Growth and transcription studies in *Bacillus cereus* ATCC 14579

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ABSTRACT

Bacillus cereus ATCC 14579 was grown under different conditions, in different growth media, and the following parameters were investigated; cell number, pH, oxygen levels, glucose levels, cell morphology, proportions of live/dead bacteria and the expression of selected genes involved in energy metabolism. Flow cytometry was used in the determination of cell number and was validated by comparison to viable counts. *B. cereus* ATCC 14579 was grown with shaking in 1 L Erlenmeyer flask in seven different growth media (a defined media, 20%, 50%, 100% Luria bertani and 25%, 50%, 100% Tryptic soy broth), which contained different carbon and nitrogen levels. The composition of the growth media was shown to have a significant effect on the pH of the media and cell morphology. The pH started to decrease from approximately 7.0 to 5.5, five hours after inoculation. This could be due to the formation of acidic fermentation products. Glucose was depleted after 8-9 hours of growth and this was followed by an increase in pH, which was proportional to the amount of organic nitrogen in the media. This could be due to the utilization of the acidic fermentation products, an increase in ammonia levels from amino acid catabolism or a combination of these factors. A growth media, 50% LB that gave satisfactory growth, minimal changes in pH and no detectable changes in cell morphology was identified and used in all further experiments. *B. cereus* was also grown in 50% LB media in 1 L Erlenmeyer flask (surface area 113 cm^2) and in a large surface area (530 cm^2) culture with and without shaking. Cultures grown with shaking reaches a 10-fold higher cell density in the stationary phase, than cultures grown without shaking. The pH in the cultures grown without shaking start to decrease five hours after inoculation but continued to remain low (pH 4.5-5.5) during further growth and throughout the stationary phase. Surface area does not have a significant influence on growth and pH.

The expression levels of genes involved in fermentation, amino acid metabolism, the TCA cycle and ATP synthesis, in cultures grown with and without shaking, were measured using real-time reverse transcription PCR, 5 and 12 hours after inoculation. The following genes were selected: Fructose-6-phosphatekinase (Fru), Lactate dehydrogenase (Lac1), Acetyl-CoA synthetase (Acco1), Glutaminase (Glu2), α-ketoglutarate dehydrogenase E1 (Oxde), Citrate synthase (Cisy) and Atp synthase ε-

chain (Atp). The results show different expression of these metabolic genes in bacteria grown with and without shaking. “Atp” and “Fru” are slightly down regulated in the cultures grown with shaking compare to the cultures grown with shaking, at time 5 and 12. “ Lac1 “ was upregulated 12 hours after inoculation in both cultures but to a lesser degree in the cultures grown without shaking.“Acco1” and “Glu2” were upregulated 12 hours after inoculation in the cultures grown with shaking and down regulated in the cultures grown without shaking. “Oxde” are upregulated at time 12 in both cultures. “Cisy1” are upregulated at time 12 in both cultures but to a greater degree in the cultures grown with shaking. There appears to be a correlation between the glucose exhaustion, a requirement of alternative carbon and energy sources and the upregulation of “ Lac1”, “Acco1”, “Glu2”, “Oxde” and “Cisy1” at time 12 in the shaking culture.

ABBREVIATIONS

ATCC	The American Type Culture Collection
#/No.	number(s)
°C	degree Celcius
Acco	Acetyl-CoA synthetase
Amp.eff	Amplification efficiency
Atp	Atp synthase ε-chain
bp	base par
cDNA	complementary DNA
Cisy	Citrate synthase
cm^2	Square centimeter
Ct	crossing point
D	derived
D.f	dilution factor
DEPC	diethyl pyrocarbonate
dH_2O	destilled water
DNA	deoxynucleic acid
dNTP	deoxyribonucleosid 5`-triphsophate nucleotide
ds	double stranded
DTT	ditiotreitol
e.g.	for example
EDTA	ethylene-diamine-tetra-acetic acid
EtBr	Etidiumbromid
Fru	Fruktose-6-phosphatekinase
FSC	Forward scatter
g	gram
Glu	Glutaminase
Glyde	Glyceraldehyd-3-phosphate dehydrogenase.
KEGG	Kyoto Encyclopeida of Genes and Genomes
L or l	litre
Lac	Lactate dehydrogenase
LB	Luria Bertani
M	molar
Mm	milli Molar
mg	milli gram (10^{-3} g)
min	minutes
ml	milli litre (10^{-3} l)
MOPS	morpoline propanesulfonic acid
nm	nano meter(10^{-9} m)
OD	Optical density
ON	Over night
Oxde	α-ketoglutarate dehydrogenase E1
PCR	Polymerase chain reaction
pmol	picomol (10^{-12} mol)
ppm	parts per million, mg/L
R^2	Correlation coefficient
RFU	relative fluorescence unit
RNA	ribonucleic acid
rpm	revolutions per minutes
RT	room temperature
sec	second
SSC	side scatter
St.dev	standard deviation

TAE	Tri-acetic acid-EDTA
Tm	melting temperature
TSB	Tryptic soy broth
µg	micro gram (10^{-6} g)
µM	micro molar
µl	micro litre (10^{-6} l)
UV-light	Ultra violet light
V	Volt
vs.	versus
w/v	weight/volume
α	alfa

CONTENTS

1.INTRODUCTION

1.1 *Bacillus cereus*

1.1.1 The *Bacillus cereus* group

The *Bacillus cereus* group is a very homogeneous cluster within the Bacillus genus and consists of gram-positive, rod-shaped and endospore-forming bacteria. The group contains of six species: *B. cereus, B. thuringiensis B. anthracis, B. mycoides, B. pseudomycoides and B. weihenstephanensis.* These species are very closely related genetically and it has been suggested that members of the *B. cereus* group should be considered as belonging to the same species, and be classified as variants of *B. cereus* (Gordon et al., 1973; Priest, 1981; Ash et al., 1991 and Helgason et al., 2000). However, *B. cereus, B. thuringiensis B. anthracis* demonstrate widely different phenotypes, particularly with regard to pathogenicity. Many strains of *B. cereus* are able to produce toxins and cause distinct types of infections and food poisoning (Drobniewski, 1993; Kotiranta et al., 2000). The major concern about *B. cereus* as food poisoning strains is their ability to form spores. Heat-treatment of food will kill vegetative cells, but not spores. Later, if the food is kept at room temperature the spores will germinate and vegetative cells will start their toxin production. *B. anthracis* causes the acute and often lethal disease anthrax (Keim & Smith, 2002) and has recently been of public interest because of its potential as a weapon in biological warfare (Inglesby et al. 1999). *B. thuringiensis* is an insects pathogen and produces intracellular insecticidal crystal toxins (δ-endotoxin) during sporulation (Schnepf et al. 1998). The crystal toxins are toxic to a wide variety of insect larvae, and *B. thuringiensis* is the most commonly used biological pesticide worldwide (Höfte & Whiteley, 1989; Aronson, 1993). Insects that ingest bacteria that have produced the toxic crystal will die due to changes in the osmotic pressure in the gut of the insects. The genes encoding pathogenicity are mostly encoded on large plasmids. The phenotypical traits employed for the identification of *B. anthracis* and *B. thuringiensis* may be lost, making them indistinguishable from *B. cereus* (Thorne, 1993).

The transcription of most of the genes encoding various virulence factors in *B. cereus* and *B. thuringiensis,* are controlled by the pleiotropic regulator PlcR (Lereclus et al.,

1996; Agaisse *et al*., 1999; Økstad *et al*., 1999). Many of these genes are also present in *B. anthracis*, but *B. anthracis* does not express an active PlcR because a point mutation induces a stop codon that results in a truncated protein (Agaisse et al. 1999).

1.2.1 Bacillus cereus

B. cereus is a motile facultative aerobic bacterium that also grows well anaerobically. *B. cereus* occurs ubiquitously in soil and in many raw and processed foods such as rice, milk and dairy products, spices, and vegetables (Carlin et al., 2000; Christianson et al., 1999; Guinebretiere et al., 2003; Sarrìas et al. 2002). *B. cereus is* an opportunistic human pathogen, indicating that it is normally non-pathogenic but it can causes a variety of infections in immunosupressed or otherwise debilitated individuals. *B. cereus* infections mainly fall into six categories (Drobniewski,1993): (i) local infections, particularly in burns, sites of injury and the eye, (ii) bacteremea and septicemia, (iii) infection of the central nervous system; (iv) respiratory infections; (v) endocarditis and pericarditis; and (vi) two types of food poisoning, the diarrhoeal type and the emetic type.

The diarrhoeal type of food poisoning is caused by complex enterotoxins (Beecher & Wong, 1997; Lund & Granum, 1997) formed by vegetative *B. cereus* cells in the small intestine (Granum, 1994). Two different three components enterotoxins have been characterised: Hemolysin (Hbl), which is capable of lysing red blood cells (Thompson et al., 1984; Beecher & MacMillan, 1990; 1991; Beecher et al., 1995) and a non-haemolytic enterotoxin (Nhe) (Lund & Granum, 1996). The emetic type of food poisoning, nausea and vomiting, is caused by an emetic toxin, cerulide (Agata et al. 1994; 1995), produced by cells growing in the food. The emetic toxins are resistant to heat, extreme pH and proteases (Shinagawa et al., 1996). The ATCC 14579 strain of *B. cereus* has tested non-emetic (Andersson et al., 1998 ; Haggblom et al., 2002). Both types of food poisonings are usually mild and last for less than 24 hours (Granum, 1994). Consequently *B. cereus* food poisoning is often unreported.

1.2 Cellular differentiation in Bacillus

1.2.1 Binary fission, sporulation and germination

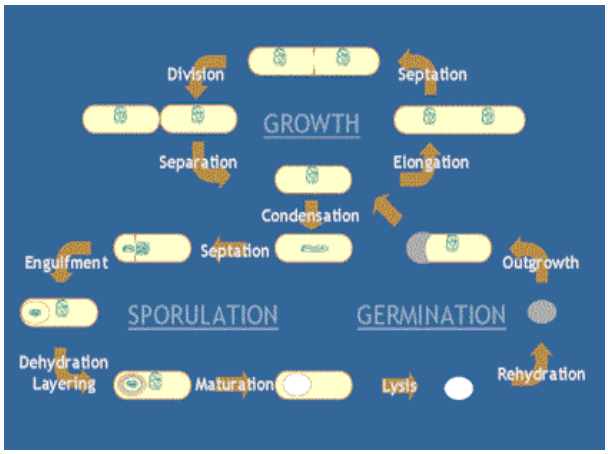


Figure 1.1: Differentiation in Bacillus. Normally bacillus divides by binary fission but under certain conditions they can form spores. When the environmental conditions improve the spores will germinate and give rise to a vegetative cell. Picture taken from www.bgsc.org/cycle.gif

Figure 1.1 shows differentiation in Bacillus. During vegetative growth *Bacillus* divides symmetrically by a process called binary fission, where DNA replicates to form two complete chromosomes. A partition, called septum, is formed and eventually separates the cells into two identical daughter cells. The septum is an inward growth of the cytoplasmic membrane and the cell wall (Madigan et al., 2003, pp 139). In response to unfavourable environmental conditions, vegetative cells can abandon vegetative growth and go through sporulation. In sporulation cell division is asymmetric, resulting in two different cell types: mother cells and a fore spore. The fore spore will form a dormant, non-growing endospore (Errington, 1993). Endospores are extremely resistant to external destructive agents like heat, drying, UV radiation and chemicals (Setlow, 1995) and can survive for long periods of time in the environment (Setlow, 1994). The DNA in spores of *Bacillus* species is covered with small acid soluble proteins (SASP), which are synthesized in the developing spore late in sporulation (Setlow, 1988). This proteins binds to DNA and protect it from variety of types of damage and provides amino acid for protein synthesis early in spore germination (Setlow, 1988; 1995). Dipicolinic acids are likely to be in a 1:1 chelated with Ca^{2+}. This complex is located in the core and appears to be important in spore core dehydration, concomitant spore heat resistance and maintenance of spore dormancy (Paidhungat et al., 2000). In response to improved environmental conditions the spores germinate and give rise to vegetative cells (Moir & Smith, 1990).

1.3 Microbial growth

Microorganisms are greatly affected by the chemical and physical conditions of their environment. The most common environmental conditions that affect microbes are temperature, pH, oxygen, light, oxygen, water and nutrients. Each bacterium has an optimum range of these conditions within which it grows at a maximum rate. Values below or above these optimum ranges can cause stressful conditions for the bacteria.

The bacteria in these experiments are grown in a batch culture, a closed system of fixed volume. The bacteria are exposed to stress due to environment changes during growth, such as: decrease in nutrient concentration, accumulation of waste product and changes in pH. In aerobic cultures oxygen availability will decrease as the number of cells in the culture increase, because the rate of oxygen consumption is faster than the rate at which it can diffuse into the medium. All organisms synthesise a set of proteins called stress proteins when they are exposed to stress. Stress response enables the bacteria to quickly adapt to changes in the environment in order to survive.

Bacteria grown in a batch culture give a classical growth curve, where number of bacteria is plotted against time (Figure 1.2). The curve can be divided into four phases: lag phase, exponential phase, stationary phase and death phase.

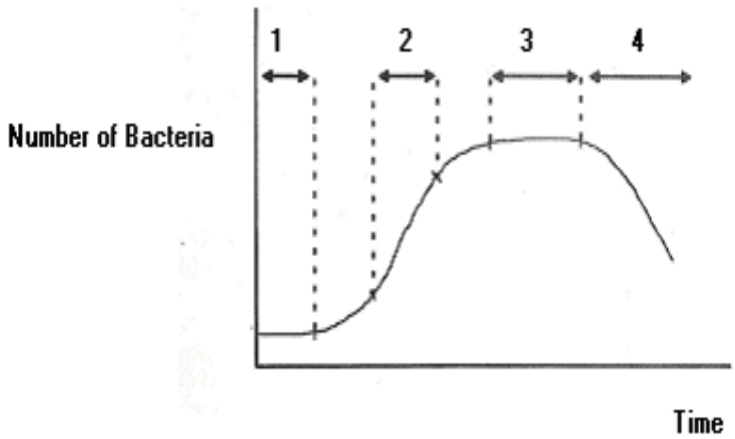


Figure 1.2: A classical growth curve where number of bacteria plotted against time.

1.3.1 Lag phase

In the Lag phase the bacteria increase in size but not in number, because the inoculated bacteria have to adapt to the new medium and synthesizes essential constituents and new enzymes. The duration of the lag phase depends not only on the size and age of the inoculum but also on the compositional change in the growth medium.

1.3.2 Exponential phase

The generation time is defined as the time it takes for the cell population to double in number. In the exponential phase the growth rate is constant and the number of cells doubles during each generation. When the cell number is plotted logarithmically it will give a straight line with a defined slope. Cells in the exponential phase are usually at their healthiest since they have an excess of nutrients and are not inhibited by waste product (Madigan et al., 2003, pp. 142-145).

1.3.3 Stationary phase

Exponential growth cannot occur indefinitely in a batch culture because essential nutrients will become limited, the supply of energy may run out or waste products will accumulate in the culture. In the stationary phase there are no net increases or decrease in number of cell, but the cells are still able to perform many cellular functions, including energy metabolism and biosynthetic processes, which the bacteria required to survive.

1.3.4 Death phase

After stationary phase the culture will after a while reach the death phase, where the generation time fall due to lack of nutrients, accumulation of waste product and limiting energy source. This causes the cells to die and results in a decrease in number of living cells.

1.4 Techniques

1.4.1 Flow cytometry and determination of number of bacteria cells

Figure 1.3: FACScan Calibur machine from Becton Dickinson used in the determined number of bacteria cells.

Flow cytometer, FACScan Calibur from Beckton Dickinson (Figure 1.3) is used to rapidly count and identify different types of cells and other biological particles that are labelled with a fluorochrome. Although the flow cytometer measures only one cell at a time, they are capable of processing thousands of cells in just a few seconds (50 000 cells/sec). The flow cytometer function by passing single cells in a flowing saline stream at high speed through a laser beam. The focused laser beam hits the moving cells and excites the fluorochrome, resulting in the emission of light at a characteristic wavelength, which is scattered in all directions. Photo detectors are placed forward (to detect forward scatter) or side-on (to detect side scatter). These photo detectors receive the pulses from scattered light and convert these pulses to electrical signal, suitable for computer analysis and interpretation. Forward scatter (FSC) is the light that passes through the cells. The amount of forward scatter light depends on cell size, organelles, water and molecular contents. Side scatter (SSC) is the light reflects of the cells and the amount is indicated by cytoplasmic density, nuclear shape or cell surface granularity.

Flow cytometry was used to determine the number of bacteria cells in the cultures at given times. The samples were diluted with filtered water and stained with a fluorescent dye, SYTO BC. SYTO BC is a nucleic acid stain that easily penetrates both gram-positive and gram-negative bacteria and results in a bright green fluorescent signal. A given volume of weakly fluorescent microspheres of known concentration was added and applied to the flow cytometer for analysis (Section 3.3.1). Analysis of the samples is done, by plotting forward scatter against green fluorescence, an example can be viewed in figure 1.4.

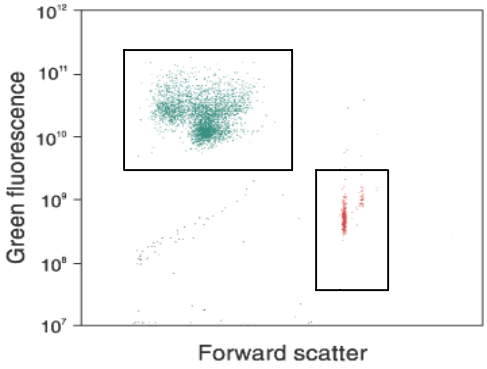


Figure 1.4: The points in the upper left hand frame represent bacteria stained with SYTO BC, while points in the lower right hand frame represent microsphere particles.

The points in the upper left hand frame represent bacteria stained with SYTO BC, where points in the lower right hand frame represent microsphere particles, which serve as a standard. Dividing the number of signals in the bacteria frame by the number of signals in the microsphere frame gives the number of bacteria per 10^{-6} mL of the sample. This can then be used to calculate the number of bacteria in the sample (Section 3.3.1).

1.4.2 Fluorescence microscope and LIVE/DEAD Staining

B. cereus was stained using LIVE/DEAD BacLight™ Bacterial Viability Kit that consists of SYTO9 and Propidium iodide. SYTO9 and Propidium iodide are fluorescent dyes that differ in their ability to penetrate bacteria cells. SYTO 9 is a green fluorescent nucleic stain that labels all bacteria in the population, those with intact membrane and those with damage membrane. Propidium iodide is a red fluorescent nucleic acid stain and can only penetrate bacteria with damage membrane, causing reduction in SYTO 9 when both dyes are present. Bacteria with intact membrane will be stained green whereas bacteria with damage membrane will be stained red. This is interpreted to indicate membrane intact = live, membrane damaged = dead.

Fluorescent dyes are used for staining microorganism and are illuminated by light provided by either a mercury or halogen lamp, and emit light of a longer wavelength. Each fluorescent has its own excitation spectrum (the range of illuminating light wavelengths that will cause it to fluoresce) and an emission spectrum (the spectrum of fluorescent light emitted) (Figure 1.5). The laser wavelength must be within the excitation spectrum to excite the fluorescent used. SYTO 9 and propidium iodide has excitation/emission maxima respectively at 480/500 and 490/635 nm.

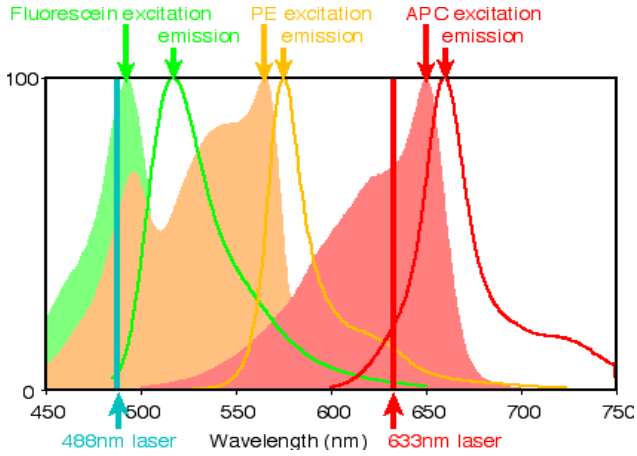


Figure 1.5:
Excitation/Emission spectrum for different fluorescent dyes.

1.4.3 The FastPrep instrument for isolation of Total RNA

The FastRNA Pro Blue Kit is designed to quickly and efficiently lyse cells and isolate total RNA from gram-positive and gram-negative bacteria. RNApro solution is mixed with the bacterial sample in a 2 ml tube containing matrix lysing particles. RNApro solution inactivates cellular RNase during cell lysis to prevent RNA degradation. The tubes are balanced and secures with locking clamps before processed in the FastPrep instrument. The FastPrep instrument is a high-speed bench top device that use vertical angular motion to lyse the cells, by simultaneous impaction of lysis matrix from multiple directions at once, complete lysis occurs in 10-40 sec. RNA is purified and isolated by chloroform extraction and ethanol precipitation. The purified RNA is ready for further applications like RT-PCR. The average RNA yields from 10^{10} bacteria should be greater than 50 µg.

1.4.4 Real-time reverse transcriptase PCR (Real time RT-PCR)

Real-time RT-PCR is a sensitive and accurate method, and can be used to estimate changes in gene expression. It is based upon two methods: Reverse transcription (RT) and the polymerase chain reaction (PCR).

1.4.4.1 Reverse transcription

Isolated total RNA is transcribed into single stranded DNA (cDNA), using reverse transcriptase and a specific oligonucleotide primer. Reverse transcriptase uses RNA as template and synthesizes the complementary DNA strand. This is first strand synthesis. Conventional PCR is then used to synthesis and amplify the second strand.

1.4.4.2 Polymerase chain reaction (PCR)

PCR is a method used to amplify DNA fragments. A PCR reaction occurs in three steps: 1) denaturation, 2) annealing of primers and 3) DNA synthesis. The double stranded DNA (dsDNA) containing the DNA sequence to be amplified is first denatured by heating to 95 °C. The reaction is cooled and the oligonucleotide primers will anneal to their complementary DNA sequence. A thermostable DNA polymerase extends the primers by adding complementary dNTPs (deoxynucloside triphosphates), results in two dsDNA fragments. Repeated cycles of denaturation and synthesis will give amplification of target sequence (Figure 1.6).

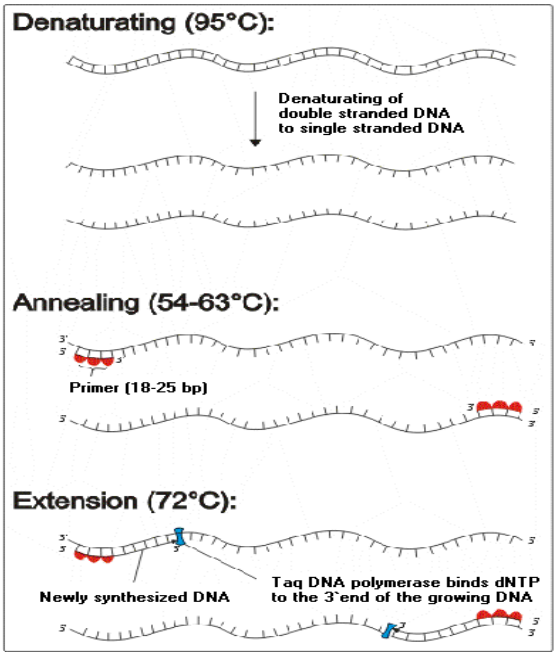


Figure 1.6: Polymerase chain reaction: The dsDNA is denatured, primers anneal and a thermostable DNA polymerase synthesises the target sequence.

The nature of the PCR reaction is exponential. The amount of target DNA theoretically doubles at each cycle, if the amplification efficiency is 100%, and after n cycle, we shall have 2^n times as much DNA as we started with. But the reaction cannot go on forever and it eventually tails off and reaches a plateau phase.

1.4.4.3 Real-time RT-PCR vs. Conventional PCR

In conventional PCR the amount of product (amplicon) of the PCR reaction can be determine by agarose gel-electrophoresis and ethidium bromide staining of the gel. This is carried out after a fixed number of cycles, normally in the plateau phase. When you look at the curves in order of dilution (Figure 1.7), quantification by agarose gel-electrophoresis and ethidium bromide staining of the gel could indicate

that some of the samples had the same level of amplification after fixed number of cycles, even though the samples differ by a factor of 10 or 100 in the amount of DNA in the logarithmic phase. Therefore, it is impossible to determine the amount of start template in conventional PCR. In real-time RT-PCR, quantitation of the amount of start template is done were the amplification reaction is logarithmic. The amount of fluorescence (Section 1.4.4.4) is measured after each amplification cycle and is proportional to the amount of amplicon produced. Fