weight, g/l

DCW_1 dry cell weight at start of exponential phase (g/l) in Eq. (2), g/l

DCW_2 dry cell weight at end of exponential phase (g/l) in Eq. (2), g/l

HPLC high-performance liquid chromatography

OD optical density

OD_1 optical density measured at 600 nm at the start of exponential phase in Eq. (1)

OD_2 optical density at end of exponential phase in Eq. (1)

PHA polyhydroxyalkanoate

PHB polyhydroxybutyrate

PHBV poly(3-hydroxybutyrateco-3-hydroxyvalerate)

PHB4B poly(3-hydroxybutyrate-co-4-hydroxybutyrate)

PHBHx poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)

PLA polylactic acid

T_d doubling time in Eq. (3), h

t_0 the time at which stationary phase started, h

t_1 time corresponding to OD_1 or DCW_1 (h) in Eq. (1) and (2), h

t_2 time corresponding to OD_2 and DCW_2 (h) in Eq. (1) and (2), h

$Y_{p/x}$ PHB yield based on dry cell weight, g PHB produced/ g dry cell mass

$Y_{p/s}$ PHB yield based on sugar consumed, g PHB produced/ g sugar consumed

μ_m maximum specific growth rate in Eq. (1), (2), (3) and (4), h^{-1}

2.1 Introduction

Polymeric materials like plastic, rubber, resin, and polyester have wide spread use in today's industrial society because of their ease of processability and amenability in providing a large variety of cost-effective commodity and specialty products ranging from plastic bottles to electronics (Khare and Deshmukh 2006). However, most plastics and synthetic polymers are synthesized from nonrenewable resources like petrochemicals and persist in the environment long after intended use. This results in problems with solid waste management and the global environment. It has been estimated that plastics accumulate in the environment at the rate of approximately 25 million tonnes per year (Nawrath et al. 1995; Patwardhan et al. 2004) and represent 12% of the total municipal solid waste stream in the USA (EPA 2008). Therefore, there is growing interest in developing biodegradable plastics, which possess desirable physical and chemical properties like conventional synthetic plastics (Patwardhan et al. 2004).

Biodegradable plastics such as polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) can be fully degraded, either by the activity of enzymes or non-enzymatic hydrolysis, leaving no harmful residues in the environment (Poirier 1995) and find applications in areas similar to conventional plastics. Polyhydroxybutyrate (PHB), which is a PHA, has received considerable attention in recent years mainly because of its unique features such as high biodegradability, thermoplastic processability and resistance to moisture (Hrabak 1992). The tensile strength (43 Mpa) and melting point (177℃) of PHB are comparable to bulk plastics like polypropylene (38 Mpa and 176℃, respectively) making it a suitable candidate for directly replacing some traditional petroleum-derived

plastics (El-Sayed et al. 2009b; Tsuge 2002). Although, copolymers of PHB like PHBV, PHB4B and PHBHx may be preferred to overcome its limitations like brittleness and thermal instability (Verlinden et al. 2007).

PHB is mainly synthesized as an energy reserve (Lee 1996; Patwardhan and Srivastava 2004) by microorganisms like *Ralstonia eutropha* (Kim et al., 1994; Ryu et al. 1997; Yu et al. 2006), *Alcaligenes latus* (Hrabak 1992; Yamane et al. 1996; Wang et al. 1997; Grothe et al. 1999), *Azotobacter vinelandii* (Page and Comish 1993), several strains of *methylotrophs* (Kim et al. 1996), and recombinant *Escherichia coli* (Fidler and Dennis 1992; Wang and Lee 1997). Commercial PHB production (under the trade name BIOPOL®) via fermentation is performed by *R. eutropha* (Zinn et al. 2003), an efficient non-growth associated PHB producer, which utilizes glucose under limitation of media nutrients such as P and N (Kim et al. 1994; Ryu et al. 1997; Saeed 2002; Yu et al. 2006). Nevertheless, compared to conventional plastics, the price of commercial grade PHB is about 10 times higher, which limits its wide spread application in industry (Mudliar et al. 2007). *A. latus* is a growth associated PHB producer, which can synthesize PHB with sucrose as carbon source and reach PHB accumulations of up to 80% of dry cell weight (Braunegg et al. 1985). Sucrose, priced at 4 dollars/kg, is a cheaper sugar compared to glucose which has a market price of about 25 dollars/kg (USDA 2010). It has been reported that nitrogen limitation in the media can enhance PHB accumulation by *A. latus* (Wang et al. 1997) by increasing final PHB content (50% to 88%), making downstream processing much easier.

Although much effort has been made by researchers to compare different fermentation strategies for producing PHB using *A. latus*, especially proving the superiority of nitrogen limitation for enhanced production, little work has been done to optimize the overall fermentation process. Efforts to optimize the time at which nitrogen limitation should be introduced for maximizing PHB yield and potentially shorten the overall length of the fermentation process are lacking. Therefore, this work focused on streamlining the fermentation process by selecting a suitable microbial strain and identifying a fermentation approach that results in the enhanced production of PHB. Selection of the microorganism was based on a comparison of growth profiles in media containing sucrose as carbon source. Two fermentation modes, two-stage batch and fed-batch, were investigated to compare dry cell weight (DCW), PHB yield, PHB content and PHB productivity as affected by differences in times at which nitrogen limitation is introduced during the growth phase.

2.2 Materials and Methods

2.2.1 Microorganisms

Intracellular PHB producing microorganisms, *Alcaligenes latus* (ATCC 29714 and ATCC 29712) and *Ralstonia eutropha* (ATCC 17699) purchased from American Type Culture Collection (ATCC) were used in this study. *A. latus* is known for its ability to utilize sucrose as a carbon source (Hrabak, 1992; Yamane et al., 1996; Wang et al., 1997; Grothe etal., 1999) while *R. eutropha* is well known for its ability to produce PHB from glucose (Kim et al., 1994; Ryu et al., 1997; Yu et al., 2006).

2.2.2 Media preparation

A variety of media types were used at various stages in this study.

Med. 1 (reviving freeze dried cultures): Freeze dried *A. latus* (ATCC 29712 and 29714) and *R. eutropha* (ATCC 17699) were revived using media 1018 as described by the ATCC catalog. The media contained (per liter of deionized water): KH_2PO_4 4 g, Na_2HPO_4 4.8 g, NH_4Cl 1.0 g, $MgSO_4 .7H_2O$ 0.5 g, and agar 20.0 g. After autoclaving the media, 5.0 ml of filter sterilized Solution A (as follows), and 10.0 ml of filter sterilized Solution B (as follows), was added to it.

Solution A: Ferric ammonium citrate 1.0 g, $CaCl_2$ 0.1 g, distilled water 100.0 ml.

Solution B: Glucose 10.0 g, distilled water 100.0 ml.

Freeze dried suspensions were incubated at 33℃ for 20 h and 20% glycerol stocks prepared for future use.

Med. 2 (maintaining cultures for study): Difco™ nutrient agar (Becton, Dickinson and Company, MD, USA) was used for maintaining active *A. latus* and *R. eutropha* cultures during the study (El-Sayed et al., 2009a) .

Med. 3 (R. eutropha growth kinetics): Media for studying growth kinetics of *R. eutropha* (Kim et al. 1994) contained (per liter): **Glucose** 10 g, $(NH_4)_2SO_4$ 1 g, KH_2PO_4 1.5 g, $Na_2HPO_4.12H_2O$ 9 g, $MgSO_4.7H_2O$ 0.2 g, TES 1 ml. The trace element solution (TES) had (per liter): $FeSO_4.7H_2O$ 10 g, $ZnSO_4.7H_2O$ 2.25 g, $CuSO_4.5H_2O$ 1 g, $MnSO_4.4\text{-}5H_2O$ 0.5 g, $CaCl_2.2H_2O$ 2 g, $Na_2B4O_7. 10H_2O$ 0.23 g, $(NH_4)_6Mo_4O_{24}$ 0.1 g, 35% HCl 10 ml.

Med. 4 (A. latus growth kinetics and nitrogen rich fermentation phase): Media used for studying *A. latus* growth kinetics and PHB production under nitrogen rich phase

(Yamane et al. 1996) contained (per liter) : **Sucrose** 20 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 1.5 g, $Na_2HPO_4.12H_2O$ 9 g, $MgSO_4.7H_2O$ 0.2 g, $FeCl_2.H_2O$ 60 mg, $CaCl_2.2H_2O$ 10 mg and TES 1 ml. Each liter of TES contained H_3BO_3 0.3 g, $CoCl_2.6H_2O$ 0.2 g, $ZnSO_4.7H_2O$ 0.1 g, $MnC1_2.4H_2O$ 30 mg, $Na_2MoO_4.2H_2O$ 30 mg, $NiSO_4.7H_2O$ 28 mg, and $CuSO_4.5H_2O$ 10 mg. pH of the formulated media was 7.0 (unadjusted).

Med. 5 (nitrogen limited A. latus fermentation phase): Media for studying *A. latus* fermentation for PHB production under nitrogen limitation (El-Sayed et al. 2009b) was similar to *Med. 4*, however only 0.2 g of $(NH_4)_2SO_4$ (10% of *Med. 4*) was added.

Med. 6: Feeding solution for fed-batch fermentation under nitrogen limitation had (per liter) 52 g sucrose.

2.2.3 Inoculum preparation

The following procedures were used to propagate frozen glycerol stocks prior to each stage of studies.

For all studies with *A.latus*, two 250 ml flasks with 30 ml of *Med. 4* were inoculated with 1.5 ml thawed 20% glycerol stocks of *A.latus* ATCC 29714 and 29712, respectively, and incubated at 33℃ for 24 h. For studying growth kinetics with glucose as carbon source, sucrose was replaced with the same amount of glucose in *Med. 4*.

For all studies with *R. eutropha* ATCC 17699, 30 ml *Med. 3* was used to propagate the microorganism at 30℃ for 24 h. When studying growth kinetics with sucrose as carbon source, glucose in *Med. 3* was replaced with the same amount of sucrose.

2.2.4 Growth measurements

A comparison of growth kinetics was done to identify the microbe most suitable for this research. Four hundred ml of *Med. 4* in 500 ml polypropylene centrifuge bottles in triplicate was inoculated with 10 ml *A. latus* culture per bottle, for ATCC 29712 and ATCC 29714 growth measurement. Another set of 500 ml polypropylene centrifuge bottles with 400 ml *Med.3* were inoculated with 10 ml *R. eutropha* (ATCC 17699) culture per bottle. Bottles inoculated with *A. latus* were incubated at 33℃, 150 rpm, while those with *R. eutropha* were incubated at 30℃, 150 rpm in a shaking water bath and microbes were cultured until they reached and maintained stationary phase for 3 h. Ten ml aliquots were drawn from each bottle at 2 h intervals to measure optical density and dry cell weight.

2.2.5 Fermentation

Studies were conducted with 2 fermentation modes, two-stage batch and fed-batch, to determine optimal conditions for PHB production by inducing the microorganism with nitrogen limitation. The effect of introducing nitrogen limitation on PHB production, either by changing to nitrogen limited media or feeding nitrogen limited media at 3 time points established based on growth measurements, was studied. Parameters including dry cell weight (DCW), PHB concentration, PHB content and PHB yield relative to biomass produced were used for comparison and selection. The time at which stationary phase started (t_0) was determined from growth measurements (dry cell weight, specific growth rate and doubling time during growth on sucrose) of the chosen

microbe, which was found to be ATCC 29714 in this study. According to the results of growth measurements, t_0 was identified to be 16 h after inoculation.

Figure 2.1 Centrifuge bottles with sponge caps used during two-stage and fed-batch culture and flasks with foil seals used for reviving culture

2.2.5.1 Two-stage nitrogen limited batch fermentation

PHB fermentation studies on introducing nitrogen limitation by replacing *Med. 4* at t_0-2, t_0 or t_0+2 h were conducted with 100 ml *Med. 4* in 250 ml centrifuge bottles. Each bottle (250 ml x 36) was inoculated with 2.5 ml inoculum prepared as described earlier and incubated at 33℃, 200 rpm in an incubator shaker (Series 25, New Brunswick Scientific CO., INC., Edison, New Jersey, U.S.A.). Samples were drawn at 4 h intervals up to and at t_0-2, t_0 or t_0+2 h. At the time point for introducing nitrogen limitation, the remaining bottles were centrifuged at 4000 rpm, 4℃ for 15 min. Residual *Med. 4* was decanted and the same volume of nitrogen limited media (*Med.5*) was added. Bottles were incubated at 33℃, 200 rpm in the incubator shaker for up to 28 h from start of

second stage. Three bottles each were taken out for analyses at 4, 8, 12, 20, 24, 26, 27 and 28 h after introduction of nitrogen limitation (El-Sayed et al. 2009a).

2.2.5.2 Nitrogen limited fed-batch fermentation

Centrifuge bottles (250ml x 36) with 100 ml *Med. 4* were inoculated with 2.5 ml *A.latus* inoculum and incubated at 33℃, 200 rpm in the incubator shaker. Samples were drawn at 4 h intervals as well as at t_0-2, t_0 or t_0+2 h for determination of OD, DCW and PHB concentration, etc. Nitrogen limitation was applied by feeding 25 ml *Med.6* starting at t_0-2, t_0 or t_0+2 h, with two more feeds being added at 4 h intervals. From the start of feeding, three bottles were taken at 4, 8, 12, 20, 24, 26, 27 and 28 h, as in two stage batch fermentation for analysis.

Figure 2.2 Shaking incubator used during two-stage and fed-batch culture and regular incubator used for reviving the culture

2.2.6 PHB extraction and quantification

A gravimetric method similar to that employed previously by Kim et al. (1994) was used to extract PHB. Dried cell powder was treated with 30% sodium hypochlorite (v/v) and chloroform to extract PHB. The amount of reagents needed was adjusted based on cell powder available to obtain a ratio equivalent to 12.5 ml chloroform and 12.5 ml 30% (v/v) sodium hypochlorite per gram of dry cell power. The mixture was vortexed (Maxi Mix Ⅱ, Barnstead Thermolyne, Type 37600 Mixer) in 50 ml glass centrifuge tubes and kept in a water bath at 30℃ for 90 min. The tubes were centrifuged for 15 min, 4000 rpm at 30℃. Three distinct phases were observed: top layer (aqueous hypochlorite solution), middle layer (cells, other biological matter), bottom layer (PHB rich chloroform). The chloroform phase was removed by pipeting after centrifugation and PHB was recovered by non-solvent precipitation. The non-solvent used was a mixture of methanol and water (7:3 v/v), the amount of non-slovent used was 1/10 volume of chloroform used for each individual sample. After extraction, uncapped tubes with PHB rich aliquots were left in a fume hood for 48 h to volatilize any excess solvent. The final PHB pellet was weighed and the various parameters calculated.

2.2.7 Analytical procedures

2.2.7.1 Optical density (OD) and Dry cell weight (DCW)

As per the method reported by Grothe et al. (1999) and Patwardhan et al. (2004), optical density (OD) of the suitably diluted cell suspension was measured at 600 nm (Grothe et al. 1999) against a media blank in a spectrophotometer (Shimadzu UV-1700,

Suzhou Instruments Manufacturing CO., Ltd., Suzhou, China). Dry cell weight was determined gravimetrically. Culture samples were centrifuged (4000 rpm, 15 min, 4 ℃) and the supernatant was refrigerated for further analysis. The cell pellet was re-suspended in deionized water, recovered after re-centrifugation (4000 rpm, 15 min, 4 ℃), dried to constant weight (90 ℃, 24 h), cooled in a desiccator, and then weighed to determine DCW.

Figure 2.3 Spectrophotometer used for OD measurement and oven used for drying the cell mass

2.2.7.2 Sugar analysis using high-performance liquid chromatography (HPLC)

Sugar analysis was performed using a HPLC (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID-10A) was used to measure the sucrose, glucose and fructose concentration of fermentation samples from each sampling points. A Biorad Aminex HPX-87P column and corresponding guard column were used for analysis with

an eluent (HPLC grade water) at a flow rate of 0.6 ml/min and column temperature of 85 ℃.

Fermentation samples in 250 ml centrifuge bottles were first centrifuged and the supernatant obtained was filtered with 0.22 μm syringe filters into HPLC vials for sample preparation. Calibration standards with sucrose concentrations of 20 g/l, 10 g/l, 5 g/l, glucose concentrations of 5g/l, 2.5g/l, 1.25 g/l and fructose concentrations of 5g/l, 2.5g/l, 1.25 g/l were prepared by diluting a stock solution containing 1 g sucrose, 0.25 g glucose and 0.25 g fructose in 50 ml HPLC grade water.

Figure 2.4 HPLC equipment used for sugar analysis

2.2.8 Parameters and Calculation

2.2.8.1 Growth kinetics

Based on the growth curves (OD vs. Time, DCW vs. Time) obtained, the start and end point of exponential phase for each strain on each carbon source was determined.

Maximum specific growth rate (μ_m), doubling time (T_d) and specific sugar consumption rate (V_c) were determined according to the method provided by Painter et al. (1963).

Maximum specific growth rate:

Based on OD:

$$\mu_m = \frac{\ln \frac{OD_2}{OD_1}}{t_2 - t_1} \qquad (1)$$

Based on DCW:

$$\mu_m = \frac{\ln \frac{DCW_2}{DCW_1}}{t_2 - t_1} \qquad (2)$$

Doubling time:

$$T_d = \frac{\ln 2}{\mu_m} \qquad (3)$$

2.2.8.2 Fermentation

Optical density (OD), dry cell weight (DCW), PHB yield coefficient relative to cell dry weight ($Y_{p/x}$, g/g, defined as gram PHB produced per gram dry cell mass produced) (Grothe et al. 1999), PHB concentration (g/l, defined as g PHB measured in 1 L culture), PHB content (g/g, defined as the ratio of PHB concentration (g/l) to dry cell concentration (g/l)) and PHB productivity (g/l.h, defined as gram PHB produced per liter media per h culture time) (Wang et al. 1997) were measured and calculated per definition during the fermentation process.

2.2.9 Statistical analysis

All experiments in this study were conducted in triplicate. Statistical analysis (T-test, $\alpha = 0.05$) using Microsoft Excel 2010® was conducted for comparing the growth of three different PHB producing microorganisms. Mixed analysis ($\alpha = 0.05$) in SAS® (version 9. 1. 3 SP4) was used to determine the effect of induction and fermentation times during two-stage batch and fed-batch fermentation.

2.3 Results

2.3.1 Selection of strain for PHB production

Growth curves obtained for the 3 PHB producing strains using sucrose as carbon source are presented in Fig 2.5. As expected, *R. eutropha* (ATCC 17699) did not show any exponential growth through 18 h of incubation indicating its inability to metabolize sucrose. Final OD of the culture was 0.02 ± 0.00. For *A. latus* (ATCC 29712) and *A. latus* (ATCC 29714), typical batch growth was observed over the 22 h incubation period and the final ODs were 1.86 ± 0.15 and 1.94 ± 0.06, respectively. Growth associated parameters for both *A. latus* strains gave similar trends, with the maximum growth rate based on DCW (μ_m) and doubling time (T_d), respectively, being 0.34 ± 0.01 h^{-1} and 2.05 ± 0.04 h for *A. latus* (ATCC 29712). The corresponding values for *A. latus* (ATCC 29714) were 0.38 ± 0.01 h^{-1} and 1.80 ± 0.05 h. ATCC 29714 was selected for subsequent studies due to its higher μ_m and lower T_d compared to ATCC 29712.

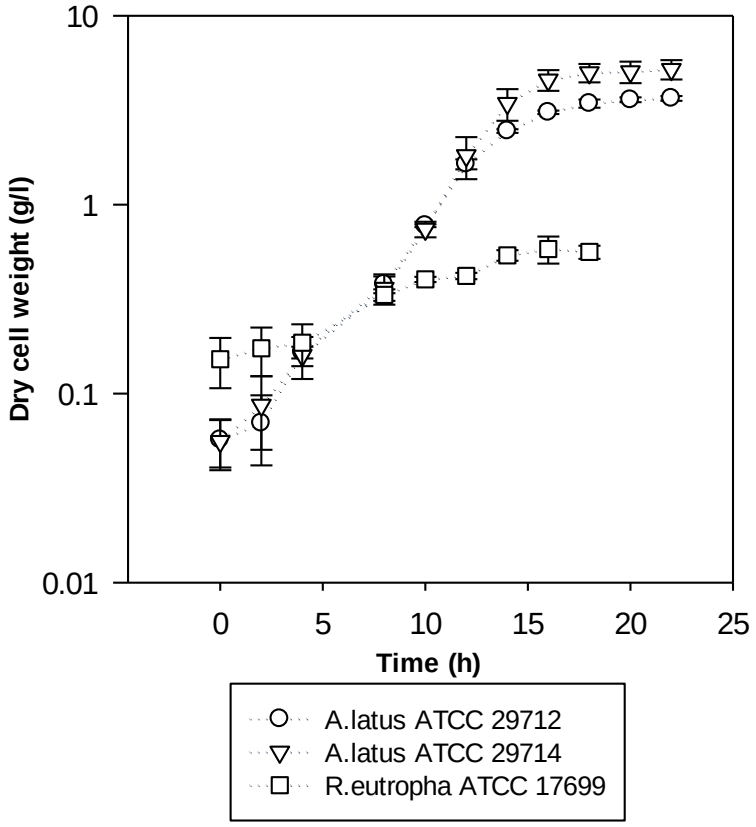

Figure 2.5 Comparison of dry cell weight (DCW, g/l) over culture time (h) during the growth of three PHB producing strains using sucrose as carbon source (*R. eutropha*: ATCC 17699; *A. latus*: ATCC 29712; *A. latus*: ATCC 29714). Dotted lines represent only the expected trend.

2.3.2 PHB production using two-stage nitrogen limited batch fermentation

Two-stage nitrogen limited batch fermentation resulted in a DCW of 3.40 ± 0.21 g/l at the end of 18 h of first stage (Fig 2.6). For 14 h and 16 h samples the value was 2.22 ± 0.04 g/l and 3.06 ± 0.08 g/l, respectively. Table 2.1 presents the changes in DCW, PHB yield coefficient based on DCW ($Y_{p/x}$) and PHB productivity during the second

stage of fermentation. DCW measurements indicated that *A. latus* (ATCC 29714) grew exponentially during 28 h of incubation with highest DCW being 6.33 ± 0.08 g/l, 7.88 ± 0.10 g/l and 7.27 ± 0.46 g/l for 14 h, 16 h and 18 h, respectively. The $Y_{p/x}$ for all three induction time points showed a similar trend as DCW and the final $Y_{p/x}$ were 0.48 ± 0.01, 0.47 ± 0.12 and 0.28 ± 0.05, respectively, which were comparable to the results obtained by El-Sayed et al. (2009a). The productivity of the process ranged from 0.05 g/l. h to 0.10 g/l.h for 14 h, 0.06 g/l.h to 0.13 g/l.h for 16 h and 0.06 g/l.h to 0.13 g/l.h for 18 h induction times, with final values being 0.10 ± 0.00 g/l.h, 0.12 ± 0.02 g/l.h and 0.09 ± 0.01 g/l.h for 14, 16 and 18h, respectively. As illustrated in Fig 2.7, PHB content increased as cultivation proceeded reaching 46.62 ± 12.24%, 48.42 ± 7.06% and 39.56 ± 5.02% at 28 h post induction of nitrogen limitation for 14 h, 16 h and 18 h, respectively. The corresponding PHB concentrations (Figure 2.8) in fermentation broth were 3.03 ± 0.01 g/l, 3.60 ± 0.06 g/l and 2.86 ± 0.02 g/l. The PHB content was comparable to what has been reported by El-Sayed et al. (2009a) during two-stage fermentation at 30℃ for 52 h using *A. latus* ATCC 29712 with sucrose as carbon source for a PHB content of 56.05%.

Based on sugar consumption analysis during the process, 5.25 ± 0.85 g/l, 5.10 ± 0.60 g/l and 10.50 ± 0.27 g/l sugar was consumed for 14 h, 16 h and 18 h, respectively. PHB yields ($Y_{p/s}$) on the basis of g of sugar consumed were obtained as 0.59 ± 0.08, 0.71 ± 0.14 and 0.27 ± 0.02 g/g for 14 – 18 h induction times.

Inorganic nitrogen consumed during the first stage was 143.33±24.01 mg/l, 74.67±39.93 mg/l, 158.67 ± 19.60 mg/l for 14 h, 16 h, 18 h, respectively. Corresponding

consumption during the second stage was 44.97 ± 2.97 mg/l, 43.38 ± 0.42 mg/l, 33.37 ± 2.11 mg/l.

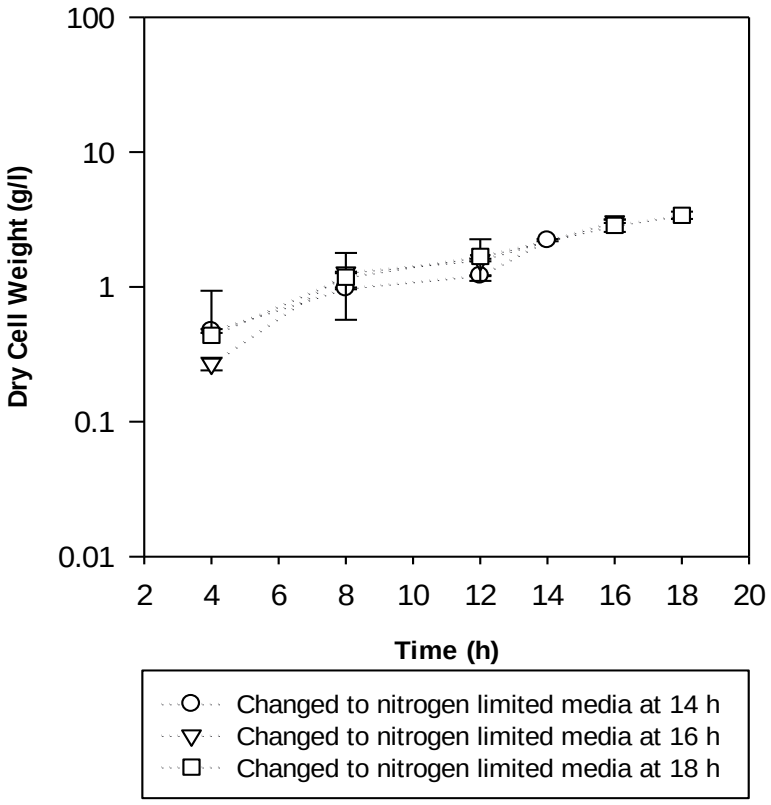

Figure 2.6 Dry cell weight during first stage of two-stage nitrogen limited batch fermentation and with *A. latus* (ATCC 29714) using sucrose as carbon. Dotted lines represent only the expected trend.

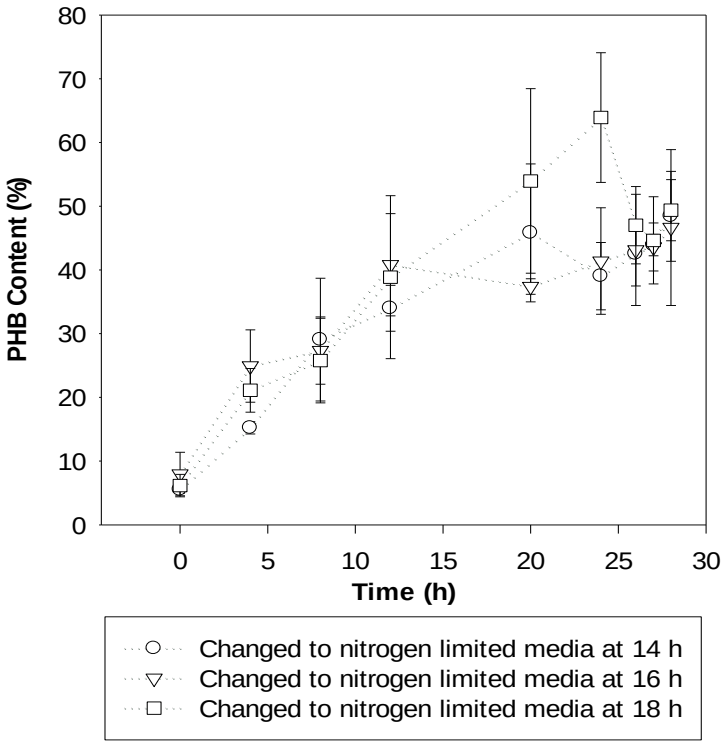

Figure 2.7 PHB content obtained from two-stage batch fermentation of *A. latus* (ATCC 29714) after initiating nitrogen limitation. Dotted lines represent only the expected trend.

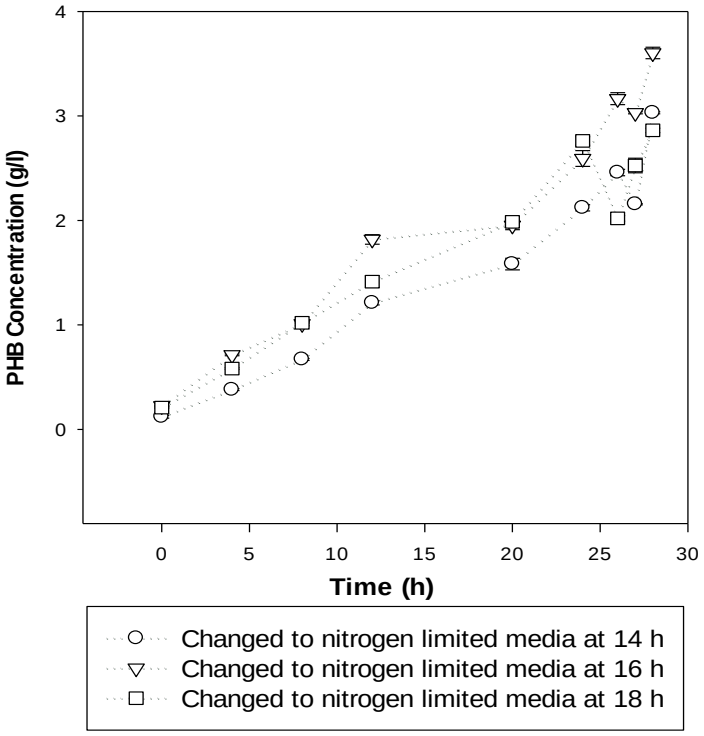

Figure 2.8 PHB concentration obtained from two-stage batch culture of *A. latus* (ATCC 29714) after initiating nitrogen limitation. Dotted lines represent only the expected trend.

Table 2.1 Growth and PHB production by *A. latus* (ATCC 29714) during second stage of two -stage fermentation

Induction by nitrogen limitation	Sample time (h)	Dry cell weight (g/l)	$Y_{p/x}$	PHB productivity (g/l.h)
14 h	4	2.53±0.07	0.15±0.01	0.07±0.02
	8	2.46±0.07	0.29±0.01	0.07±0.01
	12	3.57±0.05	0.34±0.02	0.09±0.02
	20	3.40±0.06	0.46±0.11	0.05±0.02
	24	5.45±0.06	0.39±0.05	0.07±0.01
	26	5.82±0.09	0.43±0.03	0.09±0.01
	27	4.92±0.03	0.44±0.01	0.08±0.00
	28	6.33±0.08	0.48±0.01	0.10±0.00
16 h	4	2.93±0.05	0.25±0.06	0.12±0.02
	8	3.74±0.06	0.27±0.05	0.10±0.02
	12	4.48±0.09	0.41±0.08	0.13±0.04
	20	5.22±0.11	0.37±0.01	0.06±0.02
	24	6.20±0.10	0.41±0.08	0.08±0.03
	26	7.37±0.08	0.43±0.09	0.11±0.02
	27	6.97±0.04	0.44±0.04	0.10±0.00
	28	7.88±0.10	0.47±0.12	0.12±0.02
18 h	4	3.43±0.63	0.12±0.03	0.09±0.02
	8	3.97±0.35	0.19±0.04	0.10±0.01
	12	4.75±0.40	0.21±0.15	0.10±0.05
	20	3.80±0.69	0.40±0.14	0.06±0.01
	24	4.34±0.53	0.44±0.10	0.09±0.01
	26	6.70±0.38	0.20±0.03	0.07±0.01
	27	6.87±0.80	0.21±0.13	0.09±0.03
	28	7.27±0.46	0.28±0.05	0.09±0.01

2.3.3 PHB production using fed-batch culture

Parameters similar to those studied during two-stage batch cultivation were investigated for fed-batch cultivation. Figure 2.9 presents the DCW up to the times when feeding was initiated to introduce nitrogen limitation in the media. The DCW followed an increasing trend for all three time points after initiating feeding (Table 2.2). For 16 h, the highest DCW obtained during fed-batch fermentation was 9.23 ± 0.25 g/l 28 h after initial feeding, while that for 14 h and 18 h final DCWs were 7.20 ± 1.54 g/l and 7.85 ± 0.64 g/l, respectively. $Y_{p/x}$ increased during the process with the maximum values being 0.57 ± 0.15 for 16 h induction, 0.48 ± 0.24 for 14 h and 0.26 ± 0.17 for 18 h. Productivity of the process ranged from 0.02 to 0.07 g/l.h, 0.01 to 0.14 g/l.h and 0.03 to 0.08 g/l.h for 14, 16 and 18 h, respectively, with the corresponding final values at 28 h being 0.07 ± 0.00 g/l.h, 0.12 ± 0.02 g/l.h and 0.08 ± 0.01 g/l.h. Productivity with 16 h induction was the highest at 0.12 ± 0.02 g/l.h. As shown in Fig 2.10 and 2.11, PHB content had a similar trend compared with two-stage batch fermentation. The final PHB content was 31.77 ± 6.46%, 38.77 ± 6.80% and 31.08 ± 3.15% for 14 h, 16 h and 18 h, respectively, while the corresponding PHB concentrations were 2.22 ± 0.07 g/l, 3.55 ± 0.54 g/l and 2.43 ± 0.20 g/l at 28 h.

There was 16.01 ± 8.54 g/l, 22.88 ± 11.53 g/l and 5.30 ±3.39 g/l sugar consumed during 14 h, 16 h and 18 h runs. The corresponding final PHB yield coefficients based on sugar consumed ($Y_{p/s}$) during the process were 0.16 ± 0.10, 0.19 ± 0.11 and 0.56 ± 0.38, respectively. The inorganic nitrogen consumption was 288.63 ± 12.35 mg/l for 14 h, 284.07 ± 11.98 mg/l for 16 h, and 292.27 ± 4.52 mg/l for 18 h.

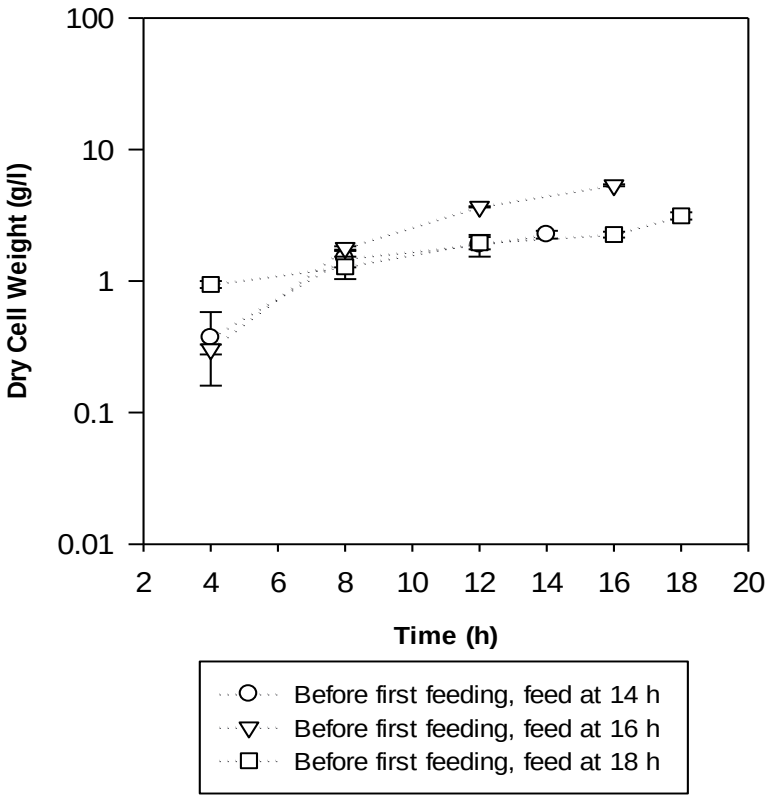

Figure 2.9 Dry cell weight before initiating feeding during fed-batch fermentation with *A.latus* (ATCC 29714) using sucrose as carbon. Dotted lines represent only the expected trend.

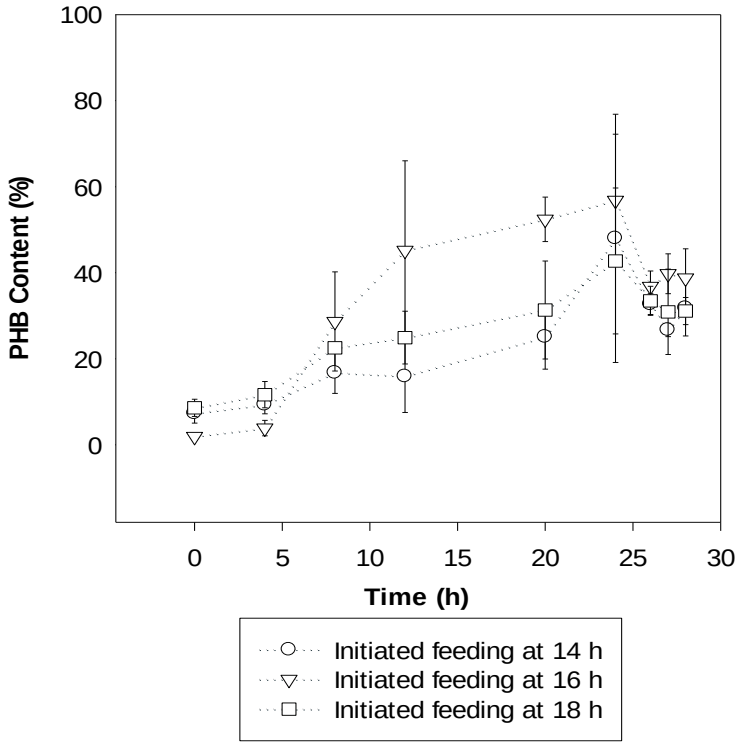

Figure 2.10 PHB content obtained from fed-batch fermentation of *A. latus* (ATCC 29714) after initiating nitrogen limitation. Dotted lines represent only the expected trend.

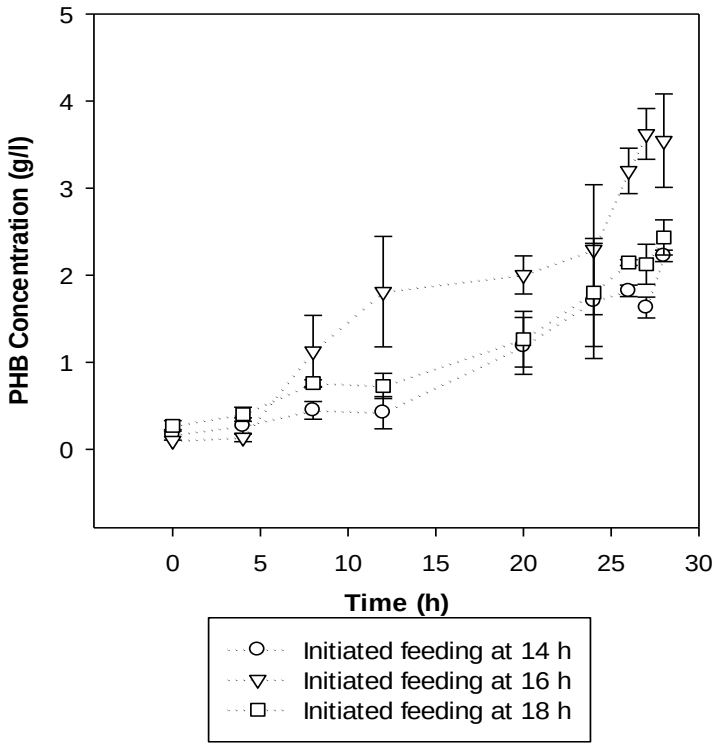

Figure 2.11 PHB concentration obtained fed-batch culture of *A. latus* (ATCC 29714) after initiating nitrogen limitation. Dotted lines represent only the expected trend.

Table 2.2 Growth and PHB production by *A. latus* (ATCC 29714) during fed-batch culture after initiating inhibition of feeding of nitrogen limited media

The time points for starting feeding	Time (h)	Dry cell weight (g/l)	$Y_{p/x}$	PHB productivity (g/l.h)[c]
14 h	4	2.82 ±0.43	0.09 ±0.02	0.02 ±0.02
	8	2.71 ±0.17	0.17 ±0.05	0.03 ±0.01
	12	2.72 ±0.23	0.16 ±0.08	0.02 ±0.02
	20	4.77 ±0.32	0.25 ±0.08	0.04 ±0.01
	24	3.91 ±0.89	0.48 ±0.29	0.05 ±0.02
	26	5.57 ±0.20	0.33 ±0.02	0.06 ±0.00
	27	6.11 ±0.59	0.27 ±0.02	0.05 ±0.00
	28	7.20 ±1.54	0.32 ±0.06	0.07 ±0.00
16 h	4	3.66 ±0.08	0.04 ±0.02	0.01 ±0.01
	8	3.96 ±0.04	0.29 ±0.12	0.13 ±0.05
	12	4.18 ±0.10	0.45 ±0.21	0.14 ±0.05
	20	3.83 ±0.06	0.52 ±0.05	0.07 ±0.01
	24	4.02 ±0.11	0.57 ±0.15	0.08 ±0.03
	26	8.71 ±0.06	0.37 ±0.04	0.12 ±0.01
	27	9.18 ±0.19	0.40 ±0.05	0.13 ±0.01
	28	9.23 ±0.25	0.39 ±0.07	0.11 ±0.02
18 h	4	3.52 ±0.41	0.12 ±0.03	0.03 ±0.02
	8	3.38 ±0.24	0.23 ±0.01	0.06 ±0.00
	12	2.95 ±0.34	0.25 ±0.06	0.04 ±0.01
	20	4.14 ±0.55	0.17 ±0.11	0.04 ±0.01
	24	4.31 ±0.50	0.26 ±0.17	0.05 ±0.02
	26	6.46 ±0.67	0.33 ±0.03	0.07 ±0.00
	27	7.20 ±1.52	0.31 ±0.10	0.07 ±0.01
	28	7.85 ±0.64	0.31 ±0.03	0.08 ±0.01

2.4 Discussion

2.4.1 Growth comparison of the three PHB producing microorganisms using sucrose as carbon source

Statistical analysis of OD values obtained during growth studies indicated that there was a significant difference ($P \leq 0.05$) in the growth of *R. eutropha* (ATCC 17699), *A. latus* (ATCC 29712) and *A. latus* (ATCC 29714) but there was no significant difference ($P > 0.05$) between *A. latus* (ATCC 29712) and *A. latus* (ATCC 29714). Maximum specific growth rate and doubling times were significantly different ($P \leq 0.05$) for all three strains. *A. latus* (ATCC 29714) had the highest μ_m and significantly ($P \leq 0.05$) lower T_d and was thus selected to perform PHB fermentation in this study. The conclusion was in line with that obtained by Yamane et al. (1997) who during their study on high-cell-density fed-batch culture of *Alcaligenes latus* indicated that *A. latus* (DSM 1123/ ATCC 29714) was the most suitable strain for PHB production from amongst three strains (DSM 1122/ATCC 29712, DSM 1123/ATCC 29714, and DSM 1124/ ATCC 29713). However, no specific data was shown in that research.

2.4.2 Effect of time of introducing nitrogen limitation (14 h, 16 h, 18 h) during two-stage batch fermentation on PHB production

Based on DCW measured at the end of first stage for three different induction times (14 h, 16 h and 18 h), 18 h samples had significantly higher DCW ($P \leq 0.05$). For second stage cultures, statistical analysis using mixed analysis (Type 3 Tests of Fixed Effects, $\alpha = 0.05$) including all parameters (DCW, PHB concentration, PHB content, $Y_{p/x}$,

PHB productivity), sampling points (4, 8, 12, 20, 24, 26, 27, 28 after introduction of nitrogen limitation) and three different induction times (14 h, 16 h and 18 h), as shown in Table 2.3, indicated that induction time had a significant ($P \leq 0.05$) effect on PHB production.

When each parameter measured during stage 2 of the study was analyzed individually, there was no significant difference ($P > 0.05$) between the DCW for 16 and 18 h runs, but they were both higher than 14 h. The 16 h samples however had the highest final DCW. PHB concentration of 16 h samples was significantly higher ($P \leq 0.05$) than 14 h and 18 h, apart from giving the highest PHB concentration at 28 h. PHB content of samples induced at 16 h was also significantly higher ($P \leq 0.05$) than those for 14 h and 18 h. PHB yield ($Y_{p/x}$, based on DCW) and productivity were significantly higher ($P \leq 0.05$) when nitrogen limitation was introduced at 16 h and 18 h, although there were no significant differences between 16 and 18 h.

Relative to reviewed parameters (PHB content, PHB yield, PHB concentration, $Y_{p/x}$ and PHB productivity), 16 h induction resulted in the superior performance compared to 14 and 18 h. During the 16 h induction process, increasing fermentation time beyond 26 h did not have a significant effect ($P > 0.05$) on DCW, PHB content, $Y_{p/x}$ and productivity. However, there were significant differences ($P \leq 0.05$) in DCW and PHB concentration between samples taken at 24 h and 26 h. Therefore, a process in which nitrogen limitation was introduced at 16 h and fermentation proceeded up to 26 h was identified as optimal for two-stage batch cultivation in this study.

Table 2.3 Mixed analysis (Type 3 Tests of Fixed Effects) for each parameter in two-stage culture including all the sampling time points (Time) and time points for media changing (Treatment)

Dependent Variable	Effect	Num DF	Den DF	F Value	Pr > F
Dry cell weight	Treatment	2	42	84.40	<.0001
	Time	6	42	483.58	<.0001
	Treatment*Time	12	42	27.41	<.0001
PHB concentration	Treatment	2	42	44.35	<.0001
	Time	6	42	112.90	<.0001
	Treatment*Time	12	42	3.96	0.0004
PHB content	Treatment	2	42	6.91	0.0025
	Time	6	42	135.35	<.0001
	Treatment*Time	12	42	4.29	0.0002
PHB yield	Treatment	2	42	7.04	0.0023
based on dry cell weight	Time	6	42	148.63	<.0001
	Treatment*Time	12	42	4.42	0.0002
PHB productivity	Treatment	2	42	44.68	<.0001
	Time	6	42	29.19	<.0001
	Treatment*Time	12	42	5.12	<.0001

2.4.3 Effect of time of introducing nitrogen limitation (14 h, 16 h, 18 h) during fed-batch cultivation on PHB production

Based on mixed analysis (Type 3 Tests of Fixed Effects, $\alpha = 0.05$) shown in Table 2.4, as a whole, considering all the parameters and time points, significant differences ($P \leq 0.05$) among the three different time points for introducing nitrogen limitation by initiating feeding of nitrogen limited media were observed. The final DCW and PHB concentration of 16 h samples obtained at 28 h was significantly higher ($P \leq 0.05$) than

those from 14 and 18 h. Although PHB content of samples induced by initiating nitrogen limitation at 16 h was highest, it did not vary significantly ($P > 0.05$) between 14 and 16 h samples. However, the values of 14 h and 16 h samples were significantly different ($P \leq 0.05$) from those obtained from 18 h initiation. Samples from the 16 h runs had significantly higher ($P \leq 0.05$) final PHB yield ($Y_{p/x}$) and productivity at the end of fermentation when a comparison was made among the three runs (14 h, 16 h and 18 h initiation).

Statistical analysis to study effect of sampling time (fermentation progression) during the 16 h run indicated there were no significant differences ($P > 0.05$) in DCW, PHB content, $Y_{p/x}$ and PHB productivity at 26, 27, and 28 h. Compared to 26 h, however, there was a significant increase ($P \leq 0.05$) in PHB concentration when fermentation was performed up to 27 h. For 14 h samples, 28 h gave significantly higher ($P \leq 0.05$) DCW and PHB concentration, but there was no significant difference ($P > 0.05$) in PHB content, $Y_{p/x}$ and productivity. For 18 h samples, also there were no significant differences ($P > 0.05$) among 26 h, 27 h and 28 h for all parameters measured. Hence, it can be inferred that 16 h post inoculation was a suitable time for initiating nitrogen limitation by feeding nitrogen limited media in a fed-batch process and the 27 h fermentation post initiation, was an optimal production time frame.

Table 2.4 Mixed analysis (Type 3 Tests of Fixed Effects) for each parameter in fed-batch culture including all the sampling time points (Time) and time points for media changing (Treatment)

Dependent Variable	Effect	Num DF	Den DF	F Value	Pr > F
Dry cell weight	Treatment	2	36	22.84	<.0001
	Time	6	36	69.54	<.0001
	Treatment*Time	12	36	0.90	0.5431
PHB concentration	Treatment	2	42	16.03	<.0001
	Time	6	42	266.80	<.0001
	Treatment*Time	12	42	3.28	0.0021
PHB content	Treatment	2	36	4.90	0.0131
	Time	6	36	14.91	<.0001
	Treatment*Time	12	36	0.57	0.8268
PHB yield	Treatment	2	36	4.67	0.0158
based on dry cell weight	Time	6	36	14.70	<.0001
	Treatment*Time	12	36	0.56	0.8349
PHB productivity	Treatment	2	36	11.41	0.0001
	Time	6	36	1.75	0.1476
	Treatment*Time	12	36	1.00	0.4632

2.4.4 Comparison of the different fermentation modes (two-stage and fed-batch) for PHB production under optimized conditions

Based on statistical analysis conducted to compare various parameters related to PHB production from optimized conditions of two-stage and fed-batch (T-test, Microsoft Excel® 2010), fed-batch samples had significantly higher ($P \leq 0.05$) DCW than two-stage. More biomass accumulation due to extended log phase is a major advantage why fed-batch fermentation has been widely applied thus far (Yamane et al. 1996). For PHB

concentration, content, productivity and $Y_{p/x}$, there were no significant differences ($P > 0.05$) between two-stage samples and fed-batch fermentation process. Apart from being easier to conduct compared to fed-batch, a two-stage process requires lower amount of sugar (5.10 ± 0.60 g/l vs. 22.88 ± 11.53 g/l), as indicated by amount of sugar consumed and total sugar left at the end of the process, thus reducing the cost (Shokri and Larsson 2004).

In conclusion, two-stage batch with introduction of nitrogen limitation at 16 h with a 26 h post induction nitrogen limited phase was observed as the optimal fermentation mode for PHB production by *A. latus* (ATCC 29714) using sucrose in this study. The final PHB concentration was higher (3.96 g/l vs. 2.97 g/l) than that obtained by Grothe et al. (1999) in 500 ml flasks with culture time set at 93 h using optimized media without nitrogen limitation. The PHB yield coefficient ($Y_{p/x}$) though slightly lower was comparable (0.47 vs. 0.56) to the one from work done by El-sayed et al..(2009a) who conducted a study under nitrogen limited conditions with the first stage lasting 24 h. This research is a step forward in the direction of reducing process time while maintaining or increasing yield of PHB and is in line with the primary goal of this study. It is expected that results of this study can be applied to use low cost sucrose based susbtrates like sugarbeet processing wastes as media for PHB fermentation to further cut the expense of PHB production.

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Chapter 3

Production of Polyhydroxybutyrate (PHB) by *Alcaligenes latus* using Sugarbeet Juice

Abstract

This research focuses on exploring the practicality of using sugarbeet juice as medium to grow *Alcaligenes latus* (ATCC 29714) for production of polyhydroxybutyrate (PHB), a kind of prospective biodegradable plastic. The culture media tested included diluted sugarbeet juice, sugarbeet juice with partial addition of other nutrients except sugar, sugarbeet juice with complete addition of other nutrients except sugar. Media with partial nutrient addition was determined to be optimal for PHB production, with final DCW as 10.30 ± 1.01 g/l, final PHB concentration as 4.01 ± 0.95 g/l, final PHB content as 38.66 ± 7.28%, $Y_{p/x}$ as 0.39 ± 0.07 and maximum PHB productivity 0.22 g/l.h. The melting temperature of extracted PHB from sugarbeet juice-grown cells supplemented with partial nutrients was measured as 151.46 ℃ with crystallinity as 43.12%, the corresponding crystallinity temperature was 45.42 ℃. Thermal degradation of extracted PHB occurred from 255.14 to 283.69 ℃ with the degradation peak at 273.86 ℃. Rheological analysis showed the linear visco-elastic region went up to 3 Pa during stress sweep, and the value was approximately 5 rad/s during frequency sweep test.

Keywords: Biodegradable plastics, Polyhydroxyalkanoate (PHA), Sucrose, Two-Stage batch fermentation

Abbreviation and Nomenclature

DCE	1,2-Dichloroethane
DCW	dry cell weight (g/l)
DSC	differential scanning calorimetry
GC-MS	gas chromatography–mass spectrometry
IC	ion chromatography
G`	storage moduli
G``	lost moduli
OD	optical density
PE	polyethylene
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
PLA	polylactic acid
PP	polypropylene
SC	sugar concentration (in °Brix) in Eq. (1)
SC_g	sugar concentration (g/l) in Eq. (1)
SG	specific gravity in Eq. (1), 10 is conversion coefficient.
TG	thermogravimetric analysis
T_c	crystallinity temperature (℃)
T_m	melting temperature (℃)
$Y_{p/x}$	PHB yield based on dry cell weight (g/g)
$Y_{p/s}$	PHB yield based on sugar consumed (g/g)

3.1 Introduction

The use of plastics has grown significantly in recent years and its application has permeated most aspects of human life and industrial production (Yu et al. 1998). The dominant raw material for current plastic production is petroleum (Yezza et al. 2007), which is nonrenewable and also the main source of energy for the world. Limited future availability of petroleum, increasing price of fossil fuel and environmental and waste management concerns due to non-biodegradability of conventional plastics have thus driven various government and industrial enterprises to look for more sustainable alternatives such as polyhydroxyalkanoates (PHAs), polylactides (PLA), aliphatic polyesters, polysaccharides, blends of starch and polypropylene and other copolymers (Lee 1996) to replace petro-derived plastics (Yu et al. 1999).

Of the various biodegradable plastics being investigated to replace petroleum-derived plastics, polyhydroxyalkanoates (PHA), especially polyhydroxybutyrate (PHB), have received special attention due to some desired mechanical properties that make them comparable to commercialized plastics such as polypropylene (PP) and polyethylene (PE) (Yu 2001). PHB is well-known for its environmental friendliness and complete decomposition to water and carbon dioxide under aerobic conditions by certain microorganisms existing in sewage, sea or soil (Lee 1996). The key applications for PHB include packaging materials, bags, containers, disposable items like one-time use cups and diapers (Lee 1996). It also has medical applications, either in surgical materials or as a slow-release carrier for long-term drug delivery (Patwardhan and Srivastava 2004).

The main obstacle inhibiting wide spread commercialization of PHB is its high production cost, which currently is approximately 10 times higher than conventional synthetic polymers (Wegan et al. 1998). The largest factor affecting PHB production is the cost of substrate, which contributes up to 50% of the processing cost (Choi and Lee 1997). Corn is a common feedstock used by various companies like Cargill Dow Polymers, LLC for biopolymer production (Lunt 2000). Besides being a major food and feed source in many regions of the world, corn is priced at $269/metric ton (USDA 2011). Thus, using cheaper feedstocks is the key to reducing production cost of PHB (Khanna and Srivastava 2005).

Soy wastes from soy milk processing facilities, malt wastes from beer breweries, agro-industrial waste water, extruded rice bran, hydrolyzed corn starch and whey from dairy processing are some resources that have been investigated for sustainable production of biodegradable plastics (Gromez et al. 1996; Huang et al. 2006; Khardenavis et al. 2007; Yang et al. 1994; Yezza et al. 2007; Yu et al. 1999). Sugarbeet, a sucrose-rich and sustainable biomass cultivated globally, is also a potential feedstock (Page 1989; Page 1992). Sugarbeet acreage planted in the US has been increasing steadily and is estimated at 1.25 million acres (NASS 2011) and the world-wide annual production of sugarbeets was about 227 million tons in 2009 (FAO Statistics, 2009). The leading sugarbeet producing countries include France, USA, Germany, and Russia (FAO Statistics, 2009). Sucrose content of dry sugarbeet powders can reach up to 80% and it contains nutrients such as N, P, K and Na which makes it highly suitable for microbial cultivation (OECD 2002). Page (1989, 1992) investigated downstream products of beet

sugar industry such as molasses and pulps for PHB production using *Azotobacter vinelandii* strain UWD. PHB concentration using molasses was however lower than that reported using sucrose-based synthetic media and fermentation (2.74 g/l vs. 7.04 g/l) by *A. latus* (El-Sayed et al. 2009) and thus calls for further investigation. Utilization of sugarbeet juice as a medium for PHB production offers a significant advantage of reduced substrate cost ($61/ton sugarbeet (USDA 2011)) especially when compared to using pure sugars or currently used resources like starch based feedstocks such as corn. Although promising, no optimization has been done by researchers on strain selection, media development and process optimization for improving PHB production using sugarbeets to make it suitable for application in industrial production. Therefore, this research focused on investigating the potential of sugarbeet juice based media with varying supplemental nutrient concentrations for PHB production in a two-stage batch fermentation process involving *A. latus. A. latus* is a growth associated PHB producer that has been reported to use sucrose as carbon source to produce PHB and has the ability to accumulate up to 80% of dry cell mass as PHB (Braunegg et al. 1985). PHB production parameters like yield, content and concentration were monitored to optimize fermentation time.

3.2 Materials and Methods

3.2.1 Microorganisms

An intracellular PHB producing microorganism, *Alcaligenes latus* (ATCC 29714) purchased from American Type Culture Collection (ATCC) was used in this study.

Selection of the strain was based on results of a previous study by Wang et al. (2011).

3.2.2 Media preparation

A variety of media types were used at various stages in this study.

Med. 1: Difco™ Nutrient agar (Becton, Dickinson and Company, MD, USA) was used for maintaining *Alcaligenes latus* (El-Sayed et al. 2009).

Med. 2: Media to prepare *A. latus* inoculum for subsequent PHB production using sugarbeet juice contained the following components (El-Sayed et al. 2009): **Sucrose 20 g/l**, **$(NH_4)_2SO_4$ 2.0 g/l**, KH_2PO_4 1.5 g, $Na_2HPO_4.12H_2O$ 9 g/l, $MgSO_4.7H_2O$ 0.2 g/l, $FeCl_2.H_2O$ 60 mg/l, $CaCl_2.2H_2O$ 10 mg/l, and 1 ml of filter sterilized trace elements solution. Each liter of trace element solution contained H_3BO_3 0.3 g/l, $CoCl_2.6H_2O$ 0.2 g/l, $ZnSO_4.7H_2O$ 0.1 g/l, $MnC1_2.4H_2O$ 30 mg/l, $Na_2MoO_4.2H_2O$ 30 mg/l, $N_iSO_4.7H_2O$ 28 mg/l and $CuSO_4.5H_2O$ 10 mg/l. The pH of the formulated media was 7.0 (unadjusted) as reported by El-Sayed et al. (2009).

Med. 3 (no nutrient addition): Sugarbeet juice obtained by extraction of sugarbeets under conditions identified as optimum in this study was diluted with deionized water to adjust the sucrose concentration to 20 g/l. The same media was used both at stage 1 and stage 2. The pH of this media was 7.03 ± 0.05(unadjusted).

Med. 4 (complete nutrient addition): Sugarbeet juice with sucrose concentration adjusted to 20 g/l (*Med. 3*) was supplemented with all the nutrients identified in *Med. 2* (except sucrose) per liter of diluted sugarbeet juice for stage 1 fermentation. For the 2nd stage, only 0.2 g $(NH_4)_2SO_4$ which was equivalent to 10% nitrogen of 1st stage was added.

The pH of this media was 7.11 ± 0.04(unadjusted) for first stage and 7.08 ± 0.04(unadjusted) for second stage.

Med. 5 (partial nutrient addition): Sugarbeet juice with sucrose concentration adjusted to 20 g/l (*Med. 3*) was supplemented with: **$(NH_4)_2SO_4$** 1.97 g/l (nitrogen rich media for first stage) or 0.17 g/l (nitrogen limited media for second stage); $Na_2HPO_4.12H_2O$ 8.6 g/l; KH_2PO_4 0.94 g/l; $FeCl_2.H_2O$ 57.84 mg/l and 1 ml of filter sterilized trace elements solution containing H_3BO_3 0.3 g/l; $CoCl_2.6H_2O$ 0.2 g/l; $Na_2MoO_4.2H_2O$ 0.03 g/l; $NiSO_4.7H_2O$ 0.03 g/l; $CuSO_4.5H_2O$ 0.01 g/l per 1 L sugarbeet juice. The level of nutrients to be added was based on elemental analysis of *Med. 3* (Table 1). When levels of inherent components were higher in *Med. 3*, the corresponding salt was not added. In other cases the difference between amount of salts added in *Med. 2* and those present in the diluted sugarbeet juice was used to estimate the amount needed for partial addition. The pH of this media was 7.23 ± 0.04(unadjusted) for first stage and 7.24 ± 0.04(unadjusted) for second stage.

3.2.3 Extraction of sugar from sugarbeets

Fresh sugarbeets harvested from Custer area of Montana were sliced and dried in a convection oven (Fisher Scientific) at 60℃ for 25 h. The dried slices were stored at 4℃ in a temperature controlled chamber during the course of the study. Dried slices were ground with a Wiley Mill to a coarse powder to pass through a 2 mm sieve with a Wiley Mill for extraction using deionized water. Sugarbeet powder and deionized water were added in 500 ml centrifuge bottle with solid loading as 1:15 (sugarbeet (w) : water (w)),

totally 12 bottles was placed in a shaking water bath (Precision, Thermo Scientific) at 60℃/150rpm for 1 h to generate 4000 ml sugarbeet juice extract for use during fermentation studies. Extraction conditions used in the study were identified as optimal based on preliminary analysis (Appendix I) looking at solid:water ratio, extraction time, and sugarbeet preparation (fresh vs. oven dry) for samples harvested from Plymouth, NC in September, 2009. After vacuum filtration, the sugarbeet juice based media was obtained.

The sugar concentration was measured in ^{o}Brix by a refractometer (ALLA FRANCE, as shown in Figure 3.1). Brix was converted to grams of sugar, using equation 1 (Bruce, 1995):

$$\mathrm{SC_g} = SC \times SG \times 10 \qquad (1)$$

The specific gravity under different ^{o}Brix was obtained from the Sucrose Conversion Table (USDA 1981).

Figure 3.1 Refractometer used during sugar concentration measurement

3.2.4 Inoculum preparation

For propagation of frozen glycerol stocks prior to starting each stage of the study, 30 ml of *Med. 2* in three 250 ml flasks was inoculated with 1.5 ml thawed *A.latus* ATCC 29714 glycerol (20%) stock and incubated at 33℃ for 24 h. Two and a half ml of inoculum (Santhanam and Sasidharan 2010) was added to 100 ml sugarbeet media for the actual fermentation process.

3.2.5 Fermentation of sugarbeet juice for PHB production

Based on the results of a previous study described in Chapter 2, two-stage batch fermentation with introduction of N limitation at 16 h by replacing spent N rich sucrose based media with fresh N limited media (10% N of original) was identified as optimal for this study. Sugarbeet juice prepared as described above had an average sucrose concentration of approx. 50 g/l, which was much higher than that normally used for *A. latus* cultivation (Yamane et al. 1996; Grothe et al. 1999). Thus, the sugarbeet juice was diluted as needed with deionized water to the desired concentration (20 g/l) before all the studies.

Fermentation studies were conducted with no additional nutrients (*Med. 3*), complete nutrient addition (*Med. 4*) and partial nutrient addition (*Med. 5*) to determine PHB production potential of the juice. Hundred ml of desired media (*Med. 3*, *Med. 4* or *Med. 5*) was added to 250 ml polypropylene centrifuge bottles (3 media types × 3 replicates × 12 sample points). Each bottle was inoculated with 2.5 ml *A. latus* inoculum and incubated at 33 ℃ in the incubator shaker (Series 25; New Brunswick Scientific CO.,

INC.; Edison, New Jersey, U.S.A.) at 200 rpm. Three bottles each were drawn at 4, 8, 12 and 16 h. At 16 h for first stage, the remaining bottles were centrifuged at 4000 rpm/30 ℃ for 10 min and residual media decanted. For N limited stage (stage 2), 100 ml of *Med. 3* was added to the no additional nutrient set, *Med. 4* with only 10% of N compared to 1st stage was added to the complete nutrient addition set and *Med. 5* with only 10% of N was added to the partial nutrient addition set. After changing to new media, the remaining bottles were incubated at 33°C, 200 rpm in the incubator shaker for up to 28 h. Similar to the first stage, three bottles each were taken out at 4, 8, 12, 20, 24, 26, 27, 28 h from start of second stage.

3.2.6 PHB extraction and quantification

The method used for PHB extraction and quantification was adapted from the gravimetric method employed by Kim et al. (1994). A mixture of sodium hypochlorite (12.5 ml, 30%, v/v) and chloroform (12.5 ml) with 1 g dry cell mass in 50 ml centrifuge bottles was vortexed and kept in a water bath at 30℃ for 90 min. It was then centrifuged for 15 min, 4000 rpm in a hanging bucket rotor (Eppendorf 5810 R, Eppendorf AG, Hamburg, Germany) at 30℃. This resulted in three different phases (as shown in Figure 3.2): top layer (aqueous hypochlorite solution), middle layer (cells, other biological matter), and bottom layer (PHB rich chloroform). The chloroform phase was pipetted out carefully after centrifugation and PHB was recovered by non-solvent precipitation using a mixture of methanol and water (7:3 v/v, 1.25 ml/g dry cell mass) and filtration. After extraction, uncapped tubes were left in a fume hood for 48 h to volatilize any excess solvent. Once

the final pellet of PHB (shown in Figure 3.3) was obtained and weighed, the yield of PHB could be estimated.

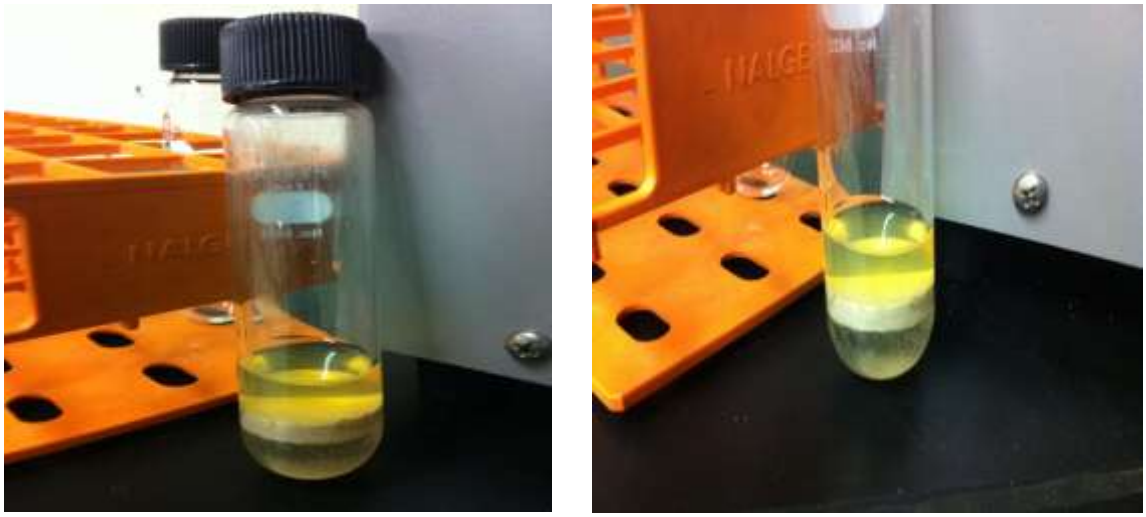


Figure 3.2 Three different phases appeared after centrifuge

Figure 3.3 PHB pellet obtained from sugarbeet juice fermentation with partial nutrient addition media

3.2.7 Analytical procedures

3.2.7.1 Optical density (OD) and Dry cell weight (DCW)

Optical Density (OD) of the suitably diluted cell suspension was measured at 600 nm against a media blank by a spectrophotometer (Shimadzu UV-1700, Suzhou Instruments Manufacturing CO., Ltd., Suzhou, China). Dry cell weight was evaluated by a gravimetric method in which fermentation samples were centrifuged (4000 rpm, 15 min, 4 ℃), decanted and dried in the oven (90 ℃, 24 h) until reached constant weight. The samples were then cooled in a desiccator and weighed to determine DCW.

3.2.7.2 Elemental Analysis

Elemental analysis including determination of N, Ca, Co, Cu,K, Mg, Mn, Ni and Zn in sugarbeet juice was performed by soil analysis lab in the department of Soil Science at North Carolina State University and Iron (II) was done by environmental analysis lab in the department of Biological and Agricultural Engineering. Iron (II) was measured by nitric acid digestion followed by direct aspiration atomic absorption spectroscopy based on the standard method 3111-B of American Public Health Association, American Water Works Association and Water Environment Federation (Clesceri et al. 1998). For inorganic nitrogen measurement, standard method 4500-NH3H provided by Greenberg et al. (2005) was applied. Standard method 3120 was used for the detection of other metals (Greenberg et al. 2005).

3.2.7.3 Sugar analysis using Ion Chromatography (IC)

An IC (Dionex ICS-5000, California, USA, as shown in Figure 3.4) equipped with a pulsed electrochemical detector (Dionex ICS-5000) was used to measure the sugar concentration of fermentation broth samples from each sampling point. The column used was CarboPac PA1 (4 x 250 mm) with eluent (18mM potassium hydroxide) at a flow rate of 0.9 ml/min and working temperature 18 ℃.

Samples in 250 ml bottles were centrifuged and the supernatant obtained was diluted 50 times before filtering through 0.22 μm syringe filters into vials with pre-split caps. Concentrations of sucrose, glucose and fructose were recorded for each sample. A stock solution containing sucrose, fructose and glucose was made by adding 0.5 each of sucrose, glucose and fructose to a 200 ml volumetric flask and dissolving in deionized water. The standards were prepared by gradient dilution to obtain sugar concentrations of 0.5 g/l, 0.25 g/l, 0.125 and 0.0625 g/l.

Figure 3.4 IC equipment used for sugar analysis

3.2.7.4 Confocal imaging of inclusion bodies of A. latus ATCC 29714

Five µl each of broth from fermentation runs resulting in enhanced PHB production by *A. latus* ATCC 29714 in synthetic and sugarbeet juice media were placed on a slide and dried in an incubator overnight at 33 ℃ (Ostle and Holt 1982). The slide was then immersed in 1% Nile blue A aqueous solution in a shallow container and stained for 40 min by placing in a shaking incubator (55 ℃, 50 rpm). The slide was dried again before 5 µl of water was put on the slide and a cover slip placed over it.

A confocal microscope (LSM 710, Carl Zeiss MicroImaging LLC., NY, U.S.A, as shown in Figure 3.5) with Zeiss Axio Observer Z1 inverted microscope and Zeiss Plan Apochromat 63x objectives (NA 1.4 oil immersion) was used to obtain images of inclusion bodies of *A. latus* at the Cellular and Molecular Imaging Facility (CMIF) in the department of Plant Biology at NCSU. The inclusion bodies were excited at 488 nm (argon laser) and fluorescence emission was determined from 492 nm to 625 nm.

Figure 3.5 Confocal microscope used for taking images for inclusion bodies of *A.latus* ATCC 29714

3.2.7.5 PHB identification using Gas Chromatography–Mass Spectrometry (GC-MS)

Samples for GC analysis were prepared as described by Riis and Mai (1988). Forty mg each of dry cell mass obtained from fermentation using synthetic media and sugarbeet juice media, both resulting in optimized PHB production, was put in 50 ml sealable glass centrifuge tubes. Two ml 1,2-Dichloroethane (DCE), 2 ml propanol mixed solution (1 volume hydrochloric acid and 4 volume propanol) and 200 µl of internal standard solution prepared by adding 2.0 g benzoic acid in 50 ml propanol were added to each sample in a 50ml sealable glass centrifuge tube. The tubes were incubated in a convection oven for 2 h at 100 ℃ and shaken once every 30 min. After cooling the tubes to room temperature, 4 ml DI water was added and the tubes were shaken for 30 s before allowing to gravity settle. The bottom organic phase was directly injected into the GC-MS. Standards were prepared by dissolving 200 mg PHB extracted from dry cell mass of the optimized run using synthetic media as described in Chapter 2, in a 10 ml volumetric flask by keeping in a convection oven for 2 h at 100 ℃. After cooling to room temperature, the solution was made up to 10 ml by adding DI water. Two hundred µl, 400 µl, 600 µl and 800 µl of this mixture were taken and treated in the same way with DCE, propanol and benzoic acid as mentioned above.

GC analysis for confirming the presence of PHB in fermentation broth was conducted at Mass Spectrometry laboratory in the department of Chemistry at NCSU. An Agilent 5975 GC-MS (as shown in Figure 3.6) in electron ionization (EI) mode equipped with a HP-5MS 30m x 250um x 0.25um column with helium at a flow of 1 ml/min was

used. Initial temperature of 50 ℃ was held for 3 min before ramping to 325 ℃ at 15 ℃/min and holding for 5 min. A 3 min solvent delay was used.

Figure 3.6 GC equipment used for PHB identification

3.2.7.6 Rheological analysis of PHB

Rheological properties of PHB samples obtained from fermentation of sugarbeet juice were analyzed. Sample discs for rheological analysis were prepared using a Carver® Press (CH-4386, Wabash, IN, USA, as shown in Figure 3.7) in the department of Chemical and Biomolecular Engineering. A 25mm die wrapped with polyimide film to prevent sticking was filled with 0.7 g extracted PHB and pressed twice at room temperature, molded at 140 ℃ by sequentially pressing the dies under 1 to 9 tons and releasing pressure/wt immediately after each application. In the end pressure was held for 2 min before cooling to room temperature. The samples (in discs) made for reological analysis were shown in Figure 3.8.

The rheological analysis was done in the department of Chemical and Biomolecular Engineering at NCSU using AR2000 Advanced Rheometer (TA Instruments, DE, USA, as shown in Figure 3.9) with 25mm ETC flat plate geometry viscoanalyzer. First, stress sweep was performed under constant temperature of 180 ℃, with pressure varying from 1 to 200 Pa to get the maximum stress within the linear viscoelastic (LVE) region. Then frequency sweep was conducted under the maximum shear stress, in a predetermined LVE region, for frequency varying from 0.6284 to 628.3 rad/s (1 to 100Hz) at 180 ℃.

Figure 3.7 Press equipment used for samples moulding

Figure 3.8 Samples (in discs) made for reological analysis

Figure 3.9 Rheometer used during reological analysis

3.2.7.7 Thermal analysis using Differential Scanning Calorimeter (DSC) and Thermogravimetric Analysis (TG)

The melting temperature and crystallinity of PHB obtained from fermentation using sugarbeet juice supplemented fermentation resulting in enhanced production was determined by Diamond DSC (PERKIN ELMER, Inc., USA, as shown in Figure 3.10) equipped with Intracooler 2P in department of Textile Engineering, Chemistry and Science. Two heating and cooling scan cycles were conducted within the temperature range from -20 to 220 ℃ with a scanning rate of 10 ℃/min. The information on thermal history of PHB samples was obtained from the first cycle and the melting temperature (T_m), crystallinity temperature (T_c) and enthalpy of fusion (ΔH) were determined from the second cycle. The crystallinity of PHB samples was calculated as the ratio of ΔH from this study to the ΔH corresponding with 100% crystallinity. The ΔH corresponding to 100% crystallinty of PHB was assumed to be 146 J/g based on that reported by Barham et al. (1984).

According to the research conducted by Yezza et al. (2007), thermogravimetric analysis (TG) was conduct using Pyris 1 TGA (PERKIN ELMER, Inc., USA, as shown in Figure 3.11) with a temperature scanning rate of 20 ℃/min from 0 to 700 ℃ under nitrogen (flow rate of 60 ml/min), to further examine the degradation process of PHB samples.

Figure 3.10 DSC equipmemt applied in thermal analysis

Figure 3.11 TGA equipment used in thermal analysis

3.2.8 Process parameters and statistical analysis

All experiments and analysis were performed in triplicate. For each run using different media, optical density (OD), dry cell weight (DCW), PHB yield coefficient relative to cell dry weight ($Y_{p/x}$, g/g, defined as gram PHB produced per gram dry cell

mass produced) (Grothe et al. 1999), PHB content (g/g, defined as the ratio of PHB concentration to dry cell concentration) and PHB productivity (g/l.h, defined as gram PHB produced per liter per hour) (Wang et al. 1997) were measured and calculated accordingly for comparison after completion of the fermentation process. ANOVA General Linear Model (GLM) analysis ($\alpha = 0.05$) and mixed analysis ($\alpha = 0.05$) using SAS® (version 9. 1. 3 SP4) were applied to compare the effect of different nutrient addition strategies used in sugarbeet juice fermentation on different parameters.

3.3 Results

3.3.1 Elemental analysis of sugarbeet juice

Elemental analysis data for synthetic media (*Med. 2*) used in PHB production by *A. latus* and sugarbeet juice based media (*Med. 3*) is presented in Table 3.1. The concentration of elements was converted to that of chemicals to make it more convenient to determine the level of nutrient addition required for partial nutrient addition based media (*Med. 5*).The results indicated that for trace elements such as Co, Cu and Ni, the amount was below the measurement range (< 0.05 mg/l) of methods used. Although N, Na and Fe were present in sugarbeet juice, the amounts were not enough compared to those in synthetic media, e.g., 25.93 ± 11.90 mg/l equivalent of $(NH_4)_2SO_4$ was present in *Med. 3* compared to 2000 mg/l required for nitrogen rich and 200 mg/l for nitrogen limited *Med. 2*. The differences (1974.07 mg/l for nitrogen rich media and 174.07 mg/l for nitrogen limited media) were the amount of corresponding salt added in *Med. 5*.

Nutrients like Ca, Mg and Mn were in excess amounts in the natural media and hence did not need to be added during fermentation with partial salt addition.

3.3.2 Effect of different nutrient supplementation strategies on PHB production from sugarbeet juice

Figure 3.12 presents the growth curves of *A. latus* ATCC 29714 over a 24 h cultivation period for the three media tested. Similar growth trends were observed for all the curves with final dry cell weights being similar ($P > 0.05$). *A latus* (ATCC 29714) showed exponential growth during first stage (16 h) under all three nutrient addition strategies (Figure 3.13). Media with partial addition resulted in the highest DCW of 7.95 $\pm$ 1.25 g/l, while the corresponding DCWs with no nutrient addition media and complete addition media were 4.75 $\pm$ 0.05 g/l and 4.07 $\pm$ 0.23 g/l, respectively.

The results for DCW, PHB yield coefficient based on DCW ($Y_{p/x}$) and PHB productivity during 28 h second stage cultivation are shown in Table 3.2, DCW data indicated that the exponential growth phase was extended for all three nutrient supplementation runs. The run using partial addition media ended at the highest DCW of 10.30 $\pm$ 1.01 g/l while it was 9.23 $\pm$ 0.06 g/l for no nutrient addition and 7.88 $\pm$ 0.76 g/l for complete addition fermentations. $Y_{p/x}$ was similar ($P > 0.05$) with partial addition and no nutrient addition, at 0.39 $\pm$ 0.07 g/l and 0.39 $\pm$ 0.14 g/l, respectively, and 0.31 $\pm$ 0.12 g/l for complete addition run. PHB productivity ranged from 0.10 to 0.13 g/l.h, 0.05 to 0.09 g/l.h and 0.12 to 0.22 g/l.h for no nutrient addition, complete nutrient addition and partial nutrient addition, respectively. Figures 3.14 and 3.15 illustrate the change in PHB

concentration and PHB content during 28 h second stage cultivation. Both parameters showed an increasing trend with ending values generally within 2.39 ± 0.78 g/l and 31.01 ± 11.81% for complete nutrient addition and 4.01 ± 0.95 g/l and 38.66 ± 7.28% for partial nutrient addition, relative to PHB concentration and content, respectively.

Sucrose consumption was 8.10 ± 6.60 g/l, 9.58 ± 0.60 g/l and 5.96 ± 1.18 g/l for no nutrient addition, complete addition and partial addition fermentations, respectively. During the second stage, corresponding PHB yield coefficients based on sugar consumed were 0.24 ±0.08, 0.25 ± 0.10 and 0.71 ± 0.28, respectively.

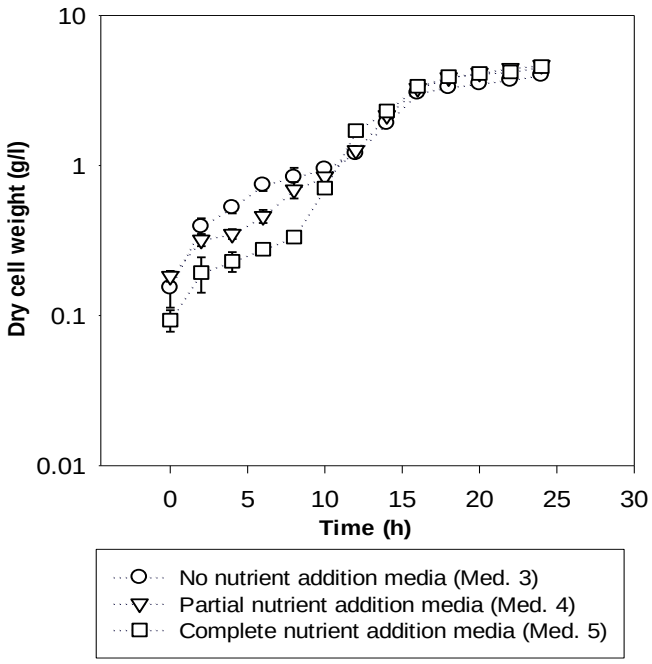


Figure 3.12 Growth of *A. latus* in three types of media with different nutrient addition strategies within 24 h

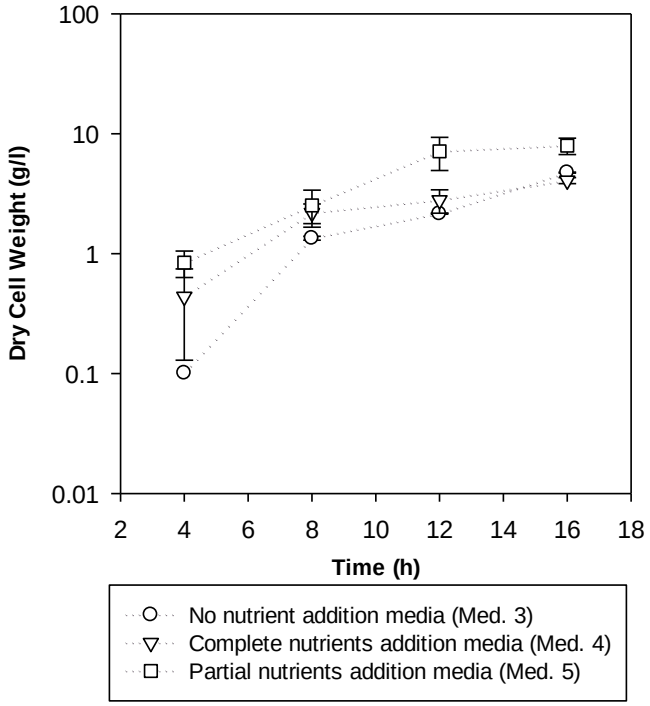


Figure 3.13 Dry cell weight obtained from the first stage of two-stage culture using sugarbeet juice with different nutrient addition strategies by *A. latus* (ATCC 29714) in 250 ml centrifuge bottles, dotted lines only represent the trend.

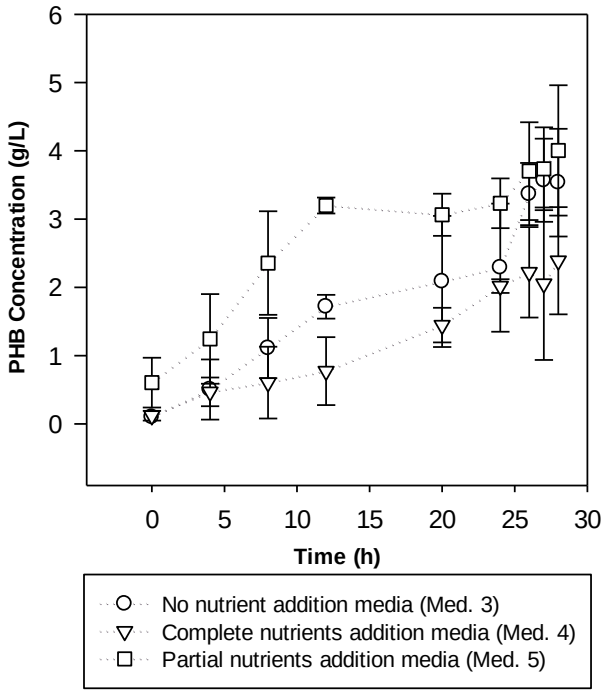


Figure 3.14 PHB Concentration obtained from two-stage batch fermentation of sugarbeet juice with different nutrient addition strategies by *A. latus* (ATCC 29714). Dotted lines only represent the trend.

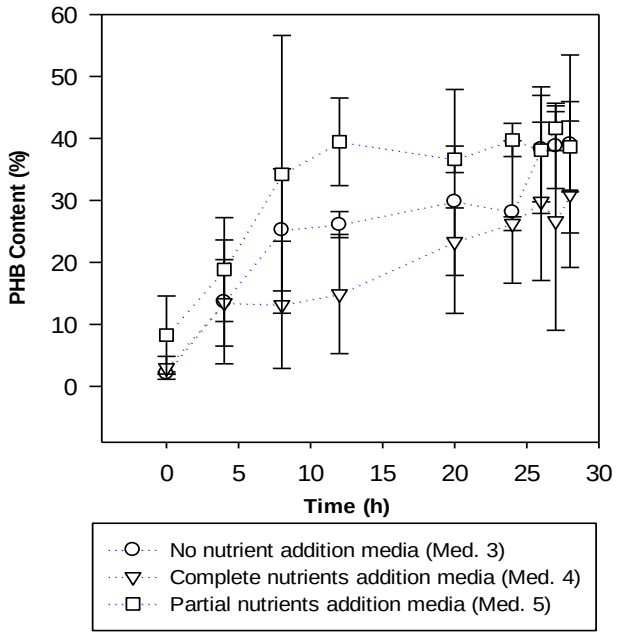


Figure 3.15 PHB Content obtained from two-stage culture using sugarbeet juice with different nutrient addition strategies by *A. latus* (ATCC 29714) in 250ml centrifuge bottles, dotted lines only represent the trend.

Table 3.1 Elemental composition analysis of Sugarbeet juice

Elements/ Chemicals	Diluted sugarbeet juice (*Med. 3*) (mg/l)[a]	*Med. 2* (mg/l)	Nutrients added (*Med. 5*) (mg/l)
N	5.50 ±2.52		
$(NH4)_2SO_4$	25.93 ±11.90	2000.00[b]	1974.07[b]
		200.00[c]	174.07[c]
Ca	3.75 ±0.04		
$CaCl_2.2H_2O$	13.68 ±0.13	10.00	
Co	<0.05		
$CoCl_2.6H_2O$	<0.05	0.20	0.20
Cu	<0.05		
$CuSO_4.5H_2O$	<0.05	0.01	0.01
K	161.33 ±1.53		
KH_2PO_4	562.60 ±5.33	1500.00	937.40
Mg	27.03 ±0.95		
$MgSO_4.7H_2O$	277.09 ±9.74	200.00	
Mn	0.31 ±0.01		
$MnCl_2.4H_2O$	1.10 ±0.02	0.03	
Na	51.63 ±1.27		
$Na_2HPO4.12H_2O$	401.84 ±9.89	9000.00	8598.16
Ni	<0.05		
$Ni_2S0_4.7H_2O$	<0.05	0.03	0.03
Zn	0.09 ±0.02		
$ZnSO_4.7H_2O$	0.40 ±0.08	0.10	
Fe	0.83 ±0.27		
$FeCl_2.H_2O$	2.16 ±0.70	60.00	57.84

[a] The sugar concentration of sugarbeet juice was first adjusted to 20 g/l. All the elemental analysis was done in triplicates to determine individual elements.
[b] Nitrogen rich media for first stage
[c] Nitrogen limited media for second stage

Table 3.2 Growth and PHB production of *A. latus* (ATCC 29714) using sugarbeet juice with three nutrient different addition strategies during two-stage batch fermentation with introduction of N limited media at 16 h[a]

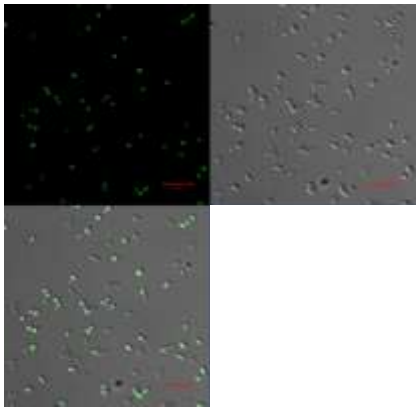
Nutrient addition strategies	Time (h)	Dry cell weight (g/l)	$Y_{p/x}$[b]	PHB productivity (g/l· h)[c]
No nutrient addition	4	3.47 ±0.05	0.14 ±0.10	0.10 ±0.11
(*Med. 3*)	8	2.46 ±0.07	0.25 ±0.10	0.13 ±0.06
	12	4.58 ±0.12	0.26 ±0.02	0.13 ±0.01
	26	6.60 ±0.08	0.38 ±0.09	0.13 ±0.02
	27	8.89 ±0.08	0.39 ±0.07	0.13 ±0.02
	28	9.23 ±0.06	0.39 ±0.14	0.12 ±0.03
Complete nutrient addition	4	3.74 ±1.36	0.13 ±0.07	0.09 ±0.05
(*Med. 4*)	8	4.41 ±0.46	0.13 ±0.10	0.06 ±0.07
	12	5.51 ±0.35	0.15 ±0.10	0.05 ±0.04
	26	7.71 ±0.94	0.30 ±0.13	0.08 ±0.03
	27	8.05 ±1.02	0.27 ±0.18	0.07 ±0.04
	28	7.88 ±0.76	0.31 ±0.12	0.08 ±0.03
Partial nutrient addition	4	6.46 ±0.71	0.19 ±0.08	0.16 ±0.16
(*Med. 5*)	8	7.88 ±2.28	0.34 ±0.22	0.22 ±0.09
	12	8.28 ±1.51	0.39 ±0.07	0.22 ±0.01
	26	9.87 ±0.84	0.38 ±0.10	0.12 ±0.03
	27	8.96 ±1.02	0.42 ±0.04	0.12 ±0.02
	28	10.30 ±1.01	0.39 ±0.07	0.12 ±0.03

[a]The second stage cultures were incubated for 28 h. Each value is an average of triplicates
[b]Gram PHB produced per gram dry cell weight
[c]Gram PHB produced per liter media per hour culture time

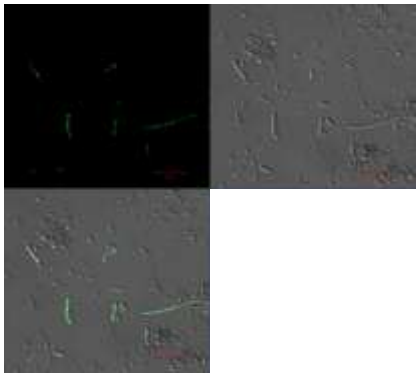
3.3.3 Identification analysis of PHB samples using confocal microscope and Gas Chromatography–Mass Spectrometry (GC-MS)

Inclusion bodies in cells from fermentation broth of synthetic media and sugarbeet juice media with partial nutrient addition was visually examined using confocal microscopy. PHB inclusion bodies can be distinguished in all the cells which appear as green fluorescence (Figure 3.16). The images in this study were similar to the ones taken by Ostle and Holt (1982), for PHB granules in *A. chroococcum* stained with Nile blue A. The size (~2 μm in diameter and 2.5 μm in length) and shape of *A. latus* is consistent with that observed by Palleroni et al. (1978) and Holt et al. (1994). Visual estimation of the percentage of cells with PHB inclusion bodies is approxmately 50% for synthetic media and 40% for sugarbeet juice media.

PHB obtained by both synthetic and sugarbeet juice media with partial nutrient addition was confirmed by GC analysis. In the chromatograms shown in Figure 3.17, three main peaks with similar retention times can be seen. As identified by comparing molecules in the GC database, the first peak represents the solvent used during sample preparation (DCE and propanol). The second peak denotes hydroxybutyric acid propyl ester and the third represents benzoic acid propyl ester. The sequence of components represented by the 3 peaks in the chromatographs is in accordance with that reported by Riis and Mai (1988). Based on the peak area, PHB content in dry cell mass was determined to be 80.15% and 65.60% for synthetic and sugarbeet juice media, respectively.

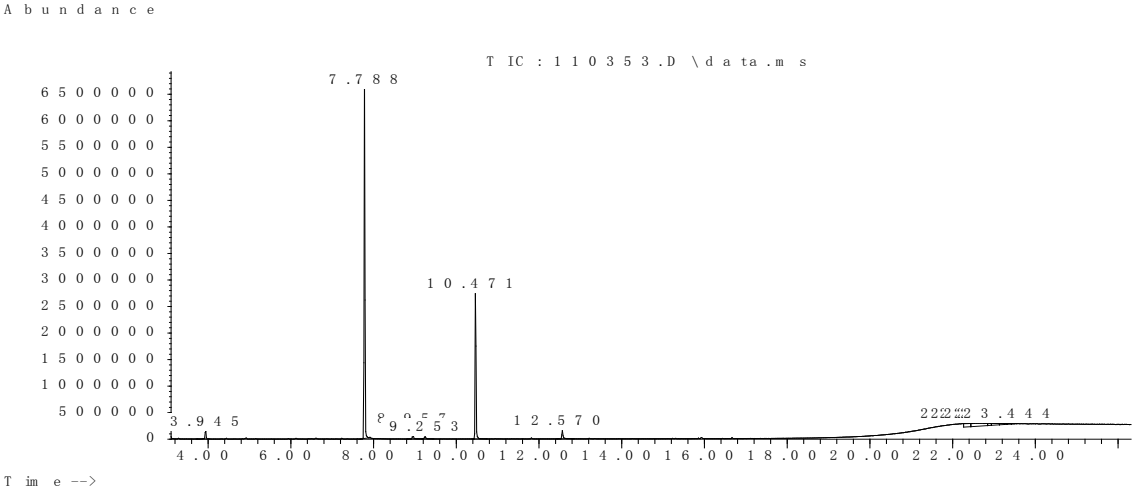


(a)

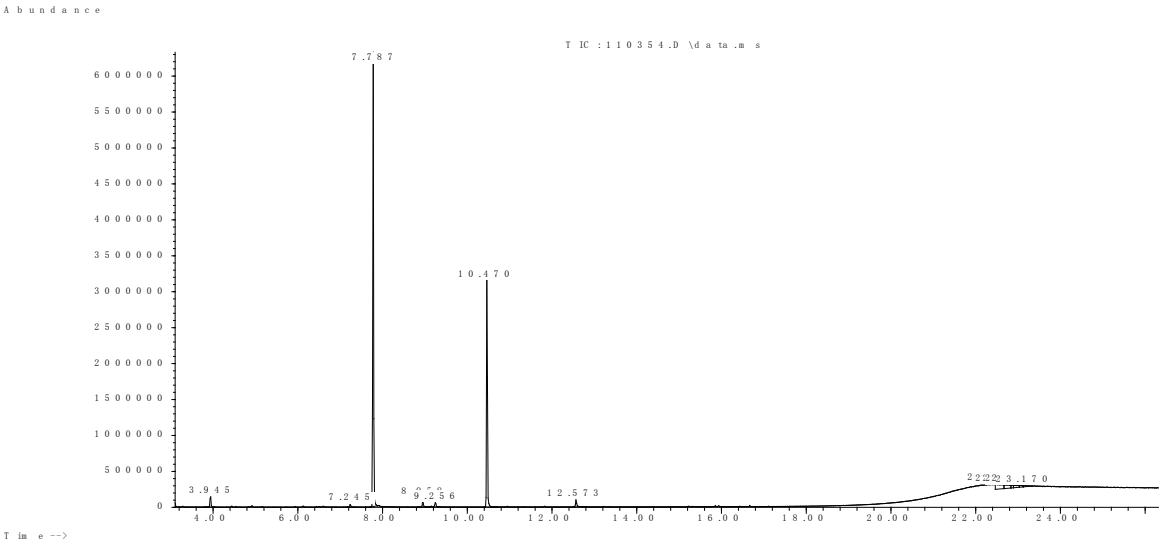


(b)

Figure 3.16 PHB inclusion bodies in *A. latus* ATCC 29714 obtained from (a) synthetic media and (b) sugarbeet juice media, stained with Nile Blue A (top left), digital image correlation (top right) and overlay of both (bottom left) observed with a confocal microscope. Scale bar, 10.0 μm.



(a)

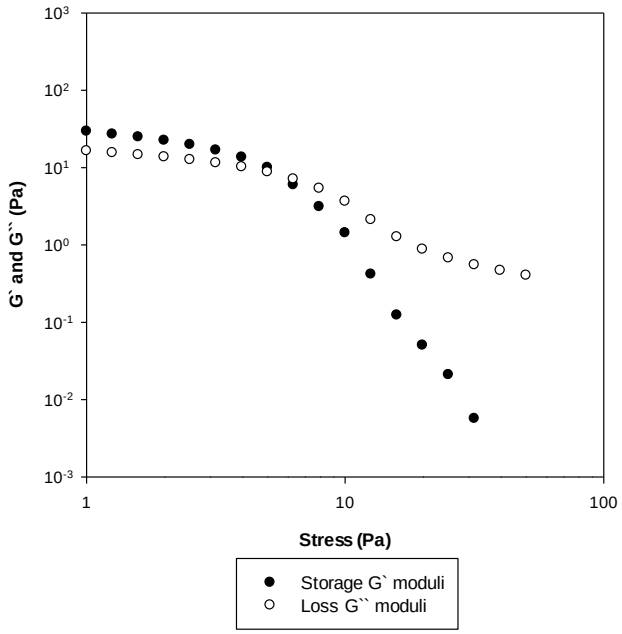


(b)

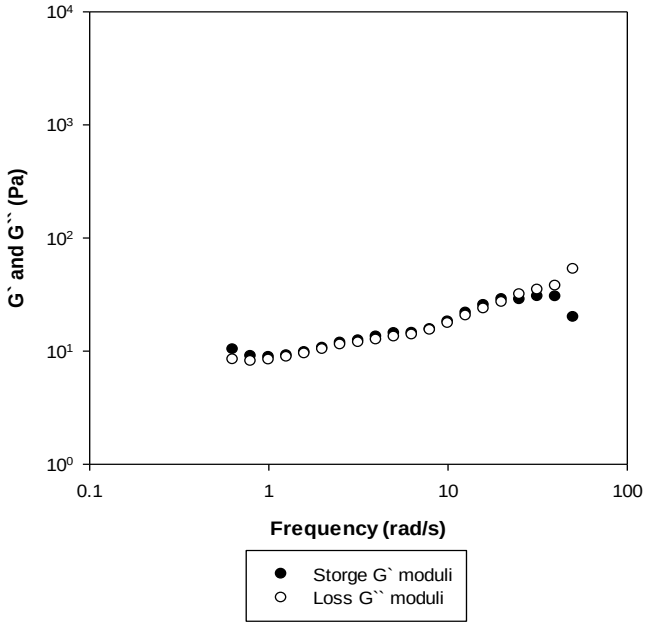
Figure 3.17 Chromatogram of sample from (a) synthetic media and (b) sugarbeet juice with partial nutrient addition. The peak at 3.945 min represents solvents (DCE and propanol), at 7.787 ±0.001 min represents hydroxybutyric acid propyl ester and at 10.470 ±0.001 min represents benzoic acid propyl ester.

3.3.4 Rheological analysis of PHB samples using AR2000 Advanced Rheometer

Maximum stress, for PHB samples generated from fermentation of sugarbeet juice with partial nutrient addition, in the linear visco-elastic region was examined by plotting , storage moduli (G`) and lost moduli (G``) versus shear stress (log-log scale), as shown in Figure 3.18a. It was observed that the linear visco-elastic region proximally ended at 3 Pa and was therefore selected as shear stress for the subsequent frequency sweep testing. During the frequency sweep test, G` and G`` were plotted relative to frequency (log-log scale), as described in Figure 3.18b, and the linear visco-elastic region extended up to approximately 5 rad/s.



(a)



(b)

Figure 3.18 Rheology analysis of PHB samples from fermentation of sugarbeet juice with partial nutrient addition, (a) Storage G` and loss G`` moduli measured at different stresses (Pa) at 180 ℃; (b) Storage G` and loss G`` moduli measured during frequency sweep analysis at 180 ℃

3.3.5 Thermal analysis using Differential Scanning Calorimeter (DSC) and Thermogravimetric Analysis (TG)

PHB samples from *A. latus* cells obtained from fermentation of sugarbeet juice with partial nutrient addition were analyzed for thermal properties. Based on results of differential scanning colorimetery, the peak representing melting point of PHB in the first scan appeared at 165.15 ℃ and the corresponding crystallization temperature was 66.28 ℃. The enthalpy of fusion was 61.161 J/g and crystallinity during melting in the

first cycle was calculated as 41.89%. In the second scan, the melting point peak appeared at 151.46 ℃ with the crystallization peak at 45.42 ℃. The corresponding enthalpy of fusion was 62.962 J/g and crystallinity during melting was calculated as 43.12%.

According to thermogravimeteric analysis (Figure 3.19), the temperature range for rapid thermal degradation of PHB was from 255.14 to 283.69 ℃ with the degradation peaking at 273.86 ℃ (the only large inverted peak), the total weight loss within this temperature range was 95.39%.

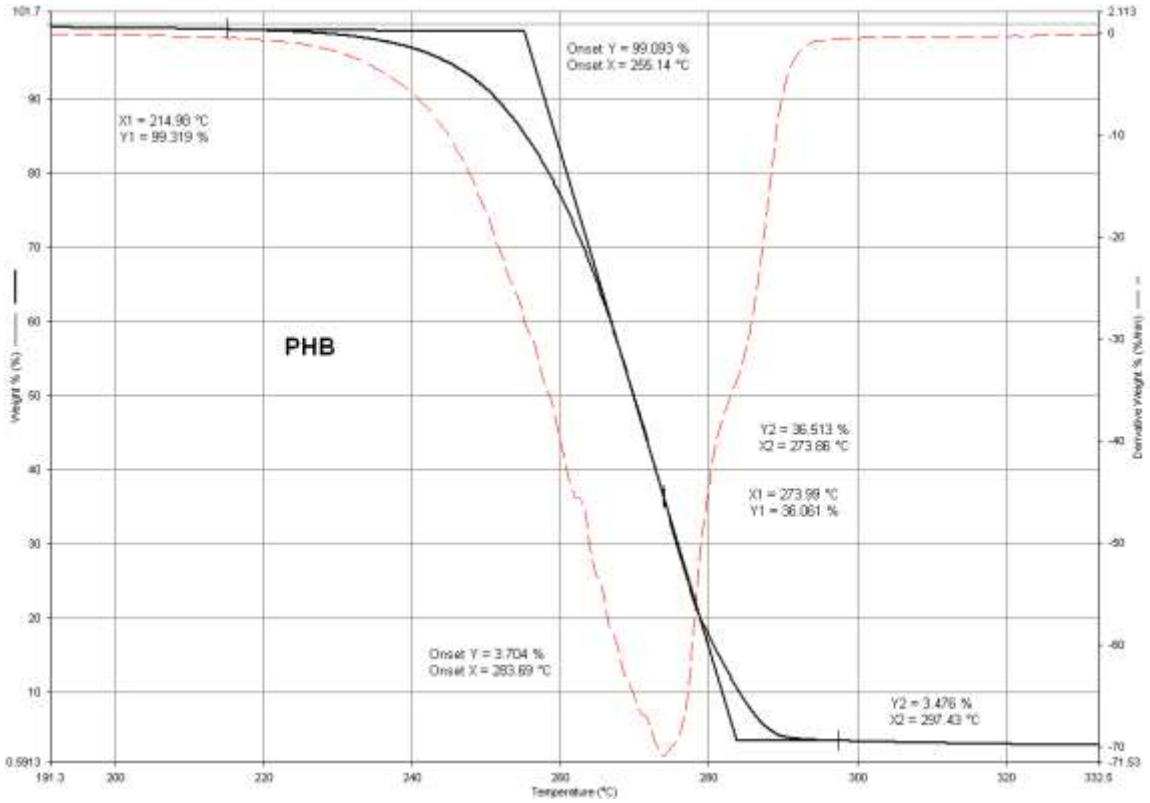


Figure 3.19 Thermogravimetric analysis of PHB samples obtained from the fermentation using sugarbeet juice with partial nutrient addition

3.4 Discussion

3.4.1 Elemental analysis of sugarbeet juice

Elemental analysis of sugarbeet juice indicated that in its natural form it is a highly complex mixture of nutrients suitable for PHB fermentation. The investigation on exploring the inhibition/promotion effect and further optimal nutrient addition strategies was thus essential to establish the foundation for the future application and development of sugarbeet juice in PHB production.

3.4.2 Effect of different nutrient supplementation strategies on PHB production from sugarbeet juice

Based on statistical analysis (ANOVA GLM analysis with $\alpha = 0.05$), DCW data obtained from the end of first stage (16 h) for each nutrient addition strategy (no addition, complete addition and partial addition) showed no significant difference ($P > 0.05$) between no addition and complete addition fermentation. Partial nutrient addition resulted in significantly higher ($P \leq 0.05$) DCW. When growth of *A. latus* within 24 h was compared among the three nutrient addition strategies, although DCW from fermentation of media with complete nutrient addition was low in the initial stage, all three media types showed no significant ($P > 0.05$) difference in DCW. Also they all entered stationary growth after 16 h.

According to statistical analysis using mixed analysis (Type 3 Tests of Fixed Effects, $\alpha = 0.05$), when data for all the parameters (DCW, PHB concentration, PHB content, $Y_{p/x}$, PHB productivity) and sampling time points (4, 8, 12, 20, 24, 26, 27, 28 h

after changing the media) for second stage was included, different nutrient addition strategies had a significant effect ($P \leq 0.05$) on PHB production, as shown in Table 3.

Table 3.3 Mixed analysis (Type 3 Tests of Fixed Effects) for each parameter in two-stage culture using sugarbeet juice including all the sampling time points (Time) and time points for media changing (Treatment)

Dependent Variable	Effect	Num DF	Den DF	F Value	Pr > F
PHB concentration	Treatment	2	28.8	31.65	<.0001
	Time	6	14.1	56.01	<.0001
	Treatment*Time	12	15.7	3.30	0.0147
Dry cell weight	Treatment	2	42	37.18	<.0001
	Time	6	42	29.93	<.0001
	Treatment*Time	12	42	1.55	0.1432
PHB content	Treatment	2	30.3	7.76	0.0019
	Time	6	14	26.43	<.0001
	Treatment*Time	12	15.6	0.86	0.5955
PHB yield based on dry cell weight	Treatment	2	30	7.63	0.0021
	Time	6	14	26.72	<.0001
	Treatment*Time	12	15.6	0.90	0.5625
PHB productivity	Treatment	2	26.2	9.11	0.0010
	Time	6	33.4	5.95	0.0003
	Treatment*Time	12	29.9	0.95	0.5138

Mixed analysis ($\alpha = 0.05$) conducted to see the differences among three nutrient addition strategies showed the same result for DCW and PHB content such that there was no significant difference ($P > 0.05$) between no addition and partial addition samples. However, they were both significantly higher than fermentations with complete addition.

For PHB concentration and productivity, fermentations with all three nutrient addition strategies gave significantly different ($P \leq 0.05$) results. The highest value was obtained from partial nutrient addition followed by no nutrient addition. It is believed that complete nutrient addition may have had an inhibitory effect during fermentation due to excess nutrients.

According to the above analysis, partial nutrient addition gave the overall best performance on PHB production among the three media types. For fermentation with partial nutrient addition, there was no significant difference in the values of various parameters (DCW, PHB concentration, PHB content, $Y_{p/x}$ and PHB productivity) among the samples taken from 26 h, 27 h and 28 h but a significant jump in PHB concentration was observed between 24 h and 26 h. This reiterated the results from a study on PHB production with sucrose based synthetic media as illustrated in Chapter 2. Therefore, the optimized two-stage fermentation strategy for using sugarbeet juice to produce PHB involves using partial nutrient addition media (*Med. 5*) with the second stage ending at 26 h. It is noteworthy that PHB content and productivity were comparable while DCW and PHB concentration obtained with fermentation of sugarbeet juice supplemented partially with nutrients were higher than those from the fermentation using synthetic media under optimized fermentation conditions.

Relative to research conducted using other renewable feedstocks, the results of PHB production obtained from partial nutrient addition were comparable and even enhanced in some cases. For instance, compared to the research by Yezza et al. (2007), which used maple sap as media for PHB production in 100 ml shake flasks over 27 h

incubation, higher PHB concentration (4.01 g/l vs. 3.41 g/l) and PHB yield coefficient based on sugar consumed (0.71 ± 0.28 vs. 0.34) were obtained. Tanamool et al. (2009) researched sweet sorghum juice as media for PHB production in 5 L bioreactor with 2 L working volume over 54 h cultivation and obtained much lower PHB concentration of 0.68 g/l and DCW of 1.73 g/l compared to this study. Another research conducted by Aremu et al. (2010) using cassava starch for PHB production in 4.2 L working volume for 84 h also resulted in much lower PHB concentration (1.25 g/l) and DCW (1.75 g/l). It can be inferred from the results of this study that scaled up fermentations under controlled environments could provide enhanced PHB production from a media source that is sustainable and economically competitive.

3.4.3 Properties of PHB obtained through fermentation of sugarbeet juice

The linear visco-elastic region obtained from rheological analysis of the PHB samples from sugarbeet juice partially supplemented with nutrients was similar to what was obtained by Park et al. (2005). They obtained values of maximum frequency value for linear region up to 10 Pa for a study conducted on pure PHB semi-crystalline powder. Our values were however less than those from similar research conducted by Choi et al. (1995) on PHB samples obtained from ICI (Imperial Chemical Industries Co.) which was synthesized by the continuous fermentation of a glucose-utilizing mutant of *Alcaligenes eutrophus*, which can be used in dynamic rheological test such as temperature sweep. The melting point and crystallinity from two DSC scans were lower than those from Yezza et al. (2007) whose work focused on exploring thermal properties of PHB produced from

maple sap by *A. latus* and El-Hadi et al. (2002) whose work focused on investigating effect of melt processing on crystallization behaviour and rheology of poly (3-hydroxybutyrate) (PHB) produced by the fermentation of molasses by *Alcaligens euthrophus*, which were 177.0 ℃ and 175.0 ℃, respectively. The degradation temperature (273.86 ℃) of PHB obtained from TG analysis was close to that (292 ℃) obtained by El-Hadi et al. (2002)

Though sugarbeets are a key feedstock for sugar production in United States, which accounted for 55% of all sugar being produced domestically (USDA 2009), this research highlights its potential for conversion to PHB. Results of this study can subsequently be applied to explore the conversion of waste from sugar industry (like sugarbeet molasses and pulps) for PHB as well as copolymer (such as PHBV and P(3HB-4HB)) production.

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Chapter 4

Conclusion and Future Scope

During this study involving three different microbial strains that produce PHB, one strain was selected for PHB production using synthetic media with two-stage batch culture and fed-batch culture. Three time points for the induction of PHB production in cells through introduction of nitrogen limitation, either by changing to a nitrogen limited media or by feeding nitrogen limited media at regular intervals, were tested. The results were used to develop a fermentation process for utilization of sugarbeet juice with and without supplemental nutrients in the natural media. The conclusions obtained from this research are listed as follows:

(1) Based on the conditions investigated during sugar extraction from sugarbeets (Sample preparation method (fresh ground and oven dried); Extraction time (30 min, 60 min, 90 min and 120 min); Solid loading (1:10, 1:15 and 1:20 dry sugarbeet : water (w/w)), the best combination of conditions involved using fresh ground sugarbeet in a 60 min extraction at a solid loading of 1:15. This resulted in a juice with a sugar content of 0.78 ± 0.02 g sucrose/g dry sugarbeet.

(2) Among the three PHB strains investigated (*R. eutropha* ATCC 17699, *A. latus* ATCC 29712 and *A. latus* ATCC 29714), *A. latus* ATCC 29714 resulted in the best performance for producing PHB using sucrose, with a maximum specific growth rate of 0.38 ± 0.01 h^{-1} and doubling time of 1.80 ± 0.05 h. Therefore, it was used for subsequent study in this research.

(3) From the study on determining the optimal time point (14, 16, or 18 h) for

induction of PHB production by introducing nitrogen limitation during two-stage batch culture, a 16 h first stage followed by a second stage cultivation up to 26 h was found to be the best combination with significantly high ($P \leq 0.05$) PHB concentration of 3.60 ± 0.06 g/l and PHB content of 48.42 ± 7.06. In a study conducted in fed-batch mode with initiation of nitrogen limitation by beginning feeding of nitrogen limited media at 14, 16, 18 h, beginning feeding at 16 h and ending fermentation at 27 h after the first feed gave optimal results with a significantly high ($P \leq 0.05$) PHB concentration of 3.55 ± 0.54 g/l and DCW of 9.23 ± 0.25 g/l. A comparison between two-stage and fed-bach indicated that the two-stage batch cultivation process was most suited for using synthetic media to produce PHB.

(4) Results of conclusion (3) were directly applied in the research on using sugarbeet juice for PHB production. Three different nutrient addition strategies including sugar content adjusted media, no nutrient addition, complete nutrients addition and partial nutrients addition, were tested. It was observed that partial nutrient addition strategy was optimal resulting in PHB yield (based on biomass) of 0.39 ± 0.07, PHB concentration of 4.01 ± 0.95 g/L and PHB content of 38.66 ± 7.28%.

As discussed previously, the main challenge of industrialized PHB production focuses on the expense of raw materials which amounts to around 40% of the total cost (Choi et al., 1999). Therefore, looking for cheaper feedstocks should be the first research direction towards the commercialized of PHB production using biomass. Although sugarbeet juice used in this research is a starting point, more feasible and economic

feedstocks including industrial or agricultural wastes, which mainly contain simple sugars, starches or other organic chemicals need to be explored. Molasses and pulps from sugar industry can be the optimal vectors for further research since not only do they have a broad range of providers and lower price, but they also contains around 50% and 10% sucrose in them, respectively (OECD, 2002).

Another issue relates to the drawbacks of PHB like its brittleness and thermal instability which greatly narrow its application. Therefore, the next step towards enhancing the applicability of biodegradable polymers is to explore the production of co-polymers, which have improved mechanical and thermal properties. For example, the incorporation of 3HV monomers to make PHBHV lowers the melting point and crystallinity of PHB, thus deceasing stiffness and increasing toughness. This makes it easier to process and acquire more favorable properties for commercial application (Poirier et al., 1995).

Another possible direction of research is the production of PHB through trans-genic plants, which are capable of producing larger amounts of PHB within the same time. The production of PHB in plants could allow synthesis of up to million ton compared to thousand ton scale which fermentation production can provide (Poirier et al., 1995). However, key barriers to be overcome for plant-based PHB production include low PHB content, more complex product recovery and downstream processing due to interference by other plant components, as well as severely stunted plant growth by the PHB produced (Nawrath et al., 1994).

In conclusion, whatever the subsequent research area, one important thing to be considered is to build a comprehensive economic analysis model for the production of PHB from renewable biomass sources. A balance between operating cost, product yield and quality is imminent to make this conversion more economically and functionally feasible.

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APPENDICES

Appendix I: The study on optimal conditions for Sugar extraction from sugarbeet

Sugarbeets harvested in September, 2010 at Plymouth, North Carolina were used for this study.

Preparation methods

For the sample preparation using oven dry sugarbeet method, first washed and cleaned sugarbeets were cut in equal halves. One half was sliced thinly and kept for drying in aluminum trays in a convection oven (Fisher Scientific) at 60℃ for 25hrs until constant weight was reached. The dry slices were weighed and ground to a coarse powder through a 2mm sieve with a Wiley Mill. The dried powder was stored in zip-locked bags at room temperature until use within 1 month.

For the sample preparation using fresh ground sugarbeet method, the other half of the sugarbeet used to prepare a dry sample was cut into medium sized pieces and place them in chopper and blended to get a coarse paste/slurry. One gram of wet slurry was placed in a convection oven at 60℃ for 25 hours for moisture content analysis.

For extraction, 2g dry sugarbeet powder or wet slurry containing 2g dry equivalent sugarbeet was placed in a 125ml flask and DI water at 60℃ was added to the flask. The volume of water needed was calculated on the basis of %moisture content (wet slurry) and %desired solid loading of 1:10, 1:15 and 1:20 (sugarbeet: water w:w). The set up was placed in a shaking water-bath at 60℃/150rpm for up to 2 hours. At 30 min intervals a well mixed 0.5ml sample was taken in a micro-centrifuge tube and centrifuged at

10000rpm for 5 min. The supernatant was analyzed for sugar concentration (in Brix) using refractometer.

Parameters and Calculation

(1) The moisture content was calculated as the difference in weight before and after drying

$$MC = \frac{W_{fs} - W_{ds}}{W_{fs}} \times 100\%$$

Where

W_{fs} = Weight of fresh slices/ Sample (g)

W_{ds} = Weight of dry slices/ Sample (g)

MC = Moisture Content (%)

(2) The weight of fresh sample needed for extraction was calculated as:

$$W_f = \frac{W_d}{MC}$$

Where

W_d = Weight of dry sugarbeet needed (g)

W_f = Weight of fresh sample needed (g)

MC = Moisture Content (%)

(3) Water needed for extraction of wet samples at the desired solid loading was calculated as:

$$W_W = W_d \times SL - W_f \times (1 - MC)$$

Where

W_d = Weight of dry sugarbeet needed (g) = 2g

W_f = Weight of fresh sample needed (g)

MC = Moisture Content (%)

W_w = Weight of water needed (g)

SL = Solid Loading (water : solid w/w)

(4) °Brix was converted to grams of sugar, using the following equation (Bruce, 1995) :

$$\mathrm{SC_g} = SC \times SG \times 10$$

*The specific gravity under different Degree Brix can be found in Sucrose Conversion Table, USDA, 1981.

Where

SC = Sugar content (in °Brix)

SC_g = Sugar content (g/L)

SG = Specific gravity

10 is conversion coefficient

(5) Sugar content g/L was converted to g/g dry sugarbeets and g/g wet sugarbeets:

$$SC_{\frac{g}{g}(dry\ sugar\ beet)} = SC_g \times \frac{V_w}{W_d}$$

$$SC_{\frac{g}{g}(fresh\ sugar\ beet)} = SC_g \times \frac{V_w}{W_f}$$

Where

SC_g = Sugar Content (g/L)

$SC_{\frac{g}{g}(dry\ sugar\ beet)}$ = Sugar Content (g/g dry sugarbeet)

$SC_{\frac{g}{g}(fresh\ sugar\ beet)}$ = Sugar Content (g/g fresh sugarbeet)

V_w = Volume of water needed (L) = W_w

W_f = Weight of fresh samples needed (g)

Statistical analysis and identification of conditions for optimal sugar extraction

Process conditions for optimal extraction of sugars were identified through statistical analysis using general linear model in SAS® (version 9.1.3). Sample preparation method (oven dry vs. wet), solid loading (1:10, 1:15, 1:20) and extraction time (30-120min) were the independent variables impacting sugar content (g/g dry sugarbeet) of extract which was set as the dependent variable. All sugar extraction studies will be done in triplicate.

Results and Discussions

Table 1 Moisture Analysis for fresh ground sugar-beet

Sample No.	Time of oven dry (h)	WT of moisture (g)	Moisture Content (%)	Moisture Content (%)
Sample 1	25	1.64	81.47	
Sample 2	25	1.28	80.98	81.28±0.27
Sample 3	25	0.68	81.41	

Table 2 Sugar content (g sugar/g dry sugarbeet) for sugarbeet using two different methods for sample preparation (fresh ground vs. oven-dried)

Extraction method	Solid loading	Extraction time(min)	Sugar content(g/g dry sugarbeet)
D	1:10	30	0.71
D	1:15	30	0.75
D	1:20	30	0.63
D	1:10	60	0.72
D	1:15	60	0.76
D	1:20	60	0.69
D	1:10	90	0.72
D	1:15	90	0.75
D	1:20	90	0.68
D	1:10	120	0.73
D	1:15	120	0.75
D	1:20	120	0.67
W	1:10	30	0.72
W	1:15	30	0.77
W	1:20	30	0.71

W	1:10	60	0.73
W	1:15	60	0.78
W	1:20	60	0.74
W	1:10	90	0.73
W	1:15	90	0.77
W	1:20	90	0.73
W	1:10	120	0.73
W	1:15	120	0.76
W	1:20	120	0.74

Based on statistical analysis using ANOVA GLM procedures, the sugarbeet juice extraction samples obtained from fresh ground sugarbeet slush after 1 h extraction with solid loading 1:15 (sugarbeet: water, w: w) resulted in significantly highest ($P \leq 0.05$) sugar content (g sugar/g dry sugarbeet) of 0.78 compared to other combinations of sample preparation methods, solid loadings and extraction times.

Appendix II: The results of Growth kinetic study

Table 1a. Growth kinetic study for *A. latus* ATCC 29712 cultured in sucrose based media

Sample ID	Time (h)	$C_{sucrose}$ (g/l)	OD[a]	DCW[b] (g/l)	μ^{c} (h^{-1})	μ_m^{d} (h^{-1})	T_d^{e} (h)	$Y_{d/s}{}^{f}$	r^{g}	$r_m{}^{h}$
1	2	21.12	0.02	0.57	N/A			N/A	N/A	
2	4	23.57	0.03	0.70	0.10			-0.05	-1.94	
3	6	23.30	0.05	1.65	0.43			3.48	0.12	
4	8	22.49	0.12	3.79	0.42			2.64	0.16	
5	10	22.11	0.28	7.74	0.36			10.39	0.03	
6	12	21.25	0.64	16.36	0.37	0.34	2.05	9.98	0.04	0.05
7	14	20.19	1.03	24.52	0.20			7.70	0.03	
8	16	19.36	1.39	30.81	0.11			7.61	0.02	
9	18	18.72	1.55	34.31	0.05			5.47	0.01	
10	20	17.84	1.72	35.88	0.02			1.78	0.01	
11	22	16.43	1.86	36.53	0.01			0.46	0.02	

Table 1b. Growth kinetic study for *A. latus* ATCC 29714 cultured in sucrose based media

Sample ID	Time (h)	$C_{sucrose}$ (g/l)	OD[a]	DCW (g/l)[b]	μ^{c} (h^{-1})	μ_m^{d} (h^{-1})	T_d^{e} (h)	$Y_{d/s}{}^{f}$	r^{g}	$r_m{}^{h}$
1	2	23.34	0.02	0.56	N/A			N/A	N/A	
2	4	23.84	0.02	0.87	0.22			-0.62	-0.36	
3	6	22.35	0.04	1.60	0.30			0.49	0.62	
4	8	21.76	0.08	3.61	0.41			3.41	0.12	
5	10	21.64	0.18	7.42	0.36			31.75	0.01	
6	12	21.23	0.42	18.20	0.45	0.38	1.81	26.29	0.02	0.03
7	14	20.17	0.90	34.33	0.32			15.26	0.02	
8	16	18.96	1.39	45.74	0.14			9.46	0.02	
9	18	17.67	1.65	49.93	0.04			3.23	0.01	
10	20	16.64	1.82	50.54	0.01			0.59	0.01	
11	22	16.12	1.94	52.03	0.01			2.87	0.01	

[a]Optical density
[b]Dry cell weight
[c]Specific growth rate on DCW
[d]Maximum specific growth rate on DCW
[e]Doubling time
[f] Biomass yield coefficient (g DCW/g sucrose consumed)
[g]Specific sucrose consumption rate on DCW (g sucrose/g DCW*h^{-1})
[h]Maximum specific sucrose consumption rate on DCW (g sucrose/g DCW*h^{-1})

Appendix III: The results of ammonia analysis

Table 1. The ammonia analysis for samples obtained from two-stage culture

Time for inducing nitrogen limitation	Sample Time (h)	N content (mg/l)	Std.
14 h (Two stage)	14	287.67	25.58
	18	<0.1	
	22	<0.1	
	26	<0.1	
	40	<0.1	
	41	<0.1	
	42	<0.1	
16 h (Two stage)	16	356.33	37.29
	20	<0.1	
	24	<0.1	
	28	<0.1	
	42	<0.1	
	43	<0.1	
	44	<0.1	
18 h (Two stage)	18	272.33	18.50
	22	55.13	65.72
	26	11.80	0.72
	30	29.30	30.92
	44	11.27	0.32
	45	21.87	19.08
	46	11.60	0.36
First stage Initial	0	431.00	10.82
Second stage Initial	0	44.97	2.47

Table 2. The ammonia analysis for samples obtained from fed-batch culture

Time for inducing nitrogen limitation	Sample Time (h)	N content (mg/l)	Std.
14 h (fed-batch)	14	361.33	1.15
	18	240.33	5.51
	22	178.33	17.01
	26	135.67	4.16
	40	78.90	12.88
	41	82.47	11.22
	42	86.03	4.72
Initial	0	374.67	11.06
16 h (fed-batch)	16	366.00	9.54
	20	176.00	66.19
	24	161.00	10.39
	28	127.67	21.13
	42	89.47	21.56
	43	105.37	11.66
	44	88.93	8.29
Initial	0	373.00	4.00
18 h (fed-batch)	18	351.33	4.93
	22	240.67	15.95
	26	157.33	17.93
	30	138.00	6.93
	42	86.27	24.98
	43	93.77	39.61
	44	99.07	5.25
Initial	0	391.33	2.08

Appendix IV: SAS Tables and Codes of ANOVA GLM and mixed analysis

Table IVa. SAS codes for sugar extraction study

```
data sugar;
   input extractionmethod solidloading extractiontime sugarconc;
cards;
1      1      30     0.7143
1      2      30     0.7469
1      3      30     0.6327
1      1      60     0.7247
1      2      60     0.7576
1      3      60     0.6873
1      1      90     0.7246
1      2      90     0.7524
1      3      90     0.6804
1      1      120    0.7281
1      2      120    0.7524
1      3      120    0.6737
2      1      30     0.7159
2      2      30     0.7700
2      3      30     0.7096
2      1      60     0.7335
2      2      60     0.7802
2      3      60     0.7370
2      1      90     0.7265
2      2      90     0.7700
```

```
2      3      90     0.7302

2      1      120    0.7300

2      2      120    0.7596

2      3      120    0.7439

;

run;

proc glm;

 class extractionmethod solidloading extractiontime;

 model sugarconc=extractionmethod solidloading extractiontime
solidloading*extractiontime extractionmethod*solidloading
extractionmethod*extractiontime;

 lsmeans extractionmethod solidloading extractiontime
solidloading*extractiontime extractionmethod*solidloading
extractionmethod*extractiontime/pdiff;

 run;
```

Note. In "extractionmethod", "1" and "2" indicate "Dry sugarbeet method" and "Fresh ground sugarbeet method"; In "solid loading", "1", "2" and "3" indicate "1:10 (sugarbeet:water/V:V)", "1:15" and "1:20"; In "extractiontime" , "30", "60", "90", "120" indicate total extraction time(minutes). In "sugarconc", it is the sugar content of sugarbeet juice(g/g dry sugarbeet).

Table IVb. SAS codes for two-stage culture on selection of best time point for inducing nitrogen limitation

```
data a14;
retain Treatment "14Hour";
do rep=1 to 3;
  input Time      Drycellweight     PHBconc     PHByield    PHBcontent
      Y_p_x PHBproductivity;
  output;
  end;
  datalines;
0      .      0.13   .      .      .      .
0      .      0.11   .      .      .      .
0      .      0.12   .      .      .      .
4      2.32   0.34   -0.19         14.78         0.15   0.06
4      1.92   0.31   0.04   16.32         0.16   0.05
4      3.33   0.48   0.05   14.55         0.15   0.09
8      3.03   0.77   0.09   25.33         0.25   0.08
8      1.62   0.65   0.08   40.00         0.40   0.07
8      2.73   0.60   0.07   21.85         0.22   0.06
12     3.23   1.21   0.16   37.50         0.38   0.09
12     3.33   1.01   0.15   30.30         0.30   0.07
12     4.14   1.41   0.20   34.15         0.34   0.11
26     5.76   2.73   0.29   47.37         0.47   0.10
26     4.95   2.12   0.25   42.86         0.43   0.08
26     6.77   2.53   0.28   37.31         0.37   0.09
27     5.25   2.22   0.18   42.31         0.42   0.08
27     4.85   2.12   0.28   43.75         0.44   0.07
27     4.65   2.12   0.23   45.65         0.46   0.07
28     6.36   2.93   0.67   46.03         0.46   0.10
28     5.56   3.13   0.60   56.36         0.56   0.11
28     7.07   3.03   0.50   42.86         0.43   0.10
;
proc print data=a14;
run;

proc sort data=a14;
  by time;
  run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
proc gplot data=a14;
  plot phbconc*time=1;
  plot2 Drycellweight*time=2;
  run;

  data a16;
retain Treatment "16Hour";
do rep=1 to 3;
  input Time      Drycellweight     PHBconc     PHByield    PHBcontent
      Y_p_x PHBproductivity ;
  output;
```

```
  end;
  datalines;
0     .     0.21  .     .     .     .
0     .     0.25  .     .     .     .
0     .     0.21  .     .     .     .
4     3.03  0.79  0.20  26.00 0.26  0.14
4     2.42  0.73  0.17  30.00 0.30  0.12
4     3.33  0.63  0.27  18.79 0.19  0.10
8     3.84  1.21  0.43  31.58 0.32  0.12
8     3.13  0.91  0.31  29.03 0.29  0.08
8     4.24  0.91  0.48  21.43 0.21  0.08
12    3.64  1.72  0.52  47.22 0.47  0.12
12    5.35  2.32  0.72  43.40 0.43  0.17
12    4.44  1.41  0.40  31.82 0.32  0.10
26    6.87  3.64  0.59  52.94 0.53  0.13
26    8.28  3.33  0.57  40.24 0.40  0.12
26    6.97  2.53  0.56  36.23 0.36  0.09
27    7.07  3.03  0.56  42.86 0.43  0.10
27    7.27  2.93  0.53  40.28 0.40  0.10
27    6.57  3.13  0.57  47.69 0.48  0.11
28    8.99  3.23  0.70  35.96 0.36  0.11
28    7.58  3.33  0.58  44.00 0.44  0.11
28    7.07  4.24  0.86  60.00 0.60  0.14
;

proc sort data=a16;
  by time;
  run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
proc gplot data=a16;
  plot phbconc*time=1;
  plot2 Drycellweight*time=2;
  run;

  data a18;
retain Treatment "18Hour";
do rep=1 to 3;
  input   Time    Drycellweight     PHBconc     PHByield    PHBcontent
      Y_p_x PHBproductivity;
  output;
  end;
  datalines;
0     .     0.27  .     .     .     .
0     .     0.19  .     .     .     .
0     .     0.16  .     .     .     .
4     3.23  0.67  0.15  20.63       0.21  0.11
4     4.14  0.59  0.11  14.15       0.14  0.09
4     2.93  0.49  0.06  16.90       0.17  0.07
8     3.64  1.01  0.11  27.78       0.28  0.10
8     3.94  1.11  0.13  28.21       0.28  0.11
8     4.34  0.94  0.11  21.63       0.22  0.09
12    5.15  0.91  0.13  17.65       0.18  0.06
```

```
12     4.34   2.02   0.26   46.51          0.47   0.15
12     4.75   1.31   0.13   27.66          0.28   0.09
26     6.97   2.32   0.48   33.33          0.33   0.08
26     6.87   1.82   0.23   26.47          0.26   0.06
26     6.26   1.92   0.18   30.65          0.31   0.07
27     7.47   2.83   0.28   37.84          0.38   0.10
27     7.17   1.72   0.22   23.94          0.24   0.06
27     5.96   3.03   0.29   50.85          0.51   0.10
28     7.17   3.03   0.29   42.25          0.42   0.10
28     6.87   2.93   0.27   42.65          0.43   0.10
28     7.78   2.63   0.25   33.77          0.34   0.09
;

proc sort data=a18;
  by time;
  run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
proc gplot data=a18;
  plot PHBconc*time=1;
  plot2 Drycellweight*time=2;
  run;

data all ;
  set a14 a16 a18;
  run;


proc sort data=all;
  by time;
  run;

   ods listing close;
   ods pdf style=journal file="results_oct292010.pdf";
  ods graphics off;
title "data";
proc print data=all;
run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
symbol3 i=rl v=square c=black;
proc gplot data=all;
  plot PHBconc*time=treatment;
  run;
proc gplot data=all;
  plot  Drycellweight*time=treatment;
  run;

  title "Mixed analysis PHB";
  proc mixed data=all;
   class  treatment  time;
   model PHBconc= treatment time treatment*time/outp=outmx residual;
```

```
   repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff;* adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;

  ***;
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
**;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  ;
  run;
```

```
data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
****;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

  title "1. Drycellweight";
  proc mixed data=all;
   class  treatment  time;
   model Drycellweight= treatment time treatment*time/outp=outmx2
residual;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time) diff;*
adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHyield";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx2;
  plot StudentResid*pred=treatment;
  run;
```

```
  ***;
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
***;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  t;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
***;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

*****;
*****;
```

```
*PHByield   ;


  title "Mixed analysis PHByield";
  proc mixed data=all;
   class  treatment  time;
   model PHByield= treatment time treatment*time/outp=outmx residual;
   *repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHyield";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;

  ***;
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
**;
```

```
proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
***;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

*****;
*PHBcontent ;


  title "Mixed analysis PHBcontent";
  proc mixed data=all;
   class  treatment  time;
   model PHBcontent= treatment time treatment*time/outp=outmx residual;
   *repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHcontent";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
```

```
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;

  ***;
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
**;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"   ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
****;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
```

```
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

  *****;
*Y_p_x      ;


  title "Mixed analysis Y_P_X";
  proc mixed data=all;
   class  treatment  time;
   model Y_p_x= treatment time treatment*time/outp=outmx residual;
  * repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS Y_p_x";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;
  ***;

title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
```

```
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
***;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
***;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"    ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"   and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
****;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);
  *****;
*************************************************************************;
*PHBproductivity;

  title "Mixed analysis PHBproductivity";
  proc mixed data=all;
   class  treatment  time;
   model PHBproductivity= treatment time treatment*time/outp=outmx
residual;
 *  repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff;* adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
```

```
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHproductivity";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;

title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
*****;


title2 "Pairwise Mean differences";
proc print data=diffds;
run;
**;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
```

```
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
***;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;
data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

  *****;
  title "1.MANOVA  Drycellweight PHBconc PHByield PHBcontent
PHBproductivity=";
  title "ANOVA";
  proc glm data=all;
   class  treatment  time;
   model Drycellweight PHBconc PHByield PHBcontent PHBproductivity=
treatment time treatment*time;
   manova h=treatment time treatment*time/printe;
   lsmeans treatment time/cl pdiff;* adjust=tukey;
   lsmeans treatment*time/cl   pdiff  slice=(treatment time);***diff
adjust=tukey;
   run;
   quit;
  ods graphics off;
  ods pdf close;
  ods listing  ;
 ;
```

Table IVc. SAS codes for fed-batch culture on selection of best time point for beginning feeding

```
data a14;
retain Treatment "14Hour";
do rep=1 to 3;
  input Time      Drycellweight     PHBconc           PHBcontent  Y_p_x
      PHBproductivity;
  output;
  end;
  datalines;
0.00  2.42  0.20  8.33  0.08  0.00
0.00  2.12  0.10  4.76  0.05  0.00
0.00  2.22  0.20  9.09  0.09  0.00
4.00  2.98  0.32  10.81       0.11  0.04
4.00  2.34  0.16  6.90  0.07  0.00
4.00  3.15  0.32  10.26       0.10  0.04
8.00  2.89  0.34  11.63       0.12  0.02
8.00  2.55  0.54  21.05       0.21  0.05
8.00  2.68  0.47  17.50       0.18  0.04
12.00       2.93  0.34  11.76       0.12  0.01
12.00       2.76  0.29  10.42       0.10  0.01
12.00       2.47  0.63  25.58       0.26  0.04
26.00       5.69  1.78  31.31       0.31  0.06
26.00       5.34  1.90  35.48       0.35  0.07
26.00       5.69  1.78  31.31       0.31  0.06
27.00       5.86  1.67  28.43       0.28  0.06
27.00       6.78  1.72  25.42       0.25  0.06
27.00       5.69  1.49  26.26       0.26  0.05
28.00       6.78  2.30  33.90       0.34  0.08
28.00       8.91  2.18  24.52       0.25  0.07
28.00       5.92  2.18  36.89       0.37  0.07
;
proc print data=a14;
run;

proc sort data=a14;
  by time;
  run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
proc gplot data=a14;
  plot phbconc*time=1;
  plot2 Drycellweight*time=2;
  run;

  data a16;
retain Treatment "16Hour";
do rep=1 to 3;
  input Time      Drycellweight     PHBconc     PHBcontent  Y_p_x
      PHBproductivity ;
  output;
```

```
  end;
  datalines;
0.00  5.35  0.10  1.89  0.02  0.00
0.00  6.26  0.10  1.61  0.02  0.00
0.00  4.44  0.10  2.27  0.02  0.00
4.00  2.93  0.16  5.56  0.06  0.02
4.00  3.98  0.16  4.08  0.04  0.02
4.00  4.07  0.08  2.00  0.02  0.00
8.00  3.72  1.49  40.00 0.40  0.17
8.00  4.19  1.22  29.03 0.29  0.14
8.00  3.99  0.68  16.95 0.17  0.07
12.00       4.22  2.02  47.95 0.48  0.16
12.00       3.58  2.31  64.52 0.65  0.18
12.00       4.74  1.10  23.17 0.23  0.08
26.00       8.32  3.18  38.19 0.38  0.12
26.00       8.79  3.47  39.47 0.39  0.13
26.00       9.02  2.95  32.69 0.33  0.11
27.00       9.37  3.35  35.79 0.36  0.12
27.00       7.99  3.58  44.86 0.45  0.13
27.00       10.17 3.93  38.64 0.39  0.14
28.00       8.91  2.95  33.09 0.33  0.10
28.00       7.99  3.70  46.31 0.46  0.13
28.00       10.80 3.99  36.91 0.37  0.14
;

proc sort data=a16;
  by time;
  run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
proc gplot data=a16;
  plot phbconc*time=1;
  plot2 Drycellweight*time=2;
  run;

  data a18;
retain Treatment "18Hour";
do rep=1 to 3;
  input   Time    Drycellweight     PHBconc     PHBcontent  Y_p_x
      PHBproductivity;
  output;
  end;
  datalines;
0.00  3.13  0.20  6.45  0.06  0.00
0.00  3.33  0.30  9.09  0.09  0.00
0.00  2.93  0.30  10.34 0.10  0.00
4.00  3.47  0.48  13.95 0.14  0.05
4.00  3.15  0.40  12.82 0.13  0.03
4.00  3.95  0.32  8.16  0.08  0.01
8.00  3.36  0.74  22.00 0.22  0.06
8.00  3.15  0.74  23.40 0.23  0.06
8.00  3.62  0.81  22.22 0.22  0.07
12.00       3.33  0.75  22.41 0.22  0.04
```

```
12.00        2.82   0.57   20.41 0.20   0.03
12.00        2.70   0.86   31.91 0.32   0.05
26.00        6.72   2.13   31.62 0.32   0.07
26.00        6.95   2.18   31.40 0.31   0.07
26.00        5.69   2.13   37.37 0.37   0.07
27.00        5.69   2.36   41.41 0.41   0.08
27.00        8.74   1.90   21.71 0.22   0.06
27.00        7.18   2.13   29.60 0.30   0.07
28.00        8.10   2.24   27.66 0.28   0.07
28.00        8.33   2.64   31.72 0.32   0.08
28.00        7.13   2.41   33.87 0.34   0.08
;

proc sort data=a18;
  by time;
  run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
proc gplot data=a18;
  plot PHBconc*time=1;
  plot2 Drycellweight*time=2;
  run;

data all ;
  set a14 a16 a18;
  run;


proc sort data=all;
  by time;
  run;

   ods listing close;
   ods pdf style=journal file="results_APR182011.pdf";
  ods graphics on;
title "data";
proc print data=all;
run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
symbol3 i=rl v=square c=black;
proc gplot data=all;
  plot PHBconc*time=treatment;
  run;
proc gplot data=all;
  plot  Drycellweight*time=treatment;
  run;

  title "1. Mixed analysis PHB concentration";
  proc mixed data=all;
   class  treatment  time;
   model PHBconc= treatment time treatment*time/outp=outmx residual;
```

```
   repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff;* adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;

  ***;
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
**;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  ;
  run;
```

```
data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
****;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

  title "2. Drycellweight";
  proc mixed data=all;
   class  treatment  time;
   model Drycellweight= treatment time treatment*time/outp=outmx2
residual;
      repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time) diff;*
adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHyield";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx2;
  plot StudentResid*pred=treatment;
```

```
  run;

  ***;
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
***;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  t;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
***;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

*****;
```

```
*****;
*PHByield   ;

  /*
  title "Mixed analysis PHByield";
  proc mixed data=all;
   class  treatment  time;
   model PHByield= treatment time treatment*time/outp=outmx residual;
   *repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHyield";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;

  ***;
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
**;
```

```
proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
***;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);
*/
*****;
*PHBcontent ;


  title "3 Mixed analysis PHBcontent";
  proc mixed data=all;
   class  treatment  time;
   model PHBcontent= treatment time treatment*time/outp=outmx residual;
    repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHcontent";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
```

```
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;

  ***;
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
**;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"   ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
****;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
```

```
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

  *****;
*Y_p_x      ;


  title "4 Mixed analysis Y_P_X";
  proc mixed data=all;
   class  treatment  time;
   model Y_p_x= treatment time treatment*time/outp=outmx residual;
    repeated  / subject= rep(treatment) group=time;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS Y_p_x";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;
  ***;

title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
```

```
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
***;

title2 "Pairwise Mean differences";
proc print data=diffds;
run;
***;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"    ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"   and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
****;

proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;   where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);
  *****;
*************************************************************************;
*PHBproductivity;

  title "5 Mixed analysis PHBproductivity";
  proc mixed data=all;
   class  treatment  time;
   model PHBproductivity= treatment time treatment*time/outp=outmx
residual;
   * repeated  / subject= rep(treatment);* group=time;
   lsmeans treatment time/cl diff;* adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
```

```
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHproductivity";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  title2 "Residuals";
symbol i=none v=star c=red;
symbol2 i=none v=circle c=blue;
symbol3 i=none v=square c=black;
proc gplot data=outmx;
  plot StudentResid*pred=treatment;
  run;

title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
*****;


title2 "Pairwise Mean differences";
proc print data=diffds;
run;
**;
proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  ;
  run;
data ppp;
  set diffds; where effect = "Treatment*Time"  and _Treatment=treatment;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
```

```
***;
proc sort data=diffds;
  by time;
  run;
title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time" and _TIME=time;
run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);
  *****;
/*
  title "1.MANOVA  Drycellweight PHBconc PHBcontent PHBproductivity=";
  title "ANOVA";
  proc glm data=all;
   class  treatment  time;
   model Drycellweight PHBconc  PHBcontent PHBproductivity= treatment
time treatment*time;
   manova h=treatment time treatment*time/printe;
   lsmeans treatment time/cl pdiff;* adjust=tukey;
   lsmeans treatment*time/cl   pdiff  slice=(treatment time);***diff
adjust=tukey;
   run;
*/
   quit;
  ods graphics off;
  ods pdf close;
  ods listing  ;
 ;
```

Table IVd. SAS codes for two-stage culture using sugarbeet juice on selection of best nutrient addition strategies

```
%include  "C:\Users\carella\Desktop\saspgm\pdmix800.sas";


/***

Time (hr)    Dry cell weight (g/l)    PHB Concentration (g/l)        PHB
content (%)        Yp/x  PHB productivity
***/

data a14;
retain Treatment "NOAddition";
do rep=1 to 3;
  input Time      Drycellweight     PHBconc     PHBcontent  Y_p_x
      PHBproductivity;
  output;
  end;
  datalines;
0.00  4.34  0.10  2.33  0.02  0
0.00  4.65  0.10  2.17  0.02  0
0.00  5.25  0.10  1.92  0.02  0
4.00  4.04  1.01  25.00 0.25  0.23
4.00  3.23  0.20  6.25  0.06  0.03
4.00  3.13  0.30  9.68  0.10  0.05
8.00  4.85  1.62  33.33 0.33  0.19
8.00  3.23  0.91  28.13 0.28  0.10
8.00  5.66  0.81  14.29 0.14  0.09
12.00 7.27  1.92  26.39 0.26  0.15
12.00 5.76  1.62  28.07 0.28  0.13
12.00 6.77  1.62  23.88 0.24  0.13
26.00 9.49  3.33  35.11 0.35  0.12
26.00 9.19  2.93  31.87 0.32  0.11
26.00 7.98  3.84  48.10 0.48  0.14
27.00 8.48  3.64  42.86 0.43  0.13
27.00 9.70  4.14  42.71 0.43  0.15
27.00 9.49  2.93  30.85 0.31  0.10
28.00 7.98  4.44  55.70 0.56  0.16
28.00 10.30 3.13  30.39 0.30  0.11
28.00 9.70  3.03  31.25 0.31  0.10

;
proc print data=a14;
run;

proc sort data=a14;
  by time;
  run;

symbol i=rl v=star c=red;
```

```
symbol2 i=rl v=circle c=blue;
proc gplot data=a14;
  plot phbconc*time=1;
  plot2 Drycellweight*time=2;
  run;

  /***
  Time (hr) Dry cell weight (g/l)   PHB Concentration (g/l)       PHB
content (%)       Yp/x  PHB productivity
  ****/

  data a16;
retain Treatment "CompleteA";
do rep=1 to 3;
  input Time      Drycellweight     PHBconc           PHBcontent  Y_p_x
      PHBproductivity ;
  output;
  end;
  datalines;
0.00  3.94  0.07  1.79  0.02  0
0.00  4.34  0.09  2.09  0.02  0
0.00  3.94  0.20  5.13  0.05  0
4.00  5.25  0.40  7.69  0.08  0.07
4.00  3.33  0.71  21.21 0.21  0.15
4.00  2.63  0.30  11.54 0.12  0.05
8.00  4.44  0.30  6.82  0.07  0.02
8.00  4.85  1.21  25.00 0.25  0.14
8.00  3.94  0.30  7.69  0.08  0.02
12.00 4.95  1.11  22.45 0.22  0.08
12.00 5.56  1.01  18.18 0.18  0.07
12.00 4.95  0.20  4.08  0.04  0.01
26.00 7.98  2.12  26.58 0.27  0.08
26.00 8.48  1.62  19.05 0.19  0.06
26.00 6.67  2.93  43.94 0.44  0.11
27.00 8.18  1.01  12.35 0.12  0.03
27.00 6.97  3.23  46.38 0.46  0.12
27.00 8.99  1.92  21.35 0.21  0.07
28.00 7.17  2.63  36.62 0.37  0.09
28.00 7.78  3.03  38.96 0.39  0.10
28.00 8.69  1.52  17.44 0.17  0.05

;

proc sort data=a16;
  by time;
  run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
proc gplot data=a16;
  plot phbconc*time=1;
  plot2 Drycellweight*time=2;
  run;
/***a18
```

```
  Time (hr) Dry cell weight (g/l)   PHB Concentration (g/l)       PHB
content (%)       Yp/x  PHB productivity
  ***/

  data a18;
retain Treatment "PartialA";
do rep=1 to 3;
  input   Time    Drycellweight     PHBconc     PHBcontent  Y_p_x
      PHBproductivity;
  output;
  end;
  datalines;
0.00  8.28  0.51  6.10  0.06  0
0.00  6.57  1.01  15.38 0.15  0
0.00  8.99  0.30  3.37  0.03  0
4.00  7.27  1.92  26.39 0.26  0.33
4.00  6.16  0.61  9.84  0.10  0.00
4.00  5.96  1.21  20.34 0.20  0.15
8.00  8.99  2.32  25.84 0.26  0.21
8.00  9.39  1.62  17.20 0.17  0.13
8.00  5.25  3.13  59.62 0.60  0.32
12.00 7.68  3.33  43.42 0.43  0.23
12.00 10.00 3.13  31.31 0.31  0.21
12.00 7.17  3.13  43.66 0.44  0.21
26.00 9.60  3.84  40.00 0.40  0.12
26.00 10.81 2.93  27.10 0.27  0.09
26.00 9.19  4.34  47.25 0.47  0.14
27.00 7.78  3.13  40.26 0.40  0.09
27.00 9.49  4.34  45.74 0.46  0.14
27.00 9.60  3.74  38.95 0.39  0.12
28.00 10.30 4.75  46.08 0.46  0.15
28.00 11.31 4.34  38.39 0.38  0.13
28.00 9.29  2.93  31.52 0.32  0.08
;

proc sort data=a18;
  by time;
  run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
proc gplot data=a18;
  plot PHBconc*time=1;
  plot2 Drycellweight*time=2;
  run;

data all ;
  set a14 a16 a18;
  *log10_Drycellweight   = log10(Drycellweight);
  *log10_PHBconc         =    log10(PHBconc );
  *log10_PHBcontent      = log10(PHBcontent);
  *log10_Y_p_x           = log10(Y_p_x);
  *log10_PHBproductivity      =     log10(PHBproductivity);
```

```
  run;

proc sort data=all;
  by time;
  run;

   ods listing close;
   ods pdf style=journal
file="results_ADDITION_OrigScale_july252011.pdf";
  ods graphics on;
title "data";
proc print data=all;
run;

symbol i=rl v=star c=red;
symbol2 i=rl v=circle c=blue;
symbol3 i=rl v=square c=black;
proc gplot data=all;
  plot PHBconc*time=treatment;
  run;
proc gplot data=all;
  plot  Drycellweight*time=treatment;
  run;
proc sort data=all;
  by treatment time rep;
  run;

  title "1. Mixed analysis PHBconc ";
  proc mixed data=all plots(only) = Studentpanel(marginal);
   class  treatment  time rep;
   model PHBconc= treatment time treatment*time/outp=outmx residual
ddfm=kr;
   repeated  / subject= rep(treatment*time)
group=time;*treatment;*;*time;*treatment;*;
   lsmeans treatment time/cl diff;* adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS  PHBconc";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;
*;

  ***;
```

```
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;


  ***;
title2 "Pairwise Mean differences -  Time ";
proc print data=diffds;  where effect = "Time";
run;
data mmm3;
  set lsmnds; where effect = "Time";
  run;

data ppp3;
  set diffds; where effect = "Time";
  run;
*** LSD test***;
%pdmix800(ppp3,mmm3,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;  where effect = "Treatment*Time"  ;
run;
**;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"   ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  ;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
****;

proc sort data=diffds;
  by time;
  run;
*title2 "Pairwise Mean differences";
```

```
*proc print data=diffds; * where effect = "Treatment*Time" and
_TIME=time;
*run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

  title "2. Drycellweight";
  proc mixed data=all plots(only) =studentpanel(marginal);
   class  treatment  time  rep;
   model Drycellweight= treatment time treatment*time/outp=outmx2
residual  ddfm=kr;
   *repeated /subject=rep(treatment*time)
group=treatment;*time;*treatment;*time;*treatment;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time) diff;*
adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS Drycellweight";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;
  ***;

  ***;
title2 "Pairwise Mean differences -  Time ";
proc print data=diffds;  where effect = "Time";
run;
data mmm3;
  set lsmnds; where effect = "Time";
  run;

data ppp3;
  set diffds; where effect = "Time";
  run;
*** LSD test***;
%pdmix800(ppp3,mmm3,alpha=.01,sort=yes);
***;
```

```
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;    where effect = "Treatment*Time"  ;
run;
***;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  ;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
***;

proc sort data=diffds;
  by time;
  run;

*title2 "Pairwise Mean differences";
*proc print data=diffds; * where effect = "Treatment*Time" and
_TIME=time;
*run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

*****;
*****;
```

```
*****;
*PHBcontent ;


  title "3. Mixed analysis PHBcontent";
  proc mixed data=all plots(only) =Studentpanel(marginal);
   class  treatment rep  time;
   model PHBcontent= treatment time treatment*time/outp=outmx residual
ddfm=kr;
   repeated  / subject= rep(treatment*time) group=time;*treatment;*time;
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHcontent";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;


  ***;
title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment"  ;
run;

  ***;
title2 "Pairwise Mean differences -  Time ";
proc print data=diffds;  where effect = "Time";
run;
data mmm3;
  set lsmnds; where effect = "Time";
  run;

data ppp3;
  set diffds; where effect = "Time";
  run;
*** LSD test***;
%pdmix800(ppp3,mmm3,alpha=.01,sort=yes);
***;

data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
```

```
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
****;

title2 "Pairwise Mean differences";
proc print data=diffds;    where effect = "Treatment*Time"  ;
run;
**;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"   ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time"  ;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
****;

proc sort data=diffds;
  by time;
  run;
*title2 "Pairwise Mean differences";
*proc print data=diffds; * where effect = "Treatment*Time" and
_TIME=time;
*run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

  *****;
*Y_p_x      ;


  title "4. Mixed analysis Y_P_X";
  proc mixed data=all plots(only) =Studentpanel(marginal);
   class  treatment  time;
   model Y_p_x= treatment time treatment*time/outp=outmx residual
ddfm=kr;
   repeated  / subject= rep(treatment*time) group=
time;*treatment;*time;
```

```
   lsmeans treatment time/cl diff ;*adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS Y_p_x";
symbol i=join v=star c=red;
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;

  ***;

  ***;
title2 "Pairwise Mean differences -  Time ";
proc print data=diffds;  where effect = "Time";
run;
data mmm3;
  set lsmnds; where effect = "Time";
  run;

data ppp3;
  set diffds; where effect = "Time";
  run;
*** LSD test***;
%pdmix800(ppp3,mmm3,alpha=.01,sort=yes);
***;

title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
***;

title2 "Pairwise Mean differences";
proc print data=diffds;   where effect = "Treatment*Time" ;
run;
***;
```

```
proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"    ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time" ;
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
****;

proc sort data=diffds;
  by time;
  run;
*title2 "Pairwise Mean differences";
*proc print data=diffds;  *where effect = "Treatment*Time" and
_TIME=time;
*run;
data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);
  *****;
*************************************************************************;
*PHBproductivity;

  title "5. Mixed analysis PHBproductivity";
  proc mixed data=all  plots(only) =StudentPanel(marginal);
   class  treatment  time;
   model PHBproductivity= treatment time treatment*time/outp=outmx
residual ddfm=kr  ;
   repeated  / subject= rep(treatment*time)
group=treatment;*time;*treatment;*time;
   lsmeans treatment time/cl diff;* adjust=tukey;
   lsmeans treatment*time/cl  slice=(treatment time)
diff ;*adjust=tukey;
   ods output lsmeans=lsmnds  diffs=diffds;
   ods exclude diffs;
   run;

proc sort data=lsmnds;
  by time;
  run;
  title2 "LSMEANS PBHproductivity";
symbol i=join v=star c=red;
```

```
symbol2 i=join v=circle c=blue;
symbol3 i=join v=square c=black;
proc gplot data=lsmnds ;where effect = "Treatment*Time";
  plot estimate*time=treatment;
  run;
 *;

  ***;
title2 "Pairwise Mean differences -  Time ";
proc print data=diffds;  where effect = "Time";
run;
data mmm3;
  set lsmnds; where effect = "Time";
  run;

data ppp3;
  set diffds; where effect = "Time";
  run;
*** LSD test***;
%pdmix800(ppp3,mmm3,alpha=.01,sort=yes);
***;

title2 "Pairwise Mean differences -  Treatment ";
proc print data=diffds;  where effect = "Treatment";
run;
data mmm1;
  set lsmnds; where effect = "Treatment";
  run;

data ppp1;
  set diffds; where effect = "Treatment";
  run;
*** LSD test***;
%pdmix800(ppp1,mmm1,alpha=.01,sort=yes);
*****;

title2 "Pairwise Mean differences";
proc print data=diffds;    where effect = "Treatment*Time" ;
run;
**;

proc sort data=diffds;
  by treatment;
  run;
data mmm;
  set lsmnds; where effect = "Treatment*Time"  ;
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;

*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=treatment);
```

```
***;

proc sort data=diffds;
  by time;
  run;

*title2 "Pairwise Mean differences";
*proc print data=diffds;  *where effect = "Treatment*Time" and
_TIME=time;
*run;

data mmm;
  set lsmnds; where effect = "Treatment*Time";
  run;

data ppp;
  set diffds; where effect = "Treatment*Time";
  run;
*** LSD test***;
%pdmix800(ppp,mmm,alpha=.01,sort=yes, slice=time);

  *****;

  title "1.MANOVA  Drycellweight PHBconc PHByield PHBcontent
PHBproductivity=";
  title "ANOVA";
  proc glm data=all plots=none;
   class  treatment rep  time;
   model Drycellweight PHBconc PHBcontent PHBproductivity= treatment
time treatment*time;
   manova h=treatment time treatment*time/printe;
   lsmeans treatment time/cl pdiff;* adjust=tukey;
   lsmeans treatment*time/cl   pdiff  slice=(treatment time);***diff
adjust=tukey;
   run;

   quit;
  ods graphics off;
  ods pdf close;
  ods listing  ;
 ;
```

Appendix V: SAS Outputs of ANOVA GLM and mixed analysis

Table Va. SAS output for sugar extraction study

The GLM Procedure

Class Level Information

Class	Levels	Values
extractionmethod	2	1 2
solidloading	3	1 2 3
extractiontime	4	30 60 90 120

Number of Observations Read	24
Number of Observations Used	24

The GLM Procedure

Dependent Variable: sugarconc

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	0.02639208	0.00155248	30.77	0.0002
Error	6	0.00030275	0.00005046		
Corrected Total	23	0.02669483			

R-Square	Coeff Var	Root MSE	sugarconc Mean
0.988659	0.975211	0.007103	0.728396

Source	DF	Type I SS	Mean Square	F Value	Pr > F
extractionmethod	1	0.00457332	0.00457332	90.64	<.0001

solidloading	2	0.01543469	0.00771735	152.95	<.0001
extractiontime	3	0.00158966	0.00052989	10.50	0.0084
solidload*extraction	6	0.00102799	0.00017133	3.40	0.0812
extractio*solidloadi	2	0.00367461	0.00183731	36.41	0.0004
extractio*extraction	3	0.00009180	0.00003060	0.61	0.6347

Source	DF	Type III SS	Mean Square	F Value	Pr > F
extractionmethod	1	0.00457332	0.00457332	90.64	<.0001
solidloading	2	0.01543469	0.00771735	152.95	<.0001
extractiontime	3	0.00158966	0.00052989	10.50	0.0084
solidload*extraction	6	0.00102799	0.00017133	3.40	0.0812
extractio*solidloadi	2	0.00367461	0.00183731	36.41	0.0004
extractio*extraction	3	0.00009180	0.00003060	0.61	0.6347

The GLM Procedure

Least Squares Means

H0:LSMean1= LSMean2

Dependent Variable: sugarconc

extractionmethod	LSMEAN	Pr > |t|
1	0.71459167	<.0001
2	0.74220000	

sugarconc LSMEAN

solidloading	LSMEAN	Number
1	0.72470000	1
2	0.76113750	2
3	0.69935000	3

Least Squares Means for effect solidloading

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: sugarconc

i/j	1	2	3
1		<.0001	0.0004
2	<.0001		<.0001
3	0.0004	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

sugarconc LSMEAN

extractiontime	LSMEAN	Number
30	0.71490000	1
60	0.73671667	2
90	0.73068333	3
120	0.73128333	4

The GLM Procedure

Least Squares Means

Least Squares Means for effect extractiontime

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: sugarconc

i/j	1	2	3	4
1		0.0018	0.0085	0.0072
2	0.0018		0.1917	0.2334
3	0.0085	0.1917		0.8885
4	0.0072	0.2334	0.8885	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

		sugarconc LSMEAN	
solidloading	extractiontime	LSMEAN	Number
1	30	0.71510000	1
1	60	0.72910000	2
1	90	0.72555000	3
1	120	0.72905000	4
2	30	0.75845000	5
2	60	0.76890000	6
2	90	0.76120000	7
2	120	0.75600000	8

3	30	0.67115000	9
3	60	0.71215000	10
3	90	0.70530000	11
3	120	0.70880000	12

The GLM Procedure

Least Squares Means

Least Squares Means for effect solidload*extraction

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: sugarconc

i/j	1	2	3	4	5	6	
1		0.0962	0.1917	0.0972	0.0009	0.0003	
2	0.0962		0.6350	0.9946	0.0061	0.0014	
3	0.1917	0.6350		0.6397	0.0036	0.0009	
4	0.0972	0.9946	0.6397		0.0061	0.0014	
5	0.0009	0.0061	0.0036	0.0061		0.1917	
6	0.0003	0.0014	0.0009	0.0014	0.1917		
7	0.0006	0.0040	0.0024	0.0040	0.7120	0.3200	
8	0.0012	0.0091	0.0052	0.0090	0.7419	0.1193	
9	0.0008	0.0002	0.0003	0.0002	<.0001	<.0001	
10	0.6924	0.0543	0.1082	0.0548	0.0006	0.0002	
11	0.2169	0.0154	0.0292	0.0155	0.0003	0.0001	
12	0.4093	0.0289	0.0564	0.0292	0.0004	0.0001	

Least Squares Means for effect solidload*extraction

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: sugarconc

i/j	7	8	9	10	11	12
1	0.0006	0.0012	0.0008	0.6924	0.2169	0.4093
2	0.0040	0.0091	0.0002	0.0543	0.0154	0.0289
3	0.0024	0.0052	0.0003	0.1082	0.0292	0.0564
4	0.0040	0.0090	0.0002	0.0548	0.0155	0.0292
5	0.7120	0.7419	<.0001	0.0006	0.0003	0.0004
6	0.3200	0.1193	<.0001	0.0002	0.0001	0.0001
7		0.4917	<.0001	0.0005	0.0002	0.0003
8	0.4917		<.0001	0.0008	0.0004	0.0006
9	<.0001	<.0001		0.0012	0.0030	0.0018
10	0.0005	0.0008	0.0012		0.3721	0.6539
11	0.0002	0.0004	0.0030	0.3721		0.6397
12	0.0003	0.0006	0.0018	0.6539	0.6397	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

		sugarconc LSMEAN	
extractionmethod	solidloading	LSMEAN	Number
1	1	0.72292500	1
1	2	0.75232500	2

1	3	0.66852500	3
2	1	0.72647500	4
2	2	0.76995000	5
2	3	0.73017500	6

Least Squares Means for effect extractio*solidloadi

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: sugarconc

i/j	1	2	3	4	5	6
1		0.0011	<.0001	0.5062	<.0001	0.1990
2	0.0011		<.0001	0.0021	0.0127	0.0045
3	<.0001	<.0001		<.0001	<.0001	<.0001
4	0.5062	0.0021	<.0001		0.0001	0.4891
5	<.0001	0.0127	<.0001	0.0001		0.0002
6	0.1990	0.0045	<.0001	0.4891	0.0002	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

		sugarconc LSMEAN	
extractionmethod	extractiontime	LSMEAN	Number
1	30	0.69796667	1

1	60	0.72320000	2
1	90	0.71913333	3
1	120	0.71806667	4
2	30	0.73183333	5
2	60	0.75023333	6
2	90	0.74223333	7
2	120	0.74450000	8

Least Squares Means for effect extractio*solidloadi

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: sugarconc

i/j	1	2	3	4	5	6
1		0.0011	<.0001	0.5062	<.0001	0.1990
2	0.0011		<.0001	0.0021	0.0127	0.0045
3	<.0001	<.0001		<.0001	<.0001	<.0001
4	0.5062	0.0021	<.0001		0.0001	0.4891
5	<.0001	0.0127	<.0001	0.0001		0.0002
6	0.1990	0.0045	<.0001	0.4891	0.0002	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

sugarconc LSMEAN

extractionmethod	extractiontime	LSMEAN	Number
1	30	0.69796667	1
1	60	0.72320000	2
1	90	0.71913333	3
1	120	0.71806667	4
2	30	0.73183333	5
2	60	0.75023333	6
2	90	0.74223333	7
2	120	0.74450000	8

Least Squares Means for effect extractio*extraction

Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: sugarconc

i/j	1	2	3	4	5	6	7	8
1		0.0048	0.0107	0.0134	0.0011	0.0001	0.0003	0.0002
2	0.0048		0.5095	0.4102	0.1872	0.0035	0.0168	0.0104
3	0.0107	0.5095		0.8601	0.0711	0.0017	0.0073	0.0047
4	0.0134	0.4102	0.8601		0.0552	0.0015	0.0059	0.0039
5	0.0011	0.1872	0.0711	0.0552		0.0193	0.1231	0.0717
6	0.0001	0.0035	0.0017	0.0015	0.0193		0.2170	0.3611
7	0.0003	0.0168	0.0073	0.0059	0.1231	0.2170		0.7094

8	0.0002	0.0104	0.0047	0.0039	0.0717	0.3611	0.7094

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Table Vb. SAS codes for two-stage culture on selection of best time point for inducing nitrogen limitation

Model Information	
Data Set	WORK.ALL
Dependent Variable	**PHBconc**
Covariance Structure	Variance Components
Subject Effect	rep(Treatment)
Group Effect	Time
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
Treatment	3	14Hour 16Hour 18Hour
Time	7	0 4 8 12 26 27 28

Mixed analysis PHB

Pairwise Mean differences

Obs	Effect	Treatment	Time	Treatment	Time	DF	t-Value	Probt	Alpha
1	Treatment	14Hour	_	16Hour	_	42	-5.30	<.0001	0.05
2	Treatment	14Hour	_	18Hour	_	42	-0.91	0.3667	0.05
3	Treatment	16Hour	_	18Hour	_	42	4.38	<.0001	0.05
4	Time		0		4	42	-11.93	<.0001	0.05
5	Time		0		8	42	-16.88	<.0001	0.05
6	Time		0		12	42	-8.89	<.0001	0.05
7	Time		0		26	42	-17.41	<.0001	0.05
8	Time		0		27	42	-17.28	<.0001	0.05
9	Time		0		28	42	-25.57	<.0001	0.05
10	Time		4		8	42	-6.83	<.0001	0.05
11	Time		4		12	42	-6.22	<.0001	0.05
12	Time		4		26	42	-14.37	<.0001	0.05
13	Time		4		27	42	-14.30	<.0001	0.05
14	Time		4		28	42	-21.79	<.0001	0.05

15	Time		8		12	42	-3.84	0.0004	0.05
16	Time		8		26	42	-11.66	<.0001	0.05
17	Time		8		27	42	-11.63	<.0001	0.05
18	Time		8		28	42	-18.41	<.0001	0.05
19	Time		12		26	42	-5.38	<.0001	0.05
20	Time		12		27	42	-5.45	<.0001	0.05
21	Time		12		28	42	-9.06	<.0001	0.05
22	Time		26		27	42	-0.11	0.9134	0.05
23	Time		26		28	42	-3.45	0.0013	0.05
24	Time		27		28	42	-3.30	0.0020	0.05
25	Treatment *Time	14Hour	0	14Hour	4	42	-4.71	<.0001	0.05
26	Treatment *Time	14Hour	0	14Hour	8	42	-7.51	<.0001	0.05
27	Treatment *Time	14Hour	0	14Hour	12	42	-4.32	<.0001	0.05
28	Treatment *Time	14Hour	0	14Hour	26	42	-9.94	<.0001	0.05
29	Treatment *Time	14Hour	0	14Hour	27	42	-8.50	<.0001	0.05
30	Treatment *Time	14Hour	0	14Hour	28	42	-14.41	<.0001	0.05
31	Treatment *Time	14Hour	0	16Hour	0	42	-3.53	0.0010	0.05
32	Treatment *Time	14Hour	0	16Hour	4	42	-10.94	<.0001	0.05
33	Treatment *Time	14Hour	0	16Hour	8	42	-12.09	<.0001	0.05
34	Treatment *Time	14Hour	0	16Hour	12	42	-6.72	<.0001	0.05
35	Treatment *Time	14Hour	0	16Hour	26	42	-12.94	<.0001	0.05
36	Treatment *Time	14Hour	0	16Hour	27	42	-12.16	<.0001	0.05
37	Treatment *Time	14Hour	0	16Hour	28	42	-17.24	<.0001	0.05
38	Treatment *Time	14Hour	0	18Hour	0	42	-2.96	0.0051	0.05
39	Treatment *Time	14Hour	0	18Hour	4	42	-8.50	<.0001	0.05
40	Treatment *Time	14Hour	0	18Hour	8	42	-12.22	<.0001	0.05
41	Treatment *Time	14Hour	0	18Hour	12	42	-5.12	<.0001	0.05
42	Treatment *Time	14Hour	0	18Hour	26	42	-8.07	<.0001	0.05
43	Treatment *Time	14Hour	0	18Hour	27	42	-10.06	<.0001	0.05
44	Treatment *Time	14Hour	0	18Hour	28	42	-13.59	<.0001	0.05

45	Treatment *Time	14Hour	4	14Hour	8	42	-3.42	0.0014	0.05
46	Treatment *Time	14Hour	4	14Hour	12	42	-3.25	0.0023	0.05
47	Treatment *Time	14Hour	4	14Hour	26	42	-8.69	<.0001	0.05
48	Treatment *Time	14Hour	4	14Hour	27	42	-7.29	<.0001	0.05
49	Treatment *Time	14Hour	4	14Hour	28	42	-12.81	<.0001	0.05
50	Treatment *Time	14Hour	4	16Hour	0	42	2.81	0.0075	0.05
51	Treatment *Time	14Hour	4	16Hour	4	42	-4.77	<.0001	0.05
52	Treatment *Time	14Hour	4	16Hour	8	42	-7.30	<.0001	0.05
53	Treatment *Time	14Hour	4	16Hour	12	42	-5.61	<.0001	0.05
54	Treatment *Time	14Hour	4	16Hour	26	42	-11.63	<.0001	0.05
55	Treatment *Time	14Hour	4	16Hour	27	42	-10.89	<.0001	0.05
56	Treatment *Time	14Hour	4	16Hour	28	42	-15.57	<.0001	0.05
57	Treatment *Time	14Hour	4	18Hour	0	42	3.12	0.0033	0.05
58	Treatment *Time	14Hour	4	18Hour	4	42	-2.90	0.0060	0.05
59	Treatment *Time	14Hour	4	18Hour	8	42	-7.41	<.0001	0.05
60	Treatment *Time	14Hour	4	18Hour	12	42	-4.04	0.0002	0.05
61	Treatment *Time	14Hour	4	18Hour	26	42	-6.85	<.0001	0.05
62	Treatment *Time	14Hour	4	18Hour	27	42	-8.82	<.0001	0.05
63	Treatment *Time	14Hour	4	18Hour	28	42	-12.01	<.0001	0.05
64	Treatment *Time	14Hour	8	14Hour	12	42	-2.05	0.0463	0.05
65	Treatment *Time	14Hour	8	14Hour	26	42	-7.30	<.0001	0.05
66	Treatment *Time	14Hour	8	14Hour	27	42	-5.95	<.0001	0.05
67	Treatment *Time	14Hour	8	14Hour	28	42	-11.07	<.0001	0.05
68	Treatment *Time	14Hour	8	16Hour	0	42	6.11	<.0001	0.05
69	Treatment *Time	14Hour	8	16Hour	4	42	-0.50	0.6203	0.05
70	Treatment *Time	14Hour	8	16Hour	8	42	-3.37	0.0016	0.05
71	Treatment	14Hour	8	16Hour	12	42	-4.37	<.0001	0.05

	*Time								
72	Treatment *Time	14Hour	8	16Hour	26	42	-10.18	<.0001	0.05
73	Treatment *Time	14Hour	8	16Hour	27	42	-9.48	<.0001	0.05
74	Treatment *Time	14Hour	8	16Hour	28	42	-13.75	<.0001	0.05
75	Treatment *Time	14Hour	8	18Hour	0	42	6.34	<.0001	0.05
76	Treatment *Time	14Hour	8	18Hour	4	42	1.04	0.3058	0.05
77		14Hour	8	18Hour	8	42	-3.47	0.0012	0.05
78		14Hour	8	18Hour	12	42	-2.83	0.0071	0.05
79		14Hour	8	18Hour	26	42	-5.50	<.0001	0.05
80		14Hour	8	18Hour	27	42	-7.45	<.0001	0.05
81		14Hour	8	18Hour	28	42	-10.29	<.0001	0.05
82		14Hour	12	14Hour	26	42	-3.63	0.0008	0.05
83		14Hour	12	14Hour	27	42	-2.72	0.0094	0.05
84		14Hour	12	14Hour	28	42	-5.65	<.0001	0.05
85		14Hour	12	16Hour	0	42	3.91	0.0003	0.05
86		14Hour	12	16Hour	4	42	1.92	0.0614	0.05
87		14Hour	12	16Hour	8	42	0.77	0.4485	0.05
88		14Hour	12	16Hour	12	42	-1.70	0.0957	0.05
89		14Hour	12	16Hour	26	42	-5.69	<.0001	0.05
90		14Hour	12	16Hour	27	42	-5.25	<.0001	0.05
91		14Hour	12	16Hour	28	42	-7.42	<.0001	0.05
92		14Hour	12	18Hour	0	42	3.97	0.0003	0.05
93		14Hour	12	18Hour	4	42	2.44	0.0189	0.05
94		14Hour	12	18Hour	8	42	0.73	0.4714	0.05
95		14Hour	12	18Hour	12	42	-0.57	0.5709	0.05
96		14Hour	12	18Hour	26	42	-2.35	0.0233	0.05
97		14Hour	12	18Hour	27	42	-3.80	0.0005	0.05
98		14Hour	12	18Hour	28	42	-5.13	<.0001	0.05
99		14Hour	26	14Hour	27	42	0.92	0.3643	0.05
100		14Hour	26	14Hour	28	42	-1.85	0.0719	0.05
101		14Hour	26	16Hour	0	42	9.50	<.0001	0.05
102		14Hour	26	16Hour	4	42	7.27	<.0001	0.05
103		14Hour	26	16Hour	8	42	5.92	<.0001	0.05
104		14Hour	26	16Hour	12	42	1.87	0.0684	0.05
105		14Hour	26	16Hour	26	42	-2.13	0.0390	0.05
106		14Hour	26	16Hour	27	42	-1.70	0.0956	0.05
107		14Hour	26	16Hour	28	42	-3.69	0.0006	0.05
108		14Hour	26	18Hour	0	42	9.57	<.0001	0.05

109	14Hour	26	18Hour	4	42	7.83	<.0001	0.05
110	14Hour	26	18Hour	8	42	5.88	<.0001	0.05
111	14Hour	26	18Hour	12	42	3.04	0.0040	0.05
112	14Hour	26	18Hour	26	42	1.33	0.1917	0.05
113	14Hour	26	18Hour	27	42	-0.20	0.8429	0.05
114	14Hour	26	18Hour	28	42	-1.31	0.1985	0.05
115	14Hour	27	14Hour	28	42	-2.81	0.0074	0.05
116	14Hour	27	16Hour	0	42	8.07	<.0001	0.05
117	14Hour	27	16Hour	4	42	5.90	<.0001	0.05
118	14Hour	27	16Hour	8	42	4.60	<.0001	0.05
119	14Hour	27	16Hour	12	42	0.97	0.3370	0.05
120	14Hour	27	16Hour	26	42	-3.03	0.0042	0.05
121	14Hour	27	16Hour	27	42	-2.60	0.0128	0.05
122	14Hour	27	16Hour	28	42	-4.64	<.0001	0.05
123	14Hour	27	18Hour	0	42	8.14	<.0001	0.05
124	14Hour	27	18Hour	4	42	6.44	<.0001	0.05
125	14Hour	27	18Hour	8	42	4.56	<.0001	0.05
126	14Hour	27	18Hour	12	42	2.13	0.0387	0.05
127	14Hour	27	18Hour	26	42	0.40	0.6921	0.05
128	14Hour	27	18Hour	27	42	-1.11	0.2744	0.05
129	14Hour	27	18Hour	28	42	-2.28	0.0279	0.05
130	14Hour	28	16Hour	0	42	13.90	<.0001	0.05
131	14Hour	28	16Hour	4	42	11.17	<.0001	0.05
132	14Hour	28	16Hour	8	42	9.49	<.0001	0.05
133	14Hour	28	16Hour	12	42	3.77	0.0005	0.05
134	14Hour	28	16Hour	26	42	-0.44	0.6603	0.05
135	14Hour	28	16Hour	27	42	0.00	1.0000	0.05
136	14Hour	28	16Hour	28	42	-2.01	0.0512	0.05
137	14Hour	28	18Hour	0	42	13.98	<.0001	0.05
138	14Hour	28	18Hour	4	42	11.82	<.0001	0.05
139	14Hour	28	18Hour	8	42	9.44	<.0001	0.05
140	14Hour	28	18Hour	12	42	5.02	<.0001	0.05
141	14Hour	28	18Hour	26	42	3.27	0.0021	0.05
142	14Hour	28	18Hour	27	42	1.61	0.1138	0.05
143	14Hour	28	18Hour	28	42	0.59	0.5605	0.05
144	16Hour	0	16Hour	4	42	-9.05	<.0001	0.05
145	16Hour	0	16Hour	8	42	-10.68	<.0001	0.05
146	16Hour	0	16Hour	12	42	-6.31	<.0001	0.05
147	16Hour	0	16Hour	26	42	-12.51	<.0001	0.05
148	16Hour	0	16Hour	27	42	-11.73	<.0001	0.05
149	16Hour	0	16Hour	28	42	-16.72	<.0001	0.05

150		16Hour	0	18Hour	0	42	0.57	0.5727	0.05
151		16Hour	0	18Hour	4	42	-6.60	<.0001	0.05
152		16Hour	0	18Hour	8	42	-10.82	<.0001	0.05
153	Treatment *Time	16Hour	0	18Hour	12	42	-4.71	<.0001	0.05
154	Treatment *Time	16Hour	0	18Hour	26	42	-7.63	<.0001	0.05
155	Treatment *Time	16Hour	0	18Hour	27	42	-9.63	<.0001	0.05
156	Treatment *Time	16Hour	0	18Hour	28	42	-13.08	<.0001	0.05
157	Treatment *Time	16Hour	4	16Hour	8	42	-3.38	0.0016	0.05
158	Treatment *Time	16Hour	4	16Hour	12	42	-4.29	0.0001	0.05
159	Treatment *Time	16Hour	4	16Hour	26	42	-10.22	<.0001	0.05
160	Treatment *Time	16Hour	4	16Hour	27	42	-9.49	<.0001	0.05
161	Treatment *Time	16Hour	4	16Hour	28	42	-13.92	<.0001	0.05
162	Treatment *Time	16Hour	4	18Hour	0	42	9.35	<.0001	0.05
163	Treatment *Time	16Hour	4	18Hour	4	42	1.87	0.0686	0.05
164	Treatment *Time	16Hour	4	18Hour	8	42	-3.49	0.0011	0.05
165	Treatment *Time	16Hour	4	18Hour	12	42	-2.71	0.0096	0.05
166	Treatment *Time	16Hour	4	18Hour	26	42	-5.43	<.0001	0.05
167	Treatment *Time	16Hour	4	18Hour	27	42	-7.43	<.0001	0.05
168	Treatment *Time	16Hour	4	18Hour	28	42	-10.37	<.0001	0.05
169	Treatment *Time	16Hour	8	16Hour	12	42	-3.09	0.0036	0.05
170	Treatment *Time	16Hour	8	16Hour	26	42	-8.81	<.0001	0.05
171	Treatment *Time	16Hour	8	16Hour	27	42	-8.12	<.0001	0.05
172	Treatment *Time	16Hour	8	16Hour	28	42	-12.16	<.0001	0.05
173	Treatment *Time	16Hour	8	18Hour	0	42	10.91	<.0001	0.05
174	Treatment *Time	16Hour	8	18Hour	4	42	4.91	<.0001	0.05
175	Treatment *Time	16Hour	8	18Hour	8	42	-0.10	0.9208	0.05
176	Treatment *Time	16Hour	8	18Hour	12	42	-1.54	0.1303	0.05
177	Treatment	16Hour	8	18Hour	26	42	-4.12	0.0002	0.05

	*Time								
178	Treatment *Time	16Hour	8	18Hour	27	42	-6.10	<.0001	0.05
179	Treatment *Time	16Hour	8	18Hour	28	42	-8.70	<.0001	0.05
180	Treatment *Time	16Hour	12	16Hour	26	42	-3.92	0.0003	0.05
181	Treatment *Time	16Hour	12	16Hour	27	42	-3.50	0.0011	0.05
182	Treatment *Time	16Hour	12	16Hour	28	42	-5.54	<.0001	0.05
183	Treatment *Time	16Hour	12	18Hour	0	42	6.38	<.0001	0.05
184	Treatment *Time	16Hour	12	18Hour	4	42	4.80	<.0001	0.05
185	Treatment *Time	16Hour	12	18Hour	8	42	3.05	0.0040	0.05
186	Treatment *Time	16Hour	12	18Hour	12	42	1.13	0.2636	0.05
187	Treatment *Time	16Hour	12	18Hour	26	42	-0.59	0.5576	0.05
188	Treatment *Time	16Hour	12	18Hour	27	42	-2.05	0.0468	0.05
189	Treatment *Time	16Hour	12	18Hour	28	42	-3.25	0.0023	0.05
190	Treatment *Time	16Hour	26	16Hour	27	42	0.41	0.6848	0.05
191	Treatment *Time	16Hour	26	16Hour	28	42	-1.40	0.1678	0.05
192	Treatment *Time	16Hour	26	18Hour	0	42	12.58	<.0001	0.05
193	Treatment *Time	16Hour	26	18Hour	4	42	10.77	<.0001	0.05
194	Treatment *Time	16Hour	26	18Hour	8	42	8.77	<.0001	0.05
195	Treatment *Time	16Hour	26	18Hour	12	42	5.10	<.0001	0.05
196	Treatment *Time	16Hour	26	18Hour	26	42	3.46	0.0013	0.05
197	Treatment *Time	16Hour	26	18Hour	27	42	1.91	0.0624	0.05
198	Treatment *Time	16Hour	26	18Hour	28	42	0.98	0.3315	0.05
199	Treatment *Time	16Hour	27	16Hour	28	42	-1.83	0.0746	0.05
200	Treatment *Time	16Hour	27	18Hour	0	42	11.80	<.0001	0.05
201	Treatment *Time	16Hour	27	18Hour	4	42	10.04	<.0001	0.05
202	Treatment *Time	16Hour	27	18Hour	8	42	8.08	<.0001	0.05
203	Treatment *Time	16Hour	27	18Hour	12	42	4.66	<.0001	0.05

204	Treatment *Time	16Hour	27	18Hour	26	42	3.02	0.0043	0.05
205	Treatment *Time	16Hour	27	18Hour	27	42	1.49	0.1429	0.05
206	Treatment *Time	16Hour	27	18Hour	28	42	0.53	0.5957	0.05
207	Treatment *Time	16Hour	28	18Hour	0	42	16.81	<.0001	0.05
208	Treatment *Time	16Hour	28	18Hour	4	42	14.57	<.0001	0.05
209	Treatment *Time	16Hour	28	18Hour	8	42	12.12	<.0001	0.05
210	Treatment *Time	16Hour	28	18Hour	12	42	6.79	<.0001	0.05
211	Treatment *Time	16Hour	28	18Hour	26	42	5.12	<.0001	0.05
212	Treatment *Time	16Hour	28	18Hour	27	42	3.44	0.0013	0.05
213	Treatment *Time	16Hour	28	18Hour	28	42	2.59	0.0130	0.05
214	Treatment *Time	18Hour	0	18Hour	4	42	-6.91	<.0001	0.05
215	Treatment *Time	18Hour	0	18Hour	8	42	-11.05	<.0001	0.05
216	Treatment *Time	18Hour	0	18Hour	12	42	-4.78	<.0001	0.05
217	Treatment *Time	18Hour	0	18Hour	26	42	-7.70	<.0001	0.05
218	Treatment *Time	18Hour	0	18Hour	27	42	-9.70	<.0001	0.05
219	Treatment *Time	18Hour	0	18Hour	28	42	-13.16	<.0001	0.05
220	Treatment *Time	18Hour	4	18Hour	8	42	-5.03	<.0001	0.05
221	Treatment *Time	18Hour	4	18Hour	12	42	-3.23	0.0024	0.05
222	Treatment *Time	18Hour	4	18Hour	26	42	-5.99	<.0001	0.05
223	Treatment *Time	18Hour	4	18Hour	27	42	-7.98	<.0001	0.05
224	Treatment *Time	18Hour	4	18Hour	28	42	-11.01	<.0001	0.05
225	Treatment *Time	18Hour	8	18Hour	12	42	-1.50	0.1399	0.05
226	Treatment *Time	18Hour	8	18Hour	26	42	-4.08	0.0002	0.05
227	Treatment *Time	18Hour	8	18Hour	27	42	-6.06	<.0001	0.05
228	Treatment *Time	18Hour	8	18Hour	28	42	-8.66	<.0001	0.05
229	Treatment *Time	18Hour	12	18Hour	26	42	-1.76	0.0850	0.05
230	Treatment	18Hour	12	18Hour	27	42	-3.21	0.0025	0.05

	*Time								
231	Treatment *Time	18Hour	12	18Hour	28	42	-4.50	<.0001	0.05
232	Treatment *Time	18Hour	26	18Hour	27	42	-1.52	0.1372	0.05
233	Treatment *Time	18Hour	26	18Hour	28	42	-2.73	0.0092	0.05
234	Treatment *Time	18Hour	27	18Hour	28	42	-1.08	0.2863	0.05

Model Information	
Data Set	WORK.ALL
Dependent Variable	**PHBcontent**
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information

Class	Levels	Values
Treatment	3	14Hour 16Hour 18Hour
Time	7	0 4 8 12 26 27 28

Mixed analysis PHB

Pairwise Mean differences

Obs	Effect	Treatment	Time	Treatment	Time	DF	tValue	Probt	Alpha
1	Treatment	14Hour	_	16Hour	_	36	-0.90	0.3763	0.05
2	Treatment	14Hour	_	18Hour	_	36	2.15	0.0384	0.05
3	Treatment	16Hour	_	18Hour	_	36	3.04	0.0043	0.05
4	Time		4		8	36	-2.36	0.0241	0.05
5	Time		4		12	36	-4.54	<.0001	0.05
6	Time		4		26	36	-5.53	<.0001	0.05
7	Time		4		27	36	-6.40	<.0001	0.05
8	Time		4		28	36	-7.31	<.0001	0.05
9	Time		8		12	36	-2.19	0.0353	0.05

10	Time		8		26	36	-3.17	0.0031	0.05
11	Time		8		27	36	-4.05	0.0003	0.05
12	Time		8		28	36	-4.95	<.0001	0.05
13	Time		12		26	36	-0.98	0.3321	0.05
14	Time		12		27	36	-1.86	0.0713	0.05
15	Time		12		28	36	-2.76	0.0089	0.05
16	Time		26		27	36	-0.88	0.3871	0.05
17	Time		26		28	36	-1.78	0.0834	0.05
18	Time		27		28	36	-0.91	0.3715	0.05
19	Treatment*Time	14Hour	4	14Hour	8	36	-2.27	0.0295	0.05
20	Treatment*Time	14Hour	4	14Hour	12	36	-3.07	0.0040	0.05
21	Treatment*Time	14Hour	4	14Hour	26	36	-4.47	<.0001	0.05
22	Treatment*Time	14Hour	4	14Hour	27	36	-4.70	<.0001	0.05
23	Treatment*Time	14Hour	4	14Hour	28	36	-5.44	<.0001	0.05
24	Treatment*Time	14Hour	4	16Hour	4	36	-1.59	0.1203	0.05
25	Treatment*Time	14Hour	4	16Hour	8	36	-1.99	0.0546	0.05
26	Treatment*Time	14Hour	4	16Hour	12	36	-4.19	0.0002	0.05
27	Treatment*Time	14Hour	4	16Hour	26	36	-4.57	<.0001	0.05
28	Treatment*Time	14Hour	4	16Hour	27	36	-4.65	<.0001	0.05
29	Treatment*Time	14Hour	4	16Hour	28	36	-5.15	<.0001	0.05
30	Treatment*Time	14Hour	4	18Hour	4	36	-0.33	0.7439	0.05
31	Treatment*Time	14Hour	4	18Hour	8	36	-1.75	0.0894	0.05
32	Treatment*Time	14Hour	4	18Hour	12	36	-2.52	0.0163	0.05
33	Treatment*Time	14Hour	4	18Hour	26	36	-2.45	0.0195	0.05
34	Treatment*Time	14Hour	4	18Hour	27	36	-3.66	0.0008	0.05
35	Treatment*Time	14Hour	4	18Hour	28	36	-3.99	0.0003	0.05
36	Treatment*Time	14Hour	8	14Hour	12	36	-0.81	0.4253	0.05
37	Treatment*Time	14Hour	8	14Hour	26	36	-2.20	0.0340	0.05
38	Treatment*Time	14Hour	8	14Hour	27	36	-2.43	0.0201	0.05
39		14Hour	8	14Hour	28	36	-3.17	0.0031	0.05

	Treatment*Time								
40	Treatment*Time	14Hour	8	16Hour	4	36	0.68	0.5030	0.05
41	Treatment*Time	14Hour	8	16Hour	8	36	0.28	0.7806	0.05
42	Treatment*Time	14Hour	8	16Hour	12	36	-1.93	0.0621	0.05
43	Treatment*Time	14Hour	8	16Hour	26	36	-2.31	0.0270	0.05
44	Treatment*Time	14Hour	8	16Hour	27	36	-2.38	0.0226	0.05
45	Treatment*Time	14Hour	8	16Hour	28	36	-2.88	0.0066	0.05
46	Treatment*Time	14Hour	8	18Hour	4	36	1.94	0.0605	0.05
47	Treatment*Time	14Hour	8	18Hour	8	36	0.52	0.6049	0.05
48	Treatment*Time	14Hour	8	18Hour	12	36	-0.25	0.8014	0.05
49	Treatment*Time	14Hour	8	18Hour	26	36	-0.18	0.8593	0.05
50	Treatment*Time	14Hour	8	18Hour	27	36	-1.39	0.1732	0.05
51	Treatment*Time	14Hour	8	18Hour	28	36	-1.72	0.0941	0.05
52	Treatment*Time	14Hour	12	14Hour	26	36	-1.40	0.1709	0.05
53	Treatment*Time	14Hour	12	14Hour	27	36	-1.62	0.1129	0.05
54	Treatment*Time	14Hour	12	14Hour	28	36	-2.36	0.0236	0.05
55	Treatment*Time	14Hour	12	16Hour	4	36	1.48	0.1468	0.05
56	Treatment*Time	14Hour	12	16Hour	8	36	1.09	0.2842	0.05
57	Treatment*Time	14Hour	12	16Hour	12	36	-1.12	0.2706	0.05
58	Treatment*Time	14Hour	12	16Hour	26	36	-1.50	0.1425	0.05
59	Treatment*Time	14Hour	12	16Hour	27	36	-1.58	0.1236	0.05
60	Treatment*Time	14Hour	12	16Hour	28	36	-2.08	0.0452	0.05
61	Treatment*Time	14Hour	12	18Hour	4	36	2.74	0.0094	0.05
62	Treatment*Time	14Hour	12	18Hour	8	36	1.33	0.1924	0.05
63	Treatment*Time	14Hour	12	18Hour	12	36	0.55	0.5836	0.05
64	Treatment*Time	14Hour	12	18Hour	26	36	0.63	0.5340	0.05
65	Treatment*Time	14Hour	12	18Hour	27	36	-0.58	0.5634	0.05

66	Treatment*Time	14Hour	12	18Hour	28	36	-0.91	0.3674	0.05
67	Treatment*Time	14Hour	26	14Hour	27	36	-0.23	0.8212	0.05
68	Treatment*Time	14Hour	26	14Hour	28	36	-0.97	0.3400	0.05
69	Treatment*Time	14Hour	26	16Hour	4	36	2.88	0.0067	0.05
70	Treatment*Time	14Hour	26	16Hour	8	36	2.48	0.0178	0.05
71	Treatment*Time	14Hour	26	16Hour	12	36	0.28	0.7822	0.05
72	Treatment*Time	14Hour	26	16Hour	26	36	-0.10	0.9192	0.05
73	Treatment*Time	14Hour	26	16Hour	27	36	-0.18	0.8584	0.05
74	Treatment*Time	14Hour	26	16Hour	28	36	-0.68	0.5020	0.05
75	Treatment*Time	14Hour	26	18Hour	4	36	4.14	0.0002	0.05
76	Treatment*Time	14Hour	26	18Hour	8	36	2.73	0.0098	0.05
77	Treatment*Time	14Hour	26	18Hour	12	36	1.95	0.0590	0.05
78	Treatment*Time	14Hour	26	18Hour	26	36	2.03	0.0503	0.05
79	Treatment*Time	14Hour	26	18Hour	27	36	0.81	0.4209	0.05
80	Treatment*Time	14Hour	26	18Hour	28	36	0.48	0.6311	0.05
81	Treatment*Time	14Hour	27	14Hour	28	36	-0.74	0.4645	0.05
82	Treatment*Time	14Hour	27	16Hour	4	36	3.11	0.0037	0.05
83	Treatment*Time	14Hour	27	16Hour	8	36	2.71	0.0102	0.05
84	Treatment*Time	14Hour	27	16Hour	12	36	0.51	0.6158	0.05
85	Treatment*Time	14Hour	27	16Hour	26	36	0.13	0.9008	0.05
86	Treatment*Time	14Hour	27	16Hour	27	36	0.05	0.9619	0.05
87	Treatment*Time	14Hour	27	16Hour	28	36	-0.45	0.6551	0.05
88	Treatment*Time	14Hour	27	18Hour	4	36	4.37	0.0001	0.05
89	Treatment*Time	14Hour	27	18Hour	8	36	2.95	0.0055	0.05
90	Treatment*Time	14Hour	27	18Hour	12	36	2.18	0.0360	0.05
91	Treatment*Time	14Hour	27	18Hour	26	36	2.25	0.0305	0.05
92		14Hour	27	18Hour	27	36	1.04	0.3045	0.05

	Treatment*Time								
93	Treatment*Time	14Hour	27	18Hour	28	36	0.71	0.4811	0.05
94	Treatment*Time	14Hour	28	16Hour	4	36	3.85	0.0005	0.05
95	Treatment*Time	14Hour	28	16Hour	8	36	3.45	0.0014	0.05
96	Treatment*Time	14Hour	28	16Hour	12	36	1.25	0.2210	0.05
97	Treatment*Time	14Hour	28	16Hour	26	36	0.86	0.3928	0.05
98	Treatment*Time	14Hour	28	16Hour	27	36	0.79	0.4362	0.05
99	Treatment*Time	14Hour	28	16Hour	28	36	0.29	0.7744	0.05
100	Treatment*Time	14Hour	28	18Hour	4	36	5.11	<.0001	0.05
101	Treatment*Time	14Hour	28	18Hour	8	36	3.69	0.0007	0.05
102	Treatment*Time	14Hour	28	18Hour	12	36	2.92	0.0060	0.05
103	Treatment*Time	14Hour	28	18Hour	26	36	2.99	0.0050	0.05
104	Treatment*Time	14Hour	28	18Hour	27	36	1.78	0.0833	0.05
105	Treatment*Time	14Hour	28	18Hour	28	36	1.45	0.1554	0.05
106	Treatment*Time	16Hour	4	16Hour	8	36	-0.40	0.6945	0.05
107	Treatment*Time	16Hour	4	16Hour	12	36	-2.60	0.0134	0.05
108	Treatment*Time	16Hour	4	16Hour	26	36	-2.98	0.0051	0.05
109	Treatment*Time	16Hour	4	16Hour	27	36	-3.06	0.0042	0.05
110	Treatment*Time	16Hour	4	16Hour	28	36	-3.56	0.0011	0.05
111	Treatment*Time	16Hour	4	18Hour	4	36	1.26	0.2151	0.05
112	Treatment*Time	16Hour	4	18Hour	8	36	-0.15	0.8781	0.05
113	Treatment*Time	16Hour	4	18Hour	12	36	-0.93	0.3586	0.05
114	Treatment*Time	16Hour	4	18Hour	26	36	-0.86	0.3982	0.05
115	Treatment*Time	16Hour	4	18Hour	27	36	-2.07	0.0461	0.05
116	Treatment*Time	16Hour	4	18Hour	28	36	-2.40	0.0219	0.05
117	Treatment*Time	16Hour	8	16Hour	12	36	-2.21	0.0339	0.05
118	Treatment*Time	16Hour	8	16Hour	26	36	-2.59	0.0139	0.05

119	Treatment*Time	16Hour	8	16Hour	27	36	-2.66	0.0115	0.05
120	Treatment*Time	16Hour	8	16Hour	28	36	-3.16	0.0032	0.05
121	Treatment*Time	16Hour	8	18Hour	4	36	1.66	0.1061	0.05
122	Treatment*Time	16Hour	8	18Hour	8	36	0.24	0.8107	0.05
123	Treatment*Time	16Hour	8	18Hour	12	36	-0.53	0.5966	0.05
124	Treatment*Time	16Hour	8	18Hour	26	36	-0.46	0.6489	0.05
125	Treatment*Time	16Hour	8	18Hour	27	36	-1.67	0.1035	0.05
126	Treatment*Time	16Hour	8	18Hour	28	36	-2.00	0.0531	0.05
127	Treatment*Time	16Hour	12	16Hour	26	36	-0.38	0.7058	0.05
128	Treatment*Time	16Hour	12	16Hour	27	36	-0.46	0.6496	0.05
129	Treatment*Time	16Hour	12	16Hour	28	36	-0.96	0.3451	0.05
130	Treatment*Time	16Hour	12	18Hour	4	36	3.86	0.0004	0.05
131	Treatment*Time	16Hour	12	18Hour	8	36	2.45	0.0194	0.05
132	Treatment*Time	16Hour	12	18Hour	12	36	1.67	0.1032	0.05
133	Treatment*Time	16Hour	12	18Hour	26	36	1.75	0.0892	0.05
134	Treatment*Time	16Hour	12	18Hour	27	36	0.54	0.5955	0.05
135	Treatment*Time	16Hour	12	18Hour	28	36	0.21	0.8381	0.05
136	Treatment*Time	16Hour	26	16Hour	27	36	-0.08	0.9386	0.05
137	Treatment*Time	16Hour	26	16Hour	28	36	-0.58	0.5682	0.05
138	Treatment*Time	16Hour	26	18Hour	4	36	4.24	0.0001	0.05
139	Treatment*Time	16Hour	26	18Hour	8	36	2.83	0.0076	0.05
140	Treatment*Time	16Hour	26	18Hour	12	36	2.05	0.0474	0.05
141	Treatment*Time	16Hour	26	18Hour	26	36	2.13	0.0403	0.05
142	Treatment*Time	16Hour	26	18Hour	27	36	0.92	0.3657	0.05
143	Treatment*Time	16Hour	26	18Hour	28	36	0.59	0.5613	0.05
144	Treatment*Time	16Hour	27	16Hour	28	36	-0.50	0.6212	0.05
145		16Hour	27	18Hour	4	36	4.32	0.0001	0.05

	Treatment*Time								
146	Treatment*Time	16Hour	27	18Hour	8	36	2.91	0.0062	0.05
147	Treatment*Time	16Hour	27	18Hour	12	36	2.13	0.0401	0.05
148	Treatment*Time	16Hour	27	18Hour	26	36	2.20	0.0339	0.05
149	Treatment*Time	16Hour	27	18Hour	27	36	0.99	0.3270	0.05
150	Treatment*Time	16Hour	27	18Hour	28	36	0.66	0.5110	0.05
151	Treatment*Time	16Hour	28	18Hour	4	36	4.82	<.0001	0.05
152	Treatment*Time	16Hour	28	18Hour	8	36	3.40	0.0016	0.05
153	Treatment*Time	16Hour	28	18Hour	12	36	2.63	0.0125	0.05
154	Treatment*Time	16Hour	28	18Hour	26	36	2.70	0.0104	0.05
155	Treatment*Time	16Hour	28	18Hour	27	36	1.49	0.1443	0.05
156	Treatment*Time	16Hour	28	18Hour	28	36	1.16	0.2527	0.05
157	Treatment*Time	18Hour	4	18Hour	8	36	-1.42	0.1653	0.05
158	Treatment*Time	18Hour	4	18Hour	12	36	-2.19	0.0350	0.05
159	Treatment*Time	18Hour	4	18Hour	26	36	-2.12	0.0412	0.05
160	Treatment*Time	18Hour	4	18Hour	27	36	-3.33	0.0020	0.05
161	Treatment*Time	18Hour	4	18Hour	28	36	-3.66	0.0008	0.05
162	Treatment*Time	18Hour	8	18Hour	12	36	-0.78	0.4432	0.05
163	Treatment*Time	18Hour	8	18Hour	26	36	-0.70	0.4881	0.05
164	Treatment*Time	18Hour	8	18Hour	27	36	-1.91	0.0639	0.05
165	Treatment*Time	18Hour	8	18Hour	28	36	-2.24	0.0313	0.05
166	Treatment*Time	18Hour	12	18Hour	26	36	0.07	0.9408	0.05
167	Treatment*Time	18Hour	12	18Hour	27	36	-1.14	0.2634	0.05
168	Treatment*Time	18Hour	12	18Hour	28	36	-1.47	0.1513	0.05
169	Treatment*Time	18Hour	26	18Hour	27	36	-1.21	0.2338	0.05
170	Treatment*Time	18Hour	26	18Hour	28	36	-1.54	0.1321	0.05
171	Treatment*Time	18Hour	27	18Hour	28	36	-0.33	0.7435	0.05

Table Vc. SAS outputs for fed-batch culture on selection of best time point for beginning feeding

Model Information	
Data Set	WORK.ALL
Dependent Variable	**PHBconc**
Covariance Structure	Variance Components
Subject Effect	rep(Treatment)
Group Effect	Time
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
Treatment	3	14Hour 16Hour 18Hour
Time	7	0 4 8 12 26 27 28

Mixed analysis PHB

Pairwise Mean differences

Obs	Effect	Treatment	Time	Treatment	Time	DF	tValue	Probt	Alpha
1	Treatment	14Hour	_	16Hour	_	42	-12.62	<.0001	0.05
2	Treatment	14Hour	_	18Hour	_	42	-3.64	0.0007	0.05
3	Treatment	16Hour	_	18Hour	_	42	8.98	<.0001	0.05
4	Time		0		4	42	-3.00	0.0045	0.05
5	Time		0		8	42	-7.22	<.0001	0.05
6	Time		0		12	42	-6.18	<.0001	0.05
7	Time		0		26	42	-40.57	<.0001	0.05
8	Time		0		27	42	-29.68	<.0001	0.05
9	Time		0		28	42	-22.77	<.0001	0.05
10	Time		4		8	42	-5.99	<.0001	0.05
11	Time		4		12	42	-5.44	<.0001	0.05
12	Time		4		26	42	-36.64	<.0001	0.05

13	Time		4		27	42	-27.64	<.0001	0.05
14	Time		4		28	42	-21.65	<.0001	0.05
15	Time		8		12	42	-1.33	0.1901	0.05
16	Time		8		26	42	-16.53	<.0001	0.05
17	Time		8		27	42	-15.07	<.0001	0.05
18	Time		8		28	42	-14.13	<.0001	0.05
19	Time		12		26	42	-10.04	<.0001	0.05
20	Time		12		27	42	-9.83	<.0001	0.05
21	Time		12		28	42	-10.23	<.0001	0.05
22	Time		26		27	42	-0.76	0.4488	0.05
23	Time		26		28	42	-2.80	0.0077	0.05
24	Time		27		28	42	-2.04	0.0479	0.05
25	Treatment*Time	14Hour	0	14Hour	4	42	-1.95	0.0582	0.05
26	Treatment*Time	14Hour	0	14Hour	8	42	-1.96	0.0570	0.05
27	Treatment*Time	14Hour	0	14Hour	12	42	-1.12	0.2693	0.05
28	Treatment*Time	14Hour	0	14Hour	26	42	-17.51	<.0001	0.05
29	Treatment*Time	14Hour	0	14Hour	27	42	-10.97	<.0001	0.05
30	Treatment*Time	14Hour	0	14Hour	28	42	-10.57	<.0001	0.05
31	Treatment*Time	14Hour	0	16Hour	0	42	1.73	0.0906	0.05
32	Treatment*Time	14Hour	0	16Hour	4	42	0.65	0.5198	0.05
33	Treatment*Time	14Hour	0	16Hour	8	42	-6.65	<.0001	0.05
34	Treatment*Time	14Hour	0	16Hour	12	42	-7.26	<.0001	0.05
35	Treatment*Time	14Hour	0	16Hour	26	42	-32.13	<.0001	0.05
36	Treatment*Time	14Hour	0	16Hour	27	42	-25.94	<.0001	0.05
37	Treatment*Time	14Hour	0	16Hour	28	42	-17.40	<.0001	0.05
38	Treatment*Time	14Hour	0	18Hour	0	42	-2.60	0.0129	0.05
39	Treatment*Time	14Hour	0	18Hour	4	42	-4.54	<.0001	0.05
40	Treatment*Time	14Hour	0	18Hour	8	42	-4.12	0.0002	0.05
41	Treatment*Time	14Hour	0	18Hour	12	42	-2.47	0.0175	0.05
42	Treatment*Time	14Hour	0	18Hour	26	42	-20.97	<.0001	0.05
43	Treatment*Time	14Hour	0	18Hour	27	42	-14.75	<.0001	0.05
44	Treatment*Time	14Hour	0	18Hour	28	42	-11.65	<.0001	0.05
45	Treatment*Time	14Hour	4	14Hour	8	42	-1.23	0.2246	0.05
46	Treatment*Time	14Hour	4	14Hour	12	42	-0.67	0.5065	0.05
47	Treatment*Time	14Hour	4	14Hour	26	42	-15.48	<.0001	0.05
48	Treatment*Time	14Hour	4	14Hour	27	42	-9.90	<.0001	0.05
49	Treatment*Time	14Hour	4	14Hour	28	42	-9.90	<.0001	0.05
50	Treatment*Time	14Hour	4	16Hour	0	42	3.25	0.0023	0.05
51	Treatment*Time	14Hour	4	16Hour	4	42	2.17	0.0361	0.05
52	Treatment*Time	14Hour	4	16Hour	8	42	-5.80	<.0001	0.05
53	Treatment*Time	14Hour	4	16Hour	12	42	-6.74	<.0001	0.05

54	Treatment*Time	14Hour	4	16Hour	26	42	-29.24	<.0001	0.05
55	Treatment*Time	14Hour	4	16Hour	27	42	-24.41	<.0001	0.05
56	Treatment*Time	14Hour	4	16Hour	28	42	-16.63	<.0001	0.05
57	Treatment*Time	14Hour	4	18Hour	0	42	-0.00	1.0000	0.05
58	Treatment*Time	14Hour	4	18Hour	4	42	-2.17	0.0361	0.05
59	Treatment*Time	14Hour	4	18Hour	8	42	-3.34	0.0018	0.05
60	Treatment*Time	14Hour	4	18Hour	12	42	-2.01	0.0509	0.05
61	Treatment*Time	14Hour	4	18Hour	26	42	-18.74	<.0001	0.05
62	Treatment*Time	14Hour	4	18Hour	27	42	-13.56	<.0001	0.05
63	Treatment*Time	14Hour	4	18Hour	28	42	-10.97	<.0001	0.05
64	Treatment*Time	14Hour	8	14Hour	12	42	0.11	0.9107	0.05
65	Treatment*Time	14Hour	8	14Hour	26	42	-8.13	<.0001	0.05
66	Treatment*Time	14Hour	8	14Hour	27	42	-6.10	<.0001	0.05
67	Treatment*Time	14Hour	8	14Hour	28	42	-7.40	<.0001	0.05
68	Treatment*Time	14Hour	8	16Hour	0	42	2.42	0.0201	0.05
69	Treatment*Time	14Hour	8	16Hour	4	42	2.13	0.0392	0.05
70	Treatment*Time	14Hour	8	16Hour	8	42	-3.38	0.0016	0.05
71	Treatment*Time	14Hour	8	16Hour	12	42	-5.11	<.0001	0.05
72	Treatment*Time	14Hour	8	16Hour	26	42	-16.32	<.0001	0.05
73	Treatment*Time	14Hour	8	16Hour	27	42	-16.43	<.0001	0.05
74	Treatment*Time	14Hour	8	16Hour	28	42	-12.94	<.0001	0.05
75	Treatment*Time	14Hour	8	18Hour	0	42	1.27	0.2124	0.05
76	Treatment*Time	14Hour	8	18Hour	4	42	0.34	0.7384	0.05
77	Treatment*Time	14Hour	8	18Hour	8	42	-1.56	0.1268	0.05
78	Treatment*Time	14Hour	8	18Hour	12	42	-1.04	0.3040	0.05
79	Treatment*Time	14Hour	8	18Hour	26	42	-10.07	<.0001	0.05
80	Treatment*Time	14Hour	8	18Hour	27	42	-8.71	<.0001	0.05
81	Treatment*Time	14Hour	8	18Hour	28	42	-8.28	<.0001	0.05
82	Treatment*Time	14Hour	12	14Hour	26	42	-5.78	<.0001	0.05
83	Treatment*Time	14Hour	12	14Hour	27	42	-4.65	<.0001	0.05
84	Treatment*Time	14Hour	12	14Hour	28	42	-6.09	<.0001	0.05
85	Treatment*Time	14Hour	12	16Hour	0	42	1.41	0.1647	0.05
86	Treatment*Time	14Hour	12	16Hour	4	42	1.25	0.2172	0.05
87	Treatment*Time	14Hour	12	16Hour	8	42	-2.67	0.0107	0.05
88	Treatment*Time	14Hour	12	16Hour	12	42	-4.38	<.0001	0.05
89	Treatment*Time	14Hour	12	16Hour	26	42	-11.48	<.0001	0.05
90	Treatment*Time	14Hour	12	16Hour	27	42	-12.32	<.0001	0.05
91	Treatment*Time	14Hour	12	16Hour	28	42	-10.57	<.0001	0.05
92	Treatment*Time	14Hour	12	18Hour	0	42	0.68	0.5018	0.05
93	Treatment*Time	14Hour	12	18Hour	4	42	0.09	0.9308	0.05
94	Treatment*Time	14Hour	12	18Hour	8	42	-1.29	0.2037	0.05

95	Treatment*Time	14Hour	12	18Hour	12	42	-0.97	0.3400	0.05
96	Treatment*Time	14Hour	12	18Hour	26	42	-7.13	<.0001	0.05
97	Treatment*Time	14Hour	12	18Hour	27	42	-6.58	<.0001	0.05
98	Treatment*Time	14Hour	12	18Hour	28	42	-6.80	<.0001	0.05
99	Treatment*Time	14Hour	26	14Hour	27	42	1.22	0.2296	0.05
100	Treatment*Time	14Hour	26	14Hour	28	42	-1.88	0.0668	0.05
101	Treatment*Time	14Hour	26	16Hour	0	42	18.22	<.0001	0.05
102	Treatment*Time	14Hour	26	16Hour	4	42	16.81	<.0001	0.05
103	Treatment*Time	14Hour	26	16Hour	8	42	4.09	0.0002	0.05
104	Treatment*Time	14Hour	26	16Hour	12	42	0.04	0.9673	0.05
105	Treatment*Time	14Hour	26	16Hour	26	42	-10.80	<.0001	0.05
106	Treatment*Time	14Hour	26	16Hour	27	42	-11.35	<.0001	0.05
107	Treatment*Time	14Hour	26	16Hour	28	42	-8.12	<.0001	0.05
108	Treatment*Time	14Hour	26	18Hour	0	42	16.46	<.0001	0.05
109	Treatment*Time	14Hour	26	18Hour	4	42	14.15	<.0001	0.05
110	Treatment*Time	14Hour	26	18Hour	8	42	6.27	<.0001	0.05
111	Treatment*Time	14Hour	26	18Hour	12	42	4.51	<.0001	0.05
112	Treatment*Time	14Hour	26	18Hour	26	42	-2.56	0.0143	0.05
113	Treatment*Time	14Hour	26	18Hour	27	42	-1.95	0.0573	0.05
114	Treatment*Time	14Hour	26	18Hour	28	42	-2.87	0.0064	0.05
115	Treatment*Time	14Hour	27	14Hour	28	42	-2.55	0.0144	0.05
116	Treatment*Time	14Hour	27	16Hour	0	42	11.47	<.0001	0.05
117	Treatment*Time	14Hour	27	16Hour	4	42	10.87	<.0001	0.05
118	Treatment*Time	14Hour	27	16Hour	8	42	2.57	0.0136	0.05
119	Treatment*Time	14Hour	27	16Hour	12	42	-0.71	0.4841	0.05
120	Treatment*Time	14Hour	27	16Hour	26	42	-9.92	<.0001	0.05
121	Treatment*Time	14Hour	27	16Hour	27	42	-10.82	<.0001	0.05
122	Treatment*Time	14Hour	27	16Hour	28	42	-8.26	<.0001	0.05
123	Treatment*Time	14Hour	27	18Hour	0	42	10.22	<.0001	0.05
124	Treatment*Time	14Hour	27	18Hour	4	42	8.93	<.0001	0.05
125	Treatment*Time	14Hour	27	18Hour	8	42	4.48	<.0001	0.05
126	Treatment*Time	14Hour	27	18Hour	12	42	3.47	0.0012	0.05
127	Treatment*Time	14Hour	27	18Hour	26	42	-3.28	0.0021	0.05
128	Treatment*Time	14Hour	27	18Hour	27	42	-2.73	0.0092	0.05
129	Treatment*Time	14Hour	27	18Hour	28	42	-3.46	0.0013	0.05
130	Treatment*Time	14Hour	28	16Hour	0	42	10.91	<.0001	0.05
131	Treatment*Time	14Hour	28	16Hour	4	42	10.58	<.0001	0.05
132	Treatment*Time	14Hour	28	16Hour	8	42	4.56	<.0001	0.05
133	Treatment*Time	14Hour	28	16Hour	12	42	1.39	0.1730	0.05
134	Treatment*Time	14Hour	28	16Hour	26	42	-4.61	<.0001	0.05
135	Treatment*Time	14Hour	28	16Hour	27	42	-6.02	<.0001	0.05

136	Treatment*Time	14Hour	28	16Hour	28	42	-4.88	<.0001	0.05
137	Treatment*Time	14Hour	28	18Hour	0	42	10.05	<.0001	0.05
138	Treatment*Time	14Hour	28	18Hour	4	42	9.23	<.0001	0.05
139	Treatment*Time	14Hour	28	18Hour	8	42	6.09	<.0001	0.05
140	Treatment*Time	14Hour	28	18Hour	12	42	5.05	<.0001	0.05
141	Treatment*Time	14Hour	28	18Hour	26	42	0.34	0.7318	0.05
142	Treatment*Time	14Hour	28	18Hour	27	42	0.39	0.7005	0.05
143	Treatment*Time	14Hour	28	18Hour	28	42	-0.77	0.4445	0.05
144	Treatment*Time	16Hour	0	16Hour	4	42	-0.65	0.5198	0.05
145	Treatment*Time	16Hour	0	16Hour	8	42	-7.11	<.0001	0.05
146	Treatment*Time	16Hour	0	16Hour	12	42	-7.56	<.0001	0.05
147	Treatment*Time	16Hour	0	16Hour	26	42	-32.84	<.0001	0.05
148	Treatment*Time	16Hour	0	16Hour	27	42	-26.44	<.0001	0.05
149	Treatment*Time	16Hour	0	16Hour	28	42	-17.74	<.0001	0.05
150	Treatment*Time	16Hour	0	18Hour	0	42	-4.33	<.0001	0.05
151	Treatment*Time	16Hour	0	18Hour	4	42	-5.84	<.0001	0.05
152	Treatment*Time	16Hour	0	18Hour	8	42	-4.58	<.0001	0.05
153	Treatment*Time	16Hour	0	18Hour	12	42	-2.77	0.0083	0.05
154	Treatment*Time	16Hour	0	18Hour	26	42	-21.68	<.0001	0.05
155	Treatment*Time	16Hour	0	18Hour	27	42	-15.25	<.0001	0.05
156	Treatment*Time	16Hour	0	18Hour	28	42	-11.99	<.0001	0.05
157	Treatment*Time	16Hour	4	16Hour	8	42	-6.70	<.0001	0.05
158	Treatment*Time	16Hour	4	16Hour	12	42	-7.33	<.0001	0.05
159	Treatment*Time	16Hour	4	16Hour	26	42	-30.56	<.0001	0.05
160	Treatment*Time	16Hour	4	16Hour	27	42	-25.38	<.0001	0.05
161	Treatment*Time	16Hour	4	16Hour	28	42	-17.30	<.0001	0.05
162	Treatment*Time	16Hour	4	18Hour	0	42	-2.60	0.0129	0.05
163	Treatment*Time	16Hour	4	18Hour	4	42	-4.33	<.0001	0.05
164	Treatment*Time	16Hour	4	18Hour	8	42	-4.24	0.0001	0.05
165	Treatment*Time	16Hour	4	18Hour	12	42	-2.59	0.0130	0.05
166	Treatment*Time	16Hour	4	18Hour	26	42	-20.07	<.0001	0.05
167	Treatment*Time	16Hour	4	18Hour	27	42	-14.53	<.0001	0.05
168	Treatment*Time	16Hour	4	18Hour	28	42	-11.64	<.0001	0.05
169	Treatment*Time	16Hour	8	16Hour	12	42	-2.56	0.0142	0.05
170	Treatment*Time	16Hour	8	16Hour	26	42	-12.28	<.0001	0.05
171	Treatment*Time	16Hour	8	16Hour	27	42	-12.91	<.0001	0.05
172	Treatment*Time	16Hour	8	16Hour	28	42	-10.10	<.0001	0.05
173	Treatment*Time	16Hour	8	18Hour	0	42	5.96	<.0001	0.05
174	Treatment*Time	16Hour	8	18Hour	4	42	4.91	<.0001	0.05
175	Treatment*Time	16Hour	8	18Hour	8	42	1.82	0.0754	0.05
176	Treatment*Time	16Hour	8	18Hour	12	42	1.52	0.1368	0.05

177	Treatment*Time	16Hour	8	18Hour	26	42	-6.03	<.0001	0.05
178	Treatment*Time	16Hour	8	18Hour	27	42	-5.18	<.0001	0.05
179	Treatment*Time	16Hour	8	18Hour	28	42	-5.43	<.0001	0.05
180	Treatment*Time	16Hour	12	16Hour	26	42	-5.74	<.0001	0.05
181	Treatment*Time	16Hour	12	16Hour	27	42	-6.97	<.0001	0.05
182	Treatment*Time	16Hour	12	16Hour	28	42	-5.87	<.0001	0.05
183	Treatment*Time	16Hour	12	18Hour	0	42	6.82	<.0001	0.05
184	Treatment*Time	16Hour	12	18Hour	4	42	6.16	<.0001	0.05
185	Treatment*Time	16Hour	12	18Hour	8	42	3.94	0.0003	0.05
186	Treatment*Time	16Hour	12	18Hour	12	42	3.41	0.0014	0.05
187	Treatment*Time	16Hour	12	18Hour	26	42	-1.39	0.1718	0.05
188	Treatment*Time	16Hour	12	18Hour	27	42	-1.23	0.2248	0.05
189	Treatment*Time	16Hour	12	18Hour	28	42	-2.10	0.0421	0.05
190	Treatment*Time	16Hour	26	16Hour	27	42	-2.65	0.0113	0.05
191	Treatment*Time	16Hour	26	16Hour	28	42	-1.63	0.1104	0.05
192	Treatment*Time	16Hour	26	18Hour	0	42	31.07	<.0001	0.05
193	Treatment*Time	16Hour	26	18Hour	4	42	27.91	<.0001	0.05
194	Treatment*Time	16Hour	26	18Hour	8	42	14.46	<.0001	0.05
195	Treatment*Time	16Hour	26	18Hour	12	42	10.21	<.0001	0.05
196	Treatment*Time	16Hour	26	18Hour	26	42	8.24	<.0001	0.05
197	Treatment*Time	16Hour	26	18Hour	27	42	6.75	<.0001	0.05
198	Treatment*Time	16Hour	26	18Hour	28	42	3.62	0.0008	0.05
199	Treatment*Time	16Hour	27	16Hour	28	42	0.32	0.7539	0.05
200	Treatment*Time	16Hour	27	18Hour	0	42	25.19	<.0001	0.05
201	Treatment*Time	16Hour	27	18Hour	4	42	23.44	<.0001	0.05
202	Treatment*Time	16Hour	27	18Hour	8	42	14.81	<.0001	0.05
203	Treatment*Time	16Hour	27	18Hour	12	42	11.14	<.0001	0.05
204	Treatment*Time	16Hour	27	18Hour	26	42	9.29	<.0001	0.05
205	Treatment*Time	16Hour	27	18Hour	27	42	8.09	<.0001	0.05
206	Treatment*Time	16Hour	27	18Hour	28	42	5.12	<.0001	0.05
207	Treatment*Time	16Hour	28	18Hour	0	42	16.88	<.0001	0.05
208	Treatment*Time	16Hour	28	18Hour	4	42	15.95	<.0001	0.05
209	Treatment*Time	16Hour	28	18Hour	8	42	11.63	<.0001	0.05
210	Treatment*Time	16Hour	28	18Hour	12	42	9.53	<.0001	0.05
211	Treatment*Time	16Hour	28	18Hour	26	42	6.59	<.0001	0.05
212	Treatment*Time	16Hour	28	18Hour	27	42	6.10	<.0001	0.05
213	Treatment*Time	16Hour	28	18Hour	28	42	4.10	0.0002	0.05
214	Treatment*Time	18Hour	0	18Hour	4	42	-2.60	0.0129	0.05
215	Treatment*Time	18Hour	0	18Hour	8	42	-3.43	0.0014	0.05
216	Treatment*Time	18Hour	0	18Hour	12	42	-2.03	0.0484	0.05
217	Treatment*Time	18Hour	0	18Hour	26	42	-19.92	<.0001	0.05

218	Treatment*Time	18Hour	0	18Hour	27	42	-14.00	<.0001	0.05
219	Treatment*Time	18Hour	0	18Hour	28	42	-11.13	<.0001	0.05
220	Treatment*Time	18Hour	4	18Hour	8	42	-2.44	0.0189	0.05
221	Treatment*Time	18Hour	4	18Hour	12	42	-1.43	0.1608	0.05
222	Treatment*Time	18Hour	4	18Hour	26	42	-17.41	<.0001	0.05
223	Treatment*Time	18Hour	4	18Hour	27	42	-12.59	<.0001	0.05
224	Treatment*Time	18Hour	4	18Hour	28	42	-10.29	<.0001	0.05
225	Treatment*Time	18Hour	8	18Hour	12	42	0.14	0.8910	0.05
226	Treatment*Time	18Hour	8	18Hour	26	42	-8.21	<.0001	0.05
227	Treatment*Time	18Hour	8	18Hour	27	42	-7.09	<.0001	0.05
228	Treatment*Time	18Hour	8	18Hour	28	42	-6.97	<.0001	0.05
229	Treatment*Time	18Hour	12	18Hour	26	42	-5.86	<.0001	0.05
230	Treatment*Time	18Hour	12	18Hour	27	42	-5.40	<.0001	0.05
231	Treatment*Time	18Hour	12	18Hour	28	42	-5.76	<.0001	0.05
232	Treatment*Time	18Hour	26	18Hour	27	42	0.11	0.9168	0.05
233	Treatment*Time	18Hour	26	18Hour	28	42	-1.33	0.1897	0.05
234	Treatment*Time	18Hour	27	18Hour	28	42	-1.29	0.2038	0.05

Model Information	
Data Set	WORK.ALL
Dependent Variable	**PHBcontent**
Covariance Structure	Variance Components
Subject Effect	rep(Treatment)
Group Effect	Time
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Between-Within

Class Level Information

Class	Levels	Values
Treatment	3	14Hour 16Hour 18Hour
Time	7	0 4 8 12 26 27 28

Mixed analysis PHB

Pairwise Mean differences

Obs	Effect	Treatment	Time	_Treatment	_Time	DF	tValue	Probt	Alpha
1	Treatment	14Hour	_	16Hour	_	42	-3.70	0.0006	0.05
2	Treatment	14Hour	_	18Hour	_	42	-1.54	0.1314	0.05
3	Treatment	16Hour	_	18Hour	_	42	2.16	0.0363	0.05
4	Time		0		4	42	-2.32	0.0251	0.05
5	Time		0		8	42	-6.73	<.0001	0.05
6	Time		0		12	42	-5.03	<.0001	0.05
7	Time		0		26	42	-23.39	<.0001	0.05
8	Time		0		27	42	-11.99	<.0001	0.05
9	Time		0		28	42	-13.99	<.0001	0.05
10	Time		4		8	42	-5.67	<.0001	0.05
11	Time		4		12	42	-4.49	<.0001	0.05
12	Time		4		26	42	-19.66	<.0001	0.05
13	Time		4		27	42	-10.64	<.0001	0.05
14	Time		4		28	42	-12.40	<.0001	0.05
15	Time		8		12	42	-1.19	0.2414	0.05
16	Time		8		26	42	-4.44	<.0001	0.05
17	Time		8		27	42	-3.06	0.0039	0.05
18	Time		8		28	42	-3.66	0.0007	0.05
19	Time		12		26	42	-1.22	0.2275	0.05
20	Time		12		27	42	-0.76	0.4504	0.05
21	Time		12		28	42	-1.07	0.2919	0.05
22	Time		26		27	42	0.78	0.4387	0.05
23	Time		26		28	42	0.20	0.8405	0.05
24	Time		27		28	42	-0.50	0.6224	0.05
25	Treatment*Time	14Hour	0	14Hour	4	42	-1.12	0.2671	0.05
26	Treatment*Time	14Hour	0	14Hour	8	42	-2.18	0.0352	0.05
27	Treatment*Time	14Hour	0	14Hour	12	42	-1.09	0.2819	0.05
28	Treatment*Time	14Hour	0	14Hour	26	42	-12.06	<.0001	0.05
29	Treatment*Time	14Hour	0	14Hour	27	42	-5.05	<.0001	0.05
30	Treatment*Time	14Hour	0	14Hour	28	42	-7.06	<.0001	0.05
31	Treatment*Time	14Hour	0	16Hour	0	42	3.78	0.0005	0.05
32	Treatment*Time	14Hour	0	16Hour	4	42	2.05	0.0469	0.05
33	Treatment*Time	14Hour	0	16Hour	8	42	-4.96	<.0001	0.05
34	Treatment*Time	14Hour	0	16Hour	12	42	-4.84	<.0001	0.05
35	Treatment*Time	14Hour	0	16Hour	26	42	-14.01	<.0001	0.05
36	Treatment*Time	14Hour	0	16Hour	27	42	-8.46	<.0001	0.05

37	Treatment*Time	14Hour	0	16Hour	28	42	-9.09	<.0001	0.05
38	Treatment*Time	14Hour	0	18Hour	0	42	-0.85	0.3983	0.05
39	Treatment*Time	14Hour	0	18Hour	4	42	-2.48	0.0174	0.05
40	Treatment*Time	14Hour	0	18Hour	8	42	-3.53	0.0010	0.05
41	Treatment*Time	14Hour	0	18Hour	12	42	-2.24	0.0305	0.05
42	Treatment*Time	14Hour	0	18Hour	26	42	-12.42	<.0001	0.05
43	Treatment*Time	14Hour	0	18Hour	27	42	-6.15	<.0001	0.05
44	Treatment*Time	14Hour	0	18Hour	28	42	-6.86	<.0001	0.05
45	Treatment*Time	14Hour	4	14Hour	8	42	-1.69	0.0989	0.05
46	Treatment*Time	14Hour	4	14Hour	12	42	-0.84	0.4070	0.05
47	Treatment*Time	14Hour	4	14Hour	26	42	-10.19	<.0001	0.05
48	Treatment*Time	14Hour	4	14Hour	27	42	-4.42	<.0001	0.05
49	Treatment*Time	14Hour	4	14Hour	28	42	-6.28	<.0001	0.05
50	Treatment*Time	14Hour	4	16Hour	0	42	4.31	<.0001	0.05
51	Treatment*Time	14Hour	4	16Hour	4	42	2.79	0.0078	0.05
52	Treatment*Time	14Hour	4	16Hour	8	42	-4.41	<.0001	0.05
53	Treatment*Time	14Hour	4	16Hour	12	42	-4.56	<.0001	0.05
54	Treatment*Time	14Hour	4	16Hour	26	42	-11.97	<.0001	0.05
55	Treatment*Time	14Hour	4	16Hour	27	42	-7.74	<.0001	0.05
56	Treatment*Time	14Hour	4	16Hour	28	42	-8.24	<.0001	0.05
57	Treatment*Time	14Hour	4	18Hour	0	42	0.41	0.6868	0.05
58	Treatment*Time	14Hour	4	18Hour	4	42	-1.19	0.2406	0.05
59	Treatment*Time	14Hour	4	18Hour	8	42	-3.01	0.0044	0.05
60	Treatment*Time	14Hour	4	18Hour	12	42	-1.98	0.0544	0.05
61	Treatment*Time	14Hour	4	18Hour	26	42	-10.53	<.0001	0.05
62	Treatment*Time	14Hour	4	18Hour	27	42	-5.49	<.0001	0.05
63	Treatment*Time	14Hour	4	18Hour	28	42	-6.09	<.0001	0.05
64	Treatment*Time	14Hour	8	14Hour	12	42	0.09	0.9274	0.05
65	Treatment*Time	14Hour	8	14Hour	26	42	-3.51	0.0011	0.05
66	Treatment*Time	14Hour	8	14Hour	27	42	-1.79	0.0800	0.05
67	Treatment*Time	14Hour	8	14Hour	28	42	-2.83	0.0071	0.05
68	Treatment*Time	14Hour	8	16Hour	0	42	3.45	0.0013	0.05
69	Treatment*Time	14Hour	8	16Hour	4	42	2.93	0.0055	0.05
70	Treatment*Time	14Hour	8	16Hour	8	42	-2.03	0.0492	0.05
71	Treatment*Time	14Hour	8	16Hour	12	42	-3.24	0.0024	0.05
72	Treatment*Time	14Hour	8	16Hour	26	42	-4.41	<.0001	0.05
73	Treatment*Time	14Hour	8	16Hour	27	42	-4.14	0.0002	0.05
74	Treatment*Time	14Hour	8	16Hour	28	42	-4.15	0.0002	0.05
75	Treatment*Time	14Hour	8	18Hour	0	42	1.89	0.0658	0.05
76	Treatment*Time	14Hour	8	18Hour	4	42	1.16	0.2531	0.05
77	Treatment*Time	14Hour	8	18Hour	8	42	-0.99	0.3293	0.05

78	Treatment*Time	14Hour	8	18Hour	12	42	-0.93	0.3579	0.05
79	Treatment*Time	14Hour	8	18Hour	26	42	-3.68	0.0007	0.05
80	Treatment*Time	14Hour	8	18Hour	27	42	-2.55	0.0145	0.05
81	Treatment*Time	14Hour	8	18Hour	28	42	-2.70	0.0099	0.05
82	Treatment*Time	14Hour	12	14Hour	26	42	-2.11	0.0412	0.05
83	Treatment*Time	14Hour	12	14Hour	27	42	-1.26	0.2161	0.05
84	Treatment*Time	14Hour	12	14Hour	28	42	-1.88	0.0669	0.05
85	Treatment*Time	14Hour	12	16Hour	0	42	1.79	0.0808	0.05
86	Treatment*Time	14Hour	12	16Hour	4	42	1.53	0.1339	0.05
87	Treatment*Time	14Hour	12	16Hour	8	42	-1.45	0.1552	0.05
88	Treatment*Time	14Hour	12	16Hour	12	42	-2.67	0.0107	0.05
89	Treatment*Time	14Hour	12	16Hour	26	42	-2.62	0.0122	0.05
90	Treatment*Time	14Hour	12	16Hour	27	42	-2.78	0.0082	0.05
91	Treatment*Time	14Hour	12	16Hour	28	42	-2.71	0.0097	0.05
92	Treatment*Time	14Hour	12	18Hour	0	42	0.93	0.3565	0.05
93	Treatment*Time	14Hour	12	18Hour	4	42	0.54	0.5900	0.05
94	Treatment*Time	14Hour	12	18Hour	8	42	-0.75	0.4562	0.05
95	Treatment*Time	14Hour	12	18Hour	12	42	-0.82	0.4170	0.05
96	Treatment*Time	14Hour	12	18Hour	26	42	-2.20	0.0332	0.05
97	Treatment*Time	14Hour	12	18Hour	27	42	-1.75	0.0882	0.05
98	Treatment*Time	14Hour	12	18Hour	28	42	-1.80	0.0792	0.05
99	Treatment*Time	14Hour	26	14Hour	27	42	1.46	0.1526	0.05
100	Treatment*Time	14Hour	26	14Hour	28	42	0.25	0.8065	0.05
101	Treatment*Time	14Hour	26	16Hour	0	42	14.67	<.0001	0.05
102	Treatment*Time	14Hour	26	16Hour	4	42	12.57	<.0001	0.05
103	Treatment*Time	14Hour	26	16Hour	8	42	0.89	0.3797	0.05
104	Treatment*Time	14Hour	26	16Hour	12	42	-1.57	0.1238	0.05
105	Treatment*Time	14Hour	26	16Hour	26	42	-1.58	0.1227	0.05
106	Treatment*Time	14Hour	26	16Hour	27	42	-1.72	0.0935	0.05
107	Treatment*Time	14Hour	26	16Hour	28	42	-1.61	0.1152	0.05
108	Treatment*Time	14Hour	26	18Hour	0	42	11.47	<.0001	0.05
109	Treatment*Time	14Hour	26	18Hour	4	42	9.18	<.0001	0.05
110	Treatment*Time	14Hour	26	18Hour	8	42	2.23	0.0310	0.05
111	Treatment*Time	14Hour	26	18Hour	12	42	0.98	0.3339	0.05
112	Treatment*Time	14Hour	26	18Hour	26	42	-0.29	0.7698	0.05
113	Treatment*Time	14Hour	26	18Hour	27	42	0.44	0.6653	0.05
114	Treatment*Time	14Hour	26	18Hour	28	42	0.43	0.6705	0.05
115	Treatment*Time	14Hour	27	14Hour	28	42	-1.02	0.3115	0.05
116	Treatment*Time	14Hour	27	16Hour	0	42	6.48	<.0001	0.05
117	Treatment*Time	14Hour	27	16Hour	4	42	5.80	<.0001	0.05
118	Treatment*Time	14Hour	27	16Hour	8	42	-0.35	0.7267	0.05

119	Treatment*Time	14Hour	27	16Hour	12	42	-2.16	0.0369	0.05
120	Treatment*Time	14Hour	27	16Hour	26	42	-2.45	0.0186	0.05
121	Treatment*Time	14Hour	27	16Hour	27	42	-2.51	0.0162	0.05
122	Treatment*Time	14Hour	27	16Hour	28	42	-2.44	0.0190	0.05
123	Treatment*Time	14Hour	27	18Hour	0	42	4.73	<.0001	0.05
124	Treatment*Time	14Hour	27	18Hour	4	42	3.83	0.0004	0.05
125	Treatment*Time	14Hour	27	18Hour	8	42	0.75	0.4583	0.05
126	Treatment*Time	14Hour	27	18Hour	12	42	0.21	0.8356	0.05
127	Treatment*Time	14Hour	27	18Hour	26	42	-1.64	0.1080	0.05
128	Treatment*Time	14Hour	27	18Hour	27	42	-0.81	0.4245	0.05
129	Treatment*Time	14Hour	27	18Hour	28	42	-0.89	0.3808	0.05
130	Treatment*Time	14Hour	28	16Hour	0	42	8.64	<.0001	0.05
131	Treatment*Time	14Hour	28	16Hour	4	42	7.80	<.0001	0.05
132	Treatment*Time	14Hour	28	16Hour	8	42	0.59	0.5614	0.05
133	Treatment*Time	14Hour	28	16Hour	12	42	-1.60	0.1182	0.05
134	Treatment*Time	14Hour	28	16Hour	26	42	-1.33	0.1911	0.05
135	Treatment*Time	14Hour	28	16Hour	27	42	-1.62	0.1135	0.05
136	Treatment*Time	14Hour	28	16Hour	28	42	-1.50	0.1409	0.05
137	Treatment*Time	14Hour	28	18Hour	0	42	6.70	<.0001	0.05
138	Treatment*Time	14Hour	28	18Hour	4	42	5.63	<.0001	0.05
139	Treatment*Time	14Hour	28	18Hour	8	42	1.74	0.0897	0.05
140	Treatment*Time	14Hour	28	18Hour	12	42	0.81	0.4202	0.05
141	Treatment*Time	14Hour	28	18Hour	26	42	-0.45	0.6559	0.05
142	Treatment*Time	14Hour	28	18Hour	27	42	0.17	0.8623	0.05
143	Treatment*Time	14Hour	28	18Hour	28	42	0.15	0.8837	0.05
144	Treatment*Time	16Hour	0	16Hour	4	42	-1.14	0.2606	0.05
145	Treatment*Time	16Hour	0	16Hour	8	42	-6.23	<.0001	0.05
146	Treatment*Time	16Hour	0	16Hour	12	42	-5.53	<.0001	0.05
147	Treatment*Time	16Hour	0	16Hour	26	42	-16.61	<.0001	0.05
148	Treatment*Time	16Hour	0	16Hour	27	42	-9.89	<.0001	0.05
149	Treatment*Time	16Hour	0	16Hour	28	42	-10.67	<.0001	0.05
150	Treatment*Time	16Hour	0	18Hour	0	42	-4.64	<.0001	0.05
151	Treatment*Time	16Hour	0	18Hour	4	42	-5.66	<.0001	0.05
152	Treatment*Time	16Hour	0	18Hour	8	42	-4.81	<.0001	0.05
153	Treatment*Time	16Hour	0	18Hour	12	42	-2.94	0.0053	0.05
154	Treatment*Time	16Hour	0	18Hour	26	42	-15.03	<.0001	0.05
155	Treatment*Time	16Hour	0	18Hour	27	42	-7.58	<.0001	0.05
156	Treatment*Time	16Hour	0	18Hour	28	42	-8.45	<.0001	0.05
157	Treatment*Time	16Hour	4	16Hour	8	42	-5.65	<.0001	0.05
158	Treatment*Time	16Hour	4	16Hour	12	42	-5.25	<.0001	0.05
159	Treatment*Time	16Hour	4	16Hour	26	42	-14.35	<.0001	0.05

160	Treatment*Time	16Hour	4	16Hour	27	42	-9.12	<.0001	0.05
161	Treatment*Time	16Hour	4	16Hour	28	42	-9.76	<.0001	0.05
162	Treatment*Time	16Hour	4	18Hour	0	42	-2.77	0.0084	0.05
163	Treatment*Time	16Hour	4	18Hour	4	42	-3.98	0.0003	0.05
164	Treatment*Time	16Hour	4	18Hour	8	42	-4.25	0.0001	0.05
165	Treatment*Time	16Hour	4	18Hour	12	42	-2.67	0.0107	0.05
166	Treatment*Time	16Hour	4	18Hour	26	42	-12.90	<.0001	0.05
167	Treatment*Time	16Hour	4	18Hour	27	42	-6.87	<.0001	0.05
168	Treatment*Time	16Hour	4	18Hour	28	42	-7.61	<.0001	0.05
169	Treatment*Time	16Hour	8	16Hour	12	42	-1.88	0.0670	0.05
170	Treatment*Time	16Hour	8	16Hour	26	42	-1.79	0.0815	0.05
171	Treatment*Time	16Hour	8	16Hour	27	42	-2.00	0.0524	0.05
172	Treatment*Time	16Hour	8	16Hour	28	42	-1.90	0.0639	0.05
173	Treatment*Time	16Hour	8	18Hour	0	42	4.67	<.0001	0.05
174	Treatment*Time	16Hour	8	18Hour	4	42	3.88	0.0004	0.05
175	Treatment*Time	16Hour	8	18Hour	8	42	1.04	0.3047	0.05
176	Treatment*Time	16Hour	8	18Hour	12	42	0.43	0.6723	0.05
177	Treatment*Time	16Hour	8	18Hour	26	42	-1.06	0.2972	0.05
178	Treatment*Time	16Hour	8	18Hour	27	42	-0.40	0.6883	0.05
179	Treatment*Time	16Hour	8	18Hour	28	42	-0.46	0.6506	0.05
180	Treatment*Time	16Hour	12	16Hour	26	42	1.06	0.2961	0.05
181	Treatment*Time	16Hour	12	16Hour	27	42	0.63	0.5290	0.05
182	Treatment*Time	16Hour	12	16Hour	28	42	0.76	0.4488	0.05
183	Treatment*Time	16Hour	12	18Hour	0	42	4.68	<.0001	0.05
184	Treatment*Time	16Hour	12	18Hour	4	42	4.26	0.0001	0.05
185	Treatment*Time	16Hour	12	18Hour	8	42	2.58	0.0136	0.05
186	Treatment*Time	16Hour	12	18Hour	12	42	1.85	0.0712	0.05
187	Treatment*Time	16Hour	12	18Hour	26	42	1.47	0.1478	0.05
188	Treatment*Time	16Hour	12	18Hour	27	42	1.67	0.1031	0.05
189	Treatment*Time	16Hour	12	18Hour	28	42	1.68	0.1010	0.05
190	Treatment*Time	16Hour	26	16Hour	27	42	-0.72	0.4731	0.05
191	Treatment*Time	16Hour	26	16Hour	28	42	-0.53	0.6013	0.05
192	Treatment*Time	16Hour	26	18Hour	0	42	13.42	<.0001	0.05
193	Treatment*Time	16Hour	26	18Hour	4	42	10.96	<.0001	0.05
194	Treatment*Time	16Hour	26	18Hour	8	42	3.13	0.0032	0.05
195	Treatment*Time	16Hour	26	18Hour	12	42	1.49	0.1437	0.05
196	Treatment*Time	16Hour	26	18Hour	26	42	1.28	0.2073	0.05
197	Treatment*Time	16Hour	26	18Hour	27	42	1.43	0.1607	0.05
198	Treatment*Time	16Hour	26	18Hour	28	42	1.51	0.1383	0.05
199	Treatment*Time	16Hour	27	16Hour	28	42	0.20	0.8418	0.05
200	Treatment*Time	16Hour	27	18Hour	0	42	8.14	<.0001	0.05

201	Treatment*Time	16Hour	27	18Hour	4	42	7.15	<.0001	0.05
202	Treatment*Time	16Hour	27	18Hour	8	42	3.10	0.0035	0.05
203	Treatment*Time	16Hour	27	18Hour	12	42	1.73	0.0910	0.05
204	Treatment*Time	16Hour	27	18Hour	26	42	1.53	0.1333	0.05
205	Treatment*Time	16Hour	27	18Hour	27	42	1.70	0.0966	0.05
206	Treatment*Time	16Hour	27	18Hour	28	42	1.76	0.0865	0.05
207	Treatment*Time	16Hour	28	18Hour	0	42	8.73	<.0001	0.05
208	Treatment*Time	16Hour	28	18Hour	4	42	7.59	<.0001	0.05
209	Treatment*Time	16Hour	28	18Hour	8	42	3.05	0.0039	0.05
210	Treatment*Time	16Hour	28	18Hour	12	42	1.64	0.1075	0.05
211	Treatment*Time	16Hour	28	18Hour	26	42	1.41	0.1669	0.05
212	Treatment*Time	16Hour	28	18Hour	27	42	1.59	0.1193	0.05
213	Treatment*Time	16Hour	28	18Hour	28	42	1.65	0.1068	0.05
214	Treatment*Time	18Hour	0	18Hour	4	42	-1.76	0.0860	0.05
215	Treatment*Time	18Hour	0	18Hour	8	42	-3.24	0.0023	0.05
216	Treatment*Time	18Hour	0	18Hour	12	42	-2.08	0.0435	0.05
217	Treatment*Time	18Hour	0	18Hour	26	42	-11.84	<.0001	0.05
218	Treatment*Time	18Hour	0	18Hour	27	42	-5.83	<.0001	0.05
219	Treatment*Time	18Hour	0	18Hour	28	42	-6.50	<.0001	0.05
220	Treatment*Time	18Hour	4	18Hour	8	42	-2.48	0.0171	0.05
221	Treatment*Time	18Hour	4	18Hour	12	42	-1.68	0.0995	0.05
222	Treatment*Time	18Hour	4	18Hour	26	42	-9.52	<.0001	0.05
223	Treatment*Time	18Hour	4	18Hour	27	42	-4.90	<.0001	0.05
224	Treatment*Time	18Hour	4	18Hour	28	42	-5.44	<.0001	0.05
225	Treatment*Time	18Hour	8	18Hour	12	42	-0.27	0.7891	0.05
226	Treatment*Time	18Hour	8	18Hour	26	42	-2.40	0.0209	0.05
227	Treatment*Time	18Hour	8	18Hour	27	42	-1.50	0.1400	0.05
228	Treatment*Time	18Hour	8	18Hour	28	42	-1.61	0.1153	0.05
229	Treatment*Time	18Hour	12	18Hour	26	42	-1.07	0.2892	0.05
230	Treatment*Time	18Hour	12	18Hour	27	42	-0.70	0.4888	0.05
231	Treatment*Time	18Hour	12	18Hour	28	42	-0.73	0.4679	0.05
232	Treatment*Time	18Hour	26	18Hour	27	42	0.62	0.5378	0.05
233	Treatment*Time	18Hour	26	18Hour	28	42	0.63	0.5316	0.05
234	Treatment*Time	18Hour	27	18Hour	28	42	-0.04	0.9717	0.05

Table IVd. SAS outputs for two-stage culture using sugarbeet juice on selection of best nutrient addition strategies

Model Information	
Data Set	WORK.ALL
Dependent Variable	**PHBconc**
Covariance Structure	Variance Components
Subject Effect	rep(Treatment*Time)
Group Effect	Time
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Class Level Information

Class	Levels	Values
Treatment	3	CompleteA NOAddition PartialA
Time	7	0 4 8 12 26 27 28
rep	3	1 2 3

Mixed analysis PHB

Pairwise Mean differences

Obs	Effect	Treatment	Time	_Treatment	_Time	DF	tValue	Probt	Alpha
25	Treatment*Time	CompleteA	0	CompleteA	4	8.37	-1.17	0.2752	0.05
26	Treatment*Time	CompleteA	0	CompleteA	8	7.56	-1.33	0.2207	0.05
27	Treatment*Time	CompleteA	0	CompleteA	12	10.6	-2.99	0.0128	0.05
28	Treatment*Time	CompleteA	0	CompleteA	26	7.41	-5.55	0.0007	0.05
29	Treatment*Time	CompleteA	0	CompleteA	27	6.83	-3.98	0.0056	0.05
30	Treatment*Time	CompleteA	0	CompleteA	28	6.77	-4.52	0.0030	0.05
31	Treatment*Time	CompleteA	0	NOAddition	0	6	0.11	0.9128	0.05
32	Treatment*Time	CompleteA	0	NOAddition	4	8.37	-1.28	0.2353	0.05
33	Treatment*Time	CompleteA	0	NOAddition	8	7.56	-2.74	0.0267	0.05
34	Treatment*Time	CompleteA	0	NOAddition	12	10.6	-7.32	<.0001	0.05

35	Treatment*Time	CompleteA	0	NOAddition	26	7.41	-8.56	<.0001	0.05
36	Treatment*Time	CompleteA	0	NOAddition	27	6.83	-7.11	0.0002	0.05
37	Treatment*Time	CompleteA	0	NOAddition	28	6.77	-6.78	0.0003	0.05
38	Treatment*Time	CompleteA	0	PartialA	0	6	-2.78	0.0320	0.05
39	Treatment*Time	CompleteA	0	PartialA	4	8.37	-3.76	0.0051	0.05
40	Treatment*Time	CompleteA	0	PartialA	8	7.56	-6.18	0.0003	0.05
41	Treatment*Time	CompleteA	0	PartialA	12	10.6	-14.07	<.0001	0.05
42	Treatment*Time	CompleteA	0	PartialA	26	7.41	-9.45	<.0001	0.05
43	Treatment*Time	CompleteA	0	PartialA	27	6.83	-7.45	0.0002	0.05
44	Treatment*Time	CompleteA	0	PartialA	28	6.77	-7.72	0.0001	0.05
45	Treatment*Time	CompleteA	4	CompleteA	8	11.5	-0.31	0.7654	0.05
46	Treatment*Time	CompleteA	4	CompleteA	12	10.4	-0.93	0.3748	0.05
47	Treatment*Time	CompleteA	4	CompleteA	26	11.2	-3.89	0.0024	0.05
48	Treatment*Time	CompleteA	4	CompleteA	27	9.65	-2.92	0.0160	0.05
49	Treatment*Time	CompleteA	4	CompleteA	28	9.42	-3.44	0.0069	0.05
50	Treatment*Time	CompleteA	4	NOAddition	0	8.37	1.23	0.2506	0.05
51	Treatment*Time	CompleteA	4	NOAddition	4	6	-0.09	0.9340	0.05
52	Treatment*Time	CompleteA	4	NOAddition	8	11.5	-1.47	0.1672	0.05
53	Treatment*Time	CompleteA	4	NOAddition	12	10.4	-3.82	0.0031	0.05
54	Treatment*Time	CompleteA	4	NOAddition	26	11.2	-6.43	<.0001	0.05
55	Treatment*Time	CompleteA	4	NOAddition	27	9.65	-5.71	0.0002	0.05
56	Treatment*Time	CompleteA	4	NOAddition	28	9.42	-5.48	0.0003	0.05
57	Treatment*Time	CompleteA	4	PartialA	0	8.37	-0.46	0.6600	0.05
58	Treatment*Time	CompleteA	4	PartialA	4	6	-2.01	0.0909	0.05
59	Treatment*Time	CompleteA	4	PartialA	8	11.5	-4.32	0.0011	0.05
60	Treatment*Time	CompleteA	4	PartialA	12	10.4	-8.34	<.0001	0.05
61	Treatment*Time	CompleteA	4	PartialA	26	11.2	-7.18	<.0001	0.05
62	Treatment*Time	CompleteA	4	PartialA	27	9.65	-6.02	0.0001	0.05
63	Treatment*Time	CompleteA	4	PartialA	28	9.42	-6.33	0.0001	0.05
64	Treatment*Time	CompleteA	8	CompleteA	12	9.12	-0.44	0.6691	0.05
65	Treatment*Time	CompleteA	8	CompleteA	26	12	-3.28	0.0066	0.05
66	Treatment*Time	CompleteA	8	CompleteA	27	10.9	-2.50	0.0295	0.05
67	Treatment*Time	CompleteA	8	CompleteA	28	10.7	-3.01	0.0122	0.05
68	Treatment*Time	CompleteA	8	NOAddition	0	7.56	1.39	0.2041	0.05
69	Treatment*Time	CompleteA	8	NOAddition	4	11.5	0.23	0.8227	0.05
70	Treatment*Time	CompleteA	8	NOAddition	8	6	-1.06	0.3300	0.05
71	Treatment*Time	CompleteA	8	NOAddition	12	9.12	-2.90	0.0174	0.05
72	Treatment*Time	CompleteA	8	NOAddition	26	12	-5.59	0.0001	0.05
73	Treatment*Time	CompleteA	8	NOAddition	27	10.9	-5.12	0.0003	0.05
74	Treatment*Time	CompleteA	8	NOAddition	28	10.7	-4.93	0.0005	0.05
75	Treatment*Time	CompleteA	8	PartialA	0	7.56	-0.01	0.9929	0.05

76	Treatment*Time	CompleteA	8	PartialA	4	11.5	-1.47	0.1672	0.05
77	Treatment*Time	CompleteA	8	PartialA	8	6	-3.64	0.0108	0.05
78	Treatment*Time	CompleteA	8	PartialA	12	9.12	-6.73	<.0001	0.05
79	Treatment*Time	CompleteA	8	PartialA	26	12	-6.27	<.0001	0.05
80	Treatment*Time	CompleteA	8	PartialA	27	10.9	-5.41	0.0002	0.05
81	Treatment*Time	CompleteA	8	PartialA	28	10.7	-5.72	0.0001	0.05
82	Treatment*Time	CompleteA	12	CompleteA	26	8.85	-3.61	0.0058	0.05
83	Treatment*Time	CompleteA	12	CompleteA	27	7.73	-2.55	0.0353	0.05
84	Treatment*Time	CompleteA	12	CompleteA	28	7.61	-3.11	0.0153	0.05
85	Treatment*Time	CompleteA	12	NOAddition	0	10.6	3.08	0.0109	0.05
86	Treatment*Time	CompleteA	12	NOAddition	4	10.4	0.83	0.4277	0.05
87	Treatment*Time	CompleteA	12	NOAddition	8	9.12	-0.88	0.3999	0.05
88	Treatment*Time	CompleteA	12	NOAddition	12	6	-3.71	0.0099	0.05
89	Treatment*Time	CompleteA	12	NOAddition	26	8.85	-6.46	0.0001	0.05
90	Treatment*Time	CompleteA	12	NOAddition	27	7.73	-5.56	0.0006	0.05
91	Treatment*Time	CompleteA	12	NOAddition	28	7.61	-5.31	0.0009	0.05
92	Treatment*Time	CompleteA	12	PartialA	0	10.6	0.76	0.4625	0.05
93	Treatment*Time	CompleteA	12	PartialA	4	10.4	-1.45	0.1774	0.05
94	Treatment*Time	CompleteA	12	PartialA	8	9.12	-4.11	0.0026	0.05
95	Treatment*Time	CompleteA	12	PartialA	12	6	-9.51	<.0001	0.05
96	Treatment*Time	CompleteA	12	PartialA	26	8.85	-7.30	<.0001	0.05
97	Treatment*Time	CompleteA	12	PartialA	27	7.73	-5.90	0.0004	0.05
98	Treatment*Time	CompleteA	12	PartialA	28	7.61	-6.22	0.0003	0.05
99	Treatment*Time	CompleteA	26	CompleteA	27	11.2	0.29	0.7786	0.05
100	Treatment*Time	CompleteA	26	CompleteA	28	11	-0.28	0.7841	0.05
101	Treatment*Time	CompleteA	26	NOAddition	0	7.41	5.60	0.0007	0.05
102	Treatment*Time	CompleteA	26	NOAddition	4	11.2	3.82	0.0028	0.05
103	Treatment*Time	CompleteA	26	NOAddition	8	12	2.25	0.0444	0.05
104	Treatment*Time	CompleteA	26	NOAddition	12	8.85	1.25	0.2417	0.05
105	Treatment*Time	CompleteA	26	NOAddition	26	6	-2.26	0.0649	0.05
106	Treatment*Time	CompleteA	26	NOAddition	27	11.2	-2.28	0.0430	0.05
107	Treatment*Time	CompleteA	26	NOAddition	28	11	-2.16	0.0533	0.05
108	Treatment*Time	CompleteA	26	PartialA	0	7.41	4.26	0.0033	0.05
109	Treatment*Time	CompleteA	26	PartialA	4	11.2	2.17	0.0525	0.05
110	Treatment*Time	CompleteA	26	PartialA	8	12	-0.27	0.7919	0.05
111	Treatment*Time	CompleteA	26	PartialA	12	8.85	-2.43	0.0386	0.05
112	Treatment*Time	CompleteA	26	PartialA	26	6	-2.92	0.0266	0.05
113	Treatment*Time	CompleteA	26	PartialA	27	11.2	-2.56	0.0260	0.05
114	Treatment*Time	CompleteA	26	PartialA	28	11	-2.95	0.0133	0.05
115	Treatment*Time	CompleteA	27	CompleteA	28	12	-0.50	0.6246	0.05
116	Treatment*Time	CompleteA	27	NOAddition	0	6.83	4.03	0.0053	0.05

117	Treatment*Time	CompleteA	27	NOAddition	4	9.65	2.86	0.0177	0.05
118	Treatment*Time	CompleteA	27	NOAddition	8	10.9	1.62	0.1333	0.05
119	Treatment*Time	CompleteA	27	NOAddition	12	7.73	0.66	0.5265	0.05
120	Treatment*Time	CompleteA	27	NOAddition	26	11.2	-2.22	0.0475	0.05
121	Treatment*Time	CompleteA	27	NOAddition	27	6	-2.29	0.0623	0.05
122	Treatment*Time	CompleteA	27	NOAddition	28	12	-2.19	0.0494	0.05
123	Treatment*Time	CompleteA	27	PartialA	0	6.83	2.98	0.0211	0.05
124	Treatment*Time	CompleteA	27	PartialA	4	9.65	1.49	0.1692	0.05
125	Treatment*Time	CompleteA	27	PartialA	8	10.9	-0.52	0.6112	0.05
126	Treatment*Time	CompleteA	27	PartialA	12	7.73	-2.27	0.0536	0.05
127	Treatment*Time	CompleteA	27	PartialA	26	11.2	-2.79	0.0171	0.05
128	Treatment*Time	CompleteA	27	PartialA	27	6	-2.54	0.0443	0.05
129	Treatment*Time	CompleteA	27	PartialA	28	12	-2.89	0.0137	0.05
130	Treatment*Time	CompleteA	28	NOAddition	0	6.77	4.56	0.0029	0.05
131	Treatment*Time	CompleteA	28	NOAddition	4	9.42	3.38	0.0076	0.05
132	Treatment*Time	CompleteA	28	NOAddition	8	10.7	2.15	0.0551	0.05
133	Treatment*Time	CompleteA	28	NOAddition	12	7.61	1.29	0.2334	0.05
134	Treatment*Time	CompleteA	28	NOAddition	26	11	-1.61	0.1362	0.05
135	Treatment*Time	CompleteA	28	NOAddition	27	12	-1.74	0.1078	0.05
136	Treatment*Time	CompleteA	28	NOAddition	28	6	-1.65	0.1496	0.05
137	Treatment*Time	CompleteA	28	PartialA	0	6.77	3.55	0.0099	0.05
138	Treatment*Time	CompleteA	28	PartialA	4	9.42	2.05	0.0691	0.05
139	Treatment*Time	CompleteA	28	PartialA	8	10.7	0.06	0.9520	0.05
140	Treatment*Time	CompleteA	28	PartialA	12	7.61	-1.54	0.1630	0.05
141	Treatment*Time	CompleteA	28	PartialA	26	11	-2.16	0.0533	0.05
142	Treatment*Time	CompleteA	28	PartialA	27	12	-1.98	0.0706	0.05
143	Treatment*Time	CompleteA	28	PartialA	28	6	-2.34	0.0580	0.05
144	Treatment*Time	NOAddition	0	NOAddition	4	8.37	-1.35	0.2138	0.05
145	Treatment*Time	NOAddition	0	NOAddition	8	7.56	-2.80	0.0246	0.05
146	Treatment*Time	NOAddition	0	NOAddition	12	10.6	-7.41	<.0001	0.05
147	Treatment*Time	NOAddition	0	NOAddition	26	7.41	-8.61	<.0001	0.05
148	Treatment*Time	NOAddition	0	NOAddition	27	6.83	-7.15	0.0002	0.05
149	Treatment*Time	NOAddition	0	NOAddition	28	6.77	-6.82	0.0003	0.05
150	Treatment*Time	NOAddition	0	PartialA	0	6	-2.89	0.0276	0.05
151	Treatment*Time	NOAddition	0	PartialA	4	8.37	-3.83	0.0046	0.05
152	Treatment*Time	NOAddition	0	PartialA	8	7.56	-6.23	0.0003	0.05
153	Treatment*Time	NOAddition	0	PartialA	12	10.6	-14.16	<.0001	0.05
154	Treatment*Time	NOAddition	0	PartialA	26	7.41	-9.50	<.0001	0.05
155	Treatment*Time	NOAddition	0	PartialA	27	6.83	-7.49	0.0002	0.05
156	Treatment*Time	NOAddition	0	PartialA	28	6.77	-7.76	0.0001	0.05
157	Treatment*Time	NOAddition	4	NOAddition	8	11.5	-1.40	0.1885	0.05

158	Treatment*Time	NOAddition	4	NOAddition	12	10.4	-3.72	0.0037	0.05
159	Treatment*Time	NOAddition	4	NOAddition	26	11.2	-6.36	<.0001	0.05
160	Treatment*Time	NOAddition	4	NOAddition	27	9.65	-5.65	0.0002	0.05
161	Treatment*Time	NOAddition	4	NOAddition	28	9.42	-5.42	0.0004	0.05
162	Treatment*Time	NOAddition	4	PartialA	0	8.37	-0.34	0.7388	0.05
163	Treatment*Time	NOAddition	4	PartialA	4	6	-1.93	0.1025	0.05
164	Treatment*Time	NOAddition	4	PartialA	8	11.5	-4.25	0.0013	0.05
165	Treatment*Time	NOAddition	4	PartialA	12	10.4	-8.23	<.0001	0.05
166	Treatment*Time	NOAddition	4	PartialA	26	11.2	-7.10	<.0001	0.05
167	Treatment*Time	NOAddition	4	PartialA	27	9.65	-5.96	0.0002	0.05
168	Treatment*Time	NOAddition	4	PartialA	28	9.42	-6.27	0.0001	0.05
169	Treatment*Time	NOAddition	8	NOAddition	12	9.12	-1.58	0.1491	0.05
170	Treatment*Time	NOAddition	8	NOAddition	26	12	-4.56	0.0007	0.05
171	Treatment*Time	NOAddition	8	NOAddition	27	10.9	-4.24	0.0014	0.05
172	Treatment*Time	NOAddition	8	NOAddition	28	10.7	-4.07	0.0020	0.05
173	Treatment*Time	NOAddition	8	PartialA	0	7.56	1.40	0.2014	0.05
174	Treatment*Time	NOAddition	8	PartialA	4	11.5	-0.31	0.7654	0.05
175	Treatment*Time	NOAddition	8	PartialA	8	6	-2.58	0.0416	0.05
176	Treatment*Time	NOAddition	8	PartialA	12	9.12	-5.41	0.0004	0.05
177	Treatment*Time	NOAddition	8	PartialA	26	12	-5.24	0.0002	0.05
178	Treatment*Time	NOAddition	8	PartialA	27	10.9	-4.53	0.0009	0.05
179	Treatment*Time	NOAddition	8	PartialA	28	10.7	-4.86	0.0005	0.05
180	Treatment*Time	NOAddition	12	NOAddition	26	8.85	-4.10	0.0028	0.05
181	Treatment*Time	NOAddition	12	NOAddition	27	7.73	-3.68	0.0066	0.05
182	Treatment*Time	NOAddition	12	NOAddition	28	7.61	-3.49	0.0089	0.05
183	Treatment*Time	NOAddition	12	PartialA	0	10.6	5.09	0.0004	0.05
184	Treatment*Time	NOAddition	12	PartialA	4	10.4	1.45	0.1774	0.05
185	Treatment*Time	NOAddition	12	PartialA	8	9.12	-1.65	0.1322	0.05
186	Treatment*Time	NOAddition	12	PartialA	12	6	-5.79	0.0012	0.05
187	Treatment*Time	NOAddition	12	PartialA	26	8.85	-4.94	0.0008	0.05
188	Treatment*Time	NOAddition	12	PartialA	27	7.73	-4.01	0.0042	0.05
189	Treatment*Time	NOAddition	12	PartialA	28	7.61	-4.40	0.0026	0.05
190	Treatment*Time	NOAddition	26	NOAddition	27	11.2	-0.34	0.7369	0.05
191	Treatment*Time	NOAddition	26	NOAddition	28	11	-0.28	0.7882	0.05
192	Treatment*Time	NOAddition	26	PartialA	0	7.41	7.28	0.0001	0.05
193	Treatment*Time	NOAddition	26	PartialA	4	11.2	4.71	0.0006	0.05
194	Treatment*Time	NOAddition	26	PartialA	8	12	2.04	0.0636	0.05
195	Treatment*Time	NOAddition	26	PartialA	12	8.85	0.42	0.6819	0.05
196	Treatment*Time	NOAddition	26	PartialA	26	6	-0.66	0.5312	0.05
197	Treatment*Time	NOAddition	26	PartialA	27	11.2	-0.63	0.5434	0.05
198	Treatment*Time	NOAddition	26	PartialA	28	11	-1.06	0.3131	0.05

199	Treatment*Time	NOAddition	27	NOAddition	28	12	0.05	0.9577	0.05
200	Treatment*Time	NOAddition	27	PartialA	0	6.83	6.11	0.0005	0.05
201	Treatment*Time	NOAddition	27	PartialA	4	9.65	4.28	0.0017	0.05
202	Treatment*Time	NOAddition	27	PartialA	8	10.9	2.09	0.0604	0.05
203	Treatment*Time	NOAddition	27	PartialA	12	7.73	0.74	0.4796	0.05
204	Treatment*Time	NOAddition	27	PartialA	26	11.2	-0.23	0.8254	0.05
205	Treatment*Time	NOAddition	27	PartialA	27	6	-0.25	0.8101	0.05
206	Treatment*Time	NOAddition	27	PartialA	28	12	-0.65	0.5310	0.05
207	Treatment*Time	NOAddition	28	PartialA	0	6.77	5.81	0.0007	0.05
208	Treatment*Time	NOAddition	28	PartialA	4	9.42	4.09	0.0025	0.05
209	Treatment*Time	NOAddition	28	PartialA	8	10.7	1.98	0.0742	0.05
210	Treatment*Time	NOAddition	28	PartialA	12	7.61	0.65	0.5365	0.05
211	Treatment*Time	NOAddition	28	PartialA	26	11	-0.28	0.7841	0.05
212	Treatment*Time	NOAddition	28	PartialA	27	12	-0.30	0.7690	0.05
213	Treatment*Time	NOAddition	28	PartialA	28	6	-0.69	0.5183	0.05
214	Treatment*Time	PartialA	0	PartialA	4	8.37	-2.14	0.0638	0.05
215	Treatment*Time	PartialA	0	PartialA	8	7.56	-4.83	0.0015	0.05
216	Treatment*Time	PartialA	0	PartialA	12	10.6	-11.85	<.0001	0.05
217	Treatment*Time	PartialA	0	PartialA	26	7.41	-8.17	<.0001	0.05
218	Treatment*Time	PartialA	0	PartialA	27	6.83	-6.45	0.0004	0.05
219	Treatment*Time	PartialA	0	PartialA	28	6.77	-6.75	0.0003	0.05
220	Treatment*Time	PartialA	4	PartialA	8	11.5	-2.54	0.0265	0.05
221	Treatment*Time	PartialA	4	PartialA	12	10.4	-5.96	0.0001	0.05
222	Treatment*Time	PartialA	4	PartialA	26	11.2	-5.45	0.0002	0.05
223	Treatment*Time	PartialA	4	PartialA	27	9.65	-4.59	0.0011	0.05
224	Treatment*Time	PartialA	4	PartialA	28	9.42	-4.94	0.0007	0.05
225	Treatment*Time	PartialA	8	PartialA	12	9.12	-2.18	0.0566	0.05
226	Treatment*Time	PartialA	8	PartialA	26	12	-2.72	0.0185	0.05
227	Treatment*Time	PartialA	8	PartialA	27	10.9	-2.38	0.0365	0.05
228	Treatment*Time	PartialA	8	PartialA	28	10.7	-2.77	0.0185	0.05
229	Treatment*Time	PartialA	12	PartialA	26	8.85	-1.26	0.2389	0.05
230	Treatment*Time	PartialA	12	PartialA	27	7.73	-1.07	0.3150	0.05
231	Treatment*Time	PartialA	12	PartialA	28	7.61	-1.56	0.1600	0.05
232	Treatment*Time	PartialA	26	PartialA	27	11.2	-0.06	0.9560	0.05
233	Treatment*Time	PartialA	26	PartialA	28	11	-0.50	0.6262	0.05
234	Treatment*Time	PartialA	27	PartialA	28	12	-0.40	0.6970	0.05

Model Information	
Data Set	WORK.ALL
Dependent Variable	**PHBcontent**
Covariance Structure	Variance Components
Subject Effect	rep(Treatment*Time)
Group Effect	Time
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Kenward-Roger
Degrees of Freedom Method	Kenward-Roger

Class Level Information

Class	Levels	Values
Treatment	3	CompleteA NOAddition PartialA
rep	3	1 2 3
Time	7	0 4 8 12 26 27 28

Mixed analysis PHB

Pairwise Mean differences

Obs	Effect	Treatment	Time	_Treatment	_Time	DF	tValue	Probt	Alpha
25	Treatment*Time	CompleteA	0	CompleteA	4	8.28	-1.94	0.0866	0.05
26	Treatment*Time	CompleteA	0	CompleteA	8	6.73	-1.12	0.3029	0.05
27	Treatment*Time	CompleteA	0	CompleteA	12	9.24	-2.59	0.0286	0.05
28	Treatment*Time	CompleteA	0	CompleteA	26	7.49	-4.11	0.0039	0.05
29	Treatment*Time	CompleteA	0	CompleteA	27	7.37	-3.49	0.0093	0.05
30	Treatment*Time	CompleteA	0	CompleteA	28	7.28	-4.00	0.0048	0.05
31	Treatment*Time	CompleteA	0	NOAddition	0	6	0.28	0.7896	0.05
32	Treatment*Time	CompleteA	0	NOAddition	4	8.28	-1.97	0.0826	0.05
33	Treatment*Time	CompleteA	0	NOAddition	8	6.73	-2.44	0.0461	0.05
34	Treatment*Time	CompleteA	0	NOAddition	12	9.24	-5.03	0.0007	0.05
35	Treatment*Time	CompleteA	0	NOAddition	26	7.49	-5.41	0.0008	0.05
36	Treatment*Time	CompleteA	0	NOAddition	27	7.37	-5.28	0.0010	0.05

37	Treatment*Time	CompleteA	0	NOAddition	28	7.28	-5.15	0.0012	0.05
38	Treatment*Time	CompleteA	0	PartialA	0	6	-1.71	0.1388	0.05
39	Treatment*Time	CompleteA	0	PartialA	4	8.28	-2.94	0.0180	0.05
40	Treatment*Time	CompleteA	0	PartialA	8	6.73	-3.43	0.0117	0.05
41	Treatment*Time	CompleteA	0	PartialA	12	9.24	-7.94	<.0001	0.05
42	Treatment*Time	CompleteA	0	PartialA	26	7.49	-5.38	0.0008	0.05
43	Treatment*Time	CompleteA	0	PartialA	27	7.37	-5.69	0.0006	0.05
44	Treatment*Time	CompleteA	0	PartialA	28	7.28	-5.09	0.0013	0.05
45	Treatment*Time	CompleteA	4	CompleteA	8	9.39	0.03	0.9762	0.05
46	Treatment*Time	CompleteA	4	CompleteA	12	11.6	-0.22	0.8271	0.05
47	Treatment*Time	CompleteA	4	CompleteA	26	11.5	-2.08	0.0610	0.05
48	Treatment*Time	CompleteA	4	CompleteA	27	11.2	-1.63	0.1303	0.05
49	Treatment*Time	CompleteA	4	CompleteA	28	11.1	-2.12	0.0578	0.05
50	Treatment*Time	CompleteA	4	NOAddition	0	8.28	2.10	0.0673	0.05
51	Treatment*Time	CompleteA	4	NOAddition	4	6	-0.02	0.9821	0.05
52	Treatment*Time	CompleteA	4	NOAddition	8	9.39	-1.16	0.2738	0.05
53	Treatment*Time	CompleteA	4	NOAddition	12	11.6	-1.98	0.0716	0.05
54	Treatment*Time	CompleteA	4	NOAddition	26	11.5	-3.16	0.0087	0.05
55	Treatment*Time	CompleteA	4	NOAddition	27	11.2	-3.13	0.0094	0.05
56	Treatment*Time	CompleteA	4	NOAddition	28	11.1	-3.10	0.0101	0.05
57	Treatment*Time	CompleteA	4	PartialA	0	8.28	0.96	0.3622	0.05
58	Treatment*Time	CompleteA	4	PartialA	4	6	-0.77	0.4695	0.05
59	Treatment*Time	CompleteA	4	PartialA	8	9.39	-2.05	0.0695	0.05
60	Treatment*Time	CompleteA	4	PartialA	12	11.6	-4.08	0.0016	0.05
61	Treatment*Time	CompleteA	4	PartialA	26	11.5	-3.13	0.0092	0.05
62	Treatment*Time	CompleteA	4	PartialA	27	11.2	-3.48	0.0050	0.05
63	Treatment*Time	CompleteA	4	PartialA	28	11.1	-3.04	0.0112	0.05
64	Treatment*Time	CompleteA	8	CompleteA	12	8.4	-0.18	0.8628	0.05
65	Treatment*Time	CompleteA	8	CompleteA	26	10.7	-1.55	0.1506	0.05
66	Treatment*Time	CompleteA	8	CompleteA	27	11	-1.24	0.2420	0.05
67	Treatment*Time	CompleteA	8	CompleteA	28	11.1	-1.61	0.1352	0.05
68	Treatment*Time	CompleteA	8	NOAddition	0	6.73	1.21	0.2670	0.05
69	Treatment*Time	CompleteA	8	NOAddition	4	9.39	-0.05	0.9637	0.05
70	Treatment*Time	CompleteA	8	NOAddition	8	6	-0.97	0.3716	0.05
71	Treatment*Time	CompleteA	8	NOAddition	12	8.4	-1.33	0.2182	0.05
72	Treatment*Time	CompleteA	8	NOAddition	26	10.7	-2.34	0.0400	0.05
73	Treatment*Time	CompleteA	8	NOAddition	27	11	-2.34	0.0389	0.05
74	Treatment*Time	CompleteA	8	NOAddition	28	11.1	-2.34	0.0387	0.05
75	Treatment*Time	CompleteA	8	PartialA	0	6.73	0.54	0.6091	0.05
76	Treatment*Time	CompleteA	8	PartialA	4	9.39	-0.56	0.5875	0.05
77	Treatment*Time	CompleteA	8	PartialA	8	6	-1.68	0.1435	0.05

78	Treatment*Time	CompleteA	8	PartialA	12	8.4	-2.70	0.0258	0.05
79	Treatment*Time	CompleteA	8	PartialA	26	10.7	-2.31	0.0416	0.05
80	Treatment*Time	CompleteA	8	PartialA	27	11	-2.60	0.0246	0.05
81	Treatment*Time	CompleteA	8	PartialA	28	11.1	-2.30	0.0416	0.05
82	Treatment*Time	CompleteA	12	CompleteA	26	10.4	-2.03	0.0687	0.05
83	Treatment*Time	CompleteA	12	CompleteA	27	10.1	-1.55	0.1510	0.05
84	Treatment*Time	CompleteA	12	CompleteA	28	9.89	-2.07	0.0658	0.05
85	Treatment*Time	CompleteA	12	NOAddition	0	9.24	2.78	0.0209	0.05
86	Treatment*Time	CompleteA	12	NOAddition	4	11.6	0.20	0.8466	0.05
87	Treatment*Time	CompleteA	12	NOAddition	8	8.4	-1.06	0.3170	0.05
88	Treatment*Time	CompleteA	12	NOAddition	12	6	-1.96	0.0973	0.05
89	Treatment*Time	CompleteA	12	NOAddition	26	10.4	-3.19	0.0093	0.05
90	Treatment*Time	CompleteA	12	NOAddition	27	10.1	-3.15	0.0102	0.05
91	Treatment*Time	CompleteA	12	NOAddition	28	9.89	-3.11	0.0112	0.05
92	Treatment*Time	CompleteA	12	PartialA	0	9.24	1.44	0.1825	0.05
93	Treatment*Time	CompleteA	12	PartialA	4	11.6	-0.62	0.5468	0.05
94	Treatment*Time	CompleteA	12	PartialA	8	8.4	-1.99	0.0806	0.05
95	Treatment*Time	CompleteA	12	PartialA	12	6	-4.30	0.0051	0.05
96	Treatment*Time	CompleteA	12	PartialA	26	10.4	-3.15	0.0098	0.05
97	Treatment*Time	CompleteA	12	PartialA	27	10.1	-3.53	0.0054	0.05
98	Treatment*Time	CompleteA	12	PartialA	28	9.89	-3.05	0.0124	0.05
99	Treatment*Time	CompleteA	26	CompleteA	27	12	0.36	0.7283	0.05
100	Treatment*Time	CompleteA	26	CompleteA	28	11.9	-0.13	0.9012	0.05
101	Treatment*Time	CompleteA	26	NOAddition	0	7.49	4.24	0.0033	0.05
102	Treatment*Time	CompleteA	26	NOAddition	4	11.5	2.06	0.0632	0.05
103	Treatment*Time	CompleteA	26	NOAddition	8	10.7	0.43	0.6775	0.05
104	Treatment*Time	CompleteA	26	NOAddition	12	10.4	0.51	0.6217	0.05
105	Treatment*Time	CompleteA	26	NOAddition	26	6	-0.98	0.3663	0.05
106	Treatment*Time	CompleteA	26	NOAddition	27	12	-1.01	0.3343	0.05
107	Treatment*Time	CompleteA	26	NOAddition	28	11.9	-1.02	0.3275	0.05
108	Treatment*Time	CompleteA	26	PartialA	0	7.49	3.30	0.0119	0.05
109	Treatment*Time	CompleteA	26	PartialA	4	11.5	1.40	0.1898	0.05
110	Treatment*Time	CompleteA	26	PartialA	8	10.7	-0.40	0.6936	0.05
111	Treatment*Time	CompleteA	26	PartialA	12	10.4	-1.31	0.2201	0.05
112	Treatment*Time	CompleteA	26	PartialA	26	6	-0.95	0.3792	0.05
113	Treatment*Time	CompleteA	26	PartialA	27	12	-1.33	0.2097	0.05
114	Treatment*Time	CompleteA	26	PartialA	28	11.9	-0.97	0.3507	0.05
115	Treatment*Time	CompleteA	27	CompleteA	28	12	-0.47	0.6494	0.05
116	Treatment*Time	CompleteA	27	NOAddition	0	7.37	3.62	0.0078	0.05
117	Treatment*Time	CompleteA	27	NOAddition	4	11.2	1.61	0.1346	0.05
118	Treatment*Time	CompleteA	27	NOAddition	8	11	0.13	0.8974	0.05

119	Treatment*Time	CompleteA	27	NOAddition	12	10.1	0.08	0.9406	0.05
120	Treatment*Time	CompleteA	27	NOAddition	26	12	-1.31	0.2143	0.05
121	Treatment*Time	CompleteA	27	NOAddition	27	6	-1.33	0.2308	0.05
122	Treatment*Time	CompleteA	27	NOAddition	28	12	-1.34	0.2043	0.05
123	Treatment*Time	CompleteA	27	PartialA	0	7.37	2.71	0.0286	0.05
124	Treatment*Time	CompleteA	27	PartialA	4	11.2	0.97	0.3533	0.05
125	Treatment*Time	CompleteA	27	PartialA	8	11	-0.69	0.5055	0.05
126	Treatment*Time	CompleteA	27	PartialA	12	10.1	-1.68	0.123	0.05
127	Treatment*Time	CompleteA	27	PartialA	26	12	-1.28	0.2234	0.05
128	Treatment*Time	CompleteA	27	PartialA	27	6	-1.65	0.1508	0.05
129	Treatment*Time	CompleteA	27	PartialA	28	12	-1.29	0.2201	0.05
130	Treatment*Time	CompleteA	28	NOAddition	0	7.28	4.12	0.0041	0.05
131	Treatment*Time	CompleteA	28	NOAddition	4	11.1	2.10	0.0598	0.05
132	Treatment*Time	CompleteA	28	NOAddition	8	11.1	0.52	0.6133	0.05
133	Treatment*Time	CompleteA	28	NOAddition	12	9.89	0.63	0.544	0.05
134	Treatment*Time	CompleteA	28	NOAddition	26	11.9	-0.81	0.4332	0.05
135	Treatment*Time	CompleteA	28	NOAddition	27	12	-0.84	0.4157	0.05
136	Treatment*Time	CompleteA	28	NOAddition	28	6	-0.86	0.4223	0.05
137	Treatment*Time	CompleteA	28	PartialA	0	7.28	3.24	0.0135	0.05
138	Treatment*Time	CompleteA	28	PartialA	4	11.1	1.47	0.1702	0.05
139	Treatment*Time	CompleteA	28	PartialA	8	11.1	-0.29	0.777	0.05
140	Treatment*Time	CompleteA	28	PartialA	12	9.89	-1.09	0.3032	0.05
141	Treatment*Time	CompleteA	28	PartialA	26	11.9	-0.78	0.4482	0.05
142	Treatment*Time	CompleteA	28	PartialA	27	12	-1.15	0.2724	0.05
143	Treatment*Time	CompleteA	28	PartialA	28	6	-0.81	0.4472	0.05
144	Treatment*Time	NOAddition	0	NOAddition	4	8.28	-2.13	0.0642	0.05
145	Treatment*Time	NOAddition	0	NOAddition	8	6.73	-2.54	0.0402	0.05
146	Treatment*Time	NOAddition	0	NOAddition	12	9.24	-5.22	0.0005	0.05
147	Treatment*Time	NOAddition	0	NOAddition	26	7.49	-5.55	0.0007	0.05
148	Treatment*Time	NOAddition	0	NOAddition	27	7.37	-5.40	0.0008	0.05
149	Treatment*Time	NOAddition	0	NOAddition	28	7.28	-5.28	0.001	0.05
150	Treatment*Time	NOAddition	0	PartialA	0	6	-1.99	0.0943	0.05
151	Treatment*Time	NOAddition	0	PartialA	4	8.28	-3.10	0.014	0.05
152	Treatment*Time	NOAddition	0	PartialA	8	6.73	-3.52	0.0104	0.05
153	Treatment*Time	NOAddition	0	PartialA	12	9.24	-8.13	<.0001	0.05
154	Treatment*Time	NOAddition	0	PartialA	26	7.49	-5.51	0.0007	0.05
155	Treatment*Time	NOAddition	0	PartialA	27	7.37	-5.82	0.0005	0.05
156	Treatment*Time	NOAddition	0	PartialA	28	7.28	-5.21	0.0011	0.05
157	Treatment*Time	NOAddition	4	NOAddition	8	9.39	-1.15	0.2801	0.05
158	Treatment*Time	NOAddition	4	NOAddition	12	11.6	-1.96	0.0748	0.05
159	Treatment*Time	NOAddition	4	NOAddition	26	11.5	-3.14	0.0091	0.05

160	Treatment*Time	NOAddition	4	NOAddition	27	11.2	-3.11	0.0097	0.05
161	Treatment*Time	NOAddition	4	NOAddition	28	11.1	-3.08	0.0105	0.05
162	Treatment*Time	NOAddition	4	PartialA	0	8.28	0.99	0.3481	0.05
163	Treatment*Time	NOAddition	4	PartialA	4	6	-0.75	0.4825	0.05
164	Treatment*Time	NOAddition	4	PartialA	8	9.39	-2.03	0.0714	0.05
165	Treatment*Time	NOAddition	4	PartialA	12	11.6	-4.05	0.0017	0.05
166	Treatment*Time	NOAddition	4	PartialA	26	11.5	-3.10	0.0096	0.05
167	Treatment*Time	NOAddition	4	PartialA	27	11.2	-3.46	0.0052	0.05
168	Treatment*Time	NOAddition	4	PartialA	28	11.1	-3.02	0.0116	0.05
169	Treatment*Time	NOAddition	8	NOAddition	12	8.4	-0.09	0.9313	0.05
170	Treatment*Time	NOAddition	8	NOAddition	26	10.7	-1.22	0.25	0.05
171	Treatment*Time	NOAddition	8	NOAddition	27	11	-1.24	0.2409	0.05
172	Treatment*Time	NOAddition	8	NOAddition	28	11.1	-1.25	0.2362	0.05
173	Treatment*Time	NOAddition	8	PartialA	0	6.73	1.86	0.1067	0.05
174	Treatment*Time	NOAddition	8	PartialA	4	9.39	0.63	0.5429	0.05
175	Treatment*Time	NOAddition	8	PartialA	8	6	-0.72	0.5004	0.05
176	Treatment*Time	NOAddition	8	PartialA	12	8.4	-1.46	0.1803	0.05
177	Treatment*Time	NOAddition	8	PartialA	26	10.7	-1.19	0.2583	0.05
178	Treatment*Time	NOAddition	8	PartialA	27	11	-1.50	0.1619	0.05
179	Treatment*Time	NOAddition	8	PartialA	28	11.1	-1.21	0.2508	0.05
180	Treatment*Time	NOAddition	12	NOAddition	26	10.4	-1.66	0.1261	0.05
181	Treatment*Time	NOAddition	12	NOAddition	27	10.1	-1.67	0.125	0.05
182	Treatment*Time	NOAddition	12	NOAddition	28	9.89	-1.67	0.1263	0.05
183	Treatment*Time	NOAddition	12	PartialA	0	9.24	3.88	0.0035	0.05
184	Treatment*Time	NOAddition	12	PartialA	4	11.6	1.14	0.2776	0.05
185	Treatment*Time	NOAddition	12	PartialA	8	8.4	-0.83	0.4276	0.05
186	Treatment*Time	NOAddition	12	PartialA	12	6	-2.34	0.058	0.05
187	Treatment*Time	NOAddition	12	PartialA	26	10.4	-1.63	0.1329	0.05
188	Treatment*Time	NOAddition	12	PartialA	27	10.1	-2.05	0.0675	0.05
189	Treatment*Time	NOAddition	12	PartialA	28	9.89	-1.61	0.1385	0.05
190	Treatment*Time	NOAddition	26	NOAddition	27	12	-0.05	0.9608	0.05
191	Treatment*Time	NOAddition	26	NOAddition	28	11.9	-0.08	0.9352	0.05
192	Treatment*Time	NOAddition	26	PartialA	0	7.49	4.60	0.0021	0.05
193	Treatment*Time	NOAddition	26	PartialA	4	11.5	2.47	0.0301	0.05
194	Treatment*Time	NOAddition	26	PartialA	8	10.7	0.38	0.7084	0.05
195	Treatment*Time	NOAddition	26	PartialA	12	10.4	-0.15	0.8837	0.05
196	Treatment*Time	NOAddition	26	PartialA	26	6	0.03	0.9786	0.05
197	Treatment*Time	NOAddition	26	PartialA	27	12	-0.37	0.718	0.05
198	Treatment*Time	NOAddition	26	PartialA	28	11.9	-0.03	0.9739	0.05
199	Treatment*Time	NOAddition	27	NOAddition	28	12	-0.03	0.9741	0.05
200	Treatment*Time	NOAddition	27	PartialA	0	7.37	4.50	0.0025	0.05

201	Treatment*Time	NOAddition	27	PartialA	4	11.2	2.46	0.031	0.05
202	Treatment*Time	NOAddition	27	PartialA	8	11	0.42	0.683	0.05
203	Treatment*Time	NOAddition	27	PartialA	12	10.1	-0.09	0.9327	0.05
204	Treatment*Time	NOAddition	27	PartialA	26	12	0.08	0.9395	0.05
205	Treatment*Time	NOAddition	27	PartialA	27	6	-0.31	0.7649	0.05
206	Treatment*Time	NOAddition	27	PartialA	28	12	0.02	0.9879	0.05
207	Treatment*Time	NOAddition	28	PartialA	0	7.28	4.40	0.0029	0.05
208	Treatment*Time	NOAddition	28	PartialA	4	11.1	2.45	0.0324	0.05
209	Treatment*Time	NOAddition	28	PartialA	8	11.1	0.44	0.667	0.05
210	Treatment*Time	NOAddition	28	PartialA	12	9.89	-0.04	0.965	0.05
211	Treatment*Time	NOAddition	28	PartialA	26	11.9	0.11	0.9143	0.05
212	Treatment*Time	NOAddition	28	PartialA	27	12	-0.27	0.7886	0.05
213	Treatment*Time	NOAddition	28	PartialA	28	6	0.05	0.9634	0.05
214	Treatment*Time	PartialA	0	PartialA	4	8.28	-1.96	0.0842	0.05
215	Treatment*Time	PartialA	0	PartialA	8	6.73	-2.85	0.0259	0.05
216	Treatment*Time	PartialA	0	PartialA	12	9.24	-6.79	<.0001	0.05
217	Treatment*Time	PartialA	0	PartialA	26	7.49	-4.57	0.0022	0.05
218	Treatment*Time	PartialA	0	PartialA	27	7.37	-4.92	0.0015	0.05
219	Treatment*Time	PartialA	0	PartialA	28	7.28	-4.33	0.0031	0.05
220	Treatment*Time	PartialA	4	PartialA	8	9.39	-1.52	0.1621	0.05
221	Treatment*Time	PartialA	4	PartialA	12	11.6	-3.24	0.0075	0.05
222	Treatment*Time	PartialA	4	PartialA	26	11.5	-2.44	0.0318	0.05
223	Treatment*Time	PartialA	4	PartialA	27	11.2	-2.82	0.0165	0.05
224	Treatment*Time	PartialA	4	PartialA	28	11.1	-2.39	0.0357	0.05
225	Treatment*Time	PartialA	8	PartialA	12	8.4	-0.54	0.6038	0.05
226	Treatment*Time	PartialA	8	PartialA	26	10.7	-0.36	0.7247	0.05
227	Treatment*Time	PartialA	8	PartialA	27	11	-0.68	0.5109	0.05
228	Treatment*Time	PartialA	8	PartialA	28	11.1	-0.40	0.6958	0.05
229	Treatment*Time	PartialA	12	PartialA	26	10.4	0.18	0.8584	0.05
230	Treatment*Time	PartialA	12	PartialA	27	10.1	-0.29	0.779	0.05
231	Treatment*Time	PartialA	12	PartialA	28	9.89	0.10	0.9202	0.05
232	Treatment*Time	PartialA	26	PartialA	27	12	-0.40	0.6982	0.05
233	Treatment*Time	PartialA	26	PartialA	28	11.9	-0.06	0.9529	0.05
234	Treatment*Time	PartialA	27	PartialA	28	12	0.32	0.7524	0.05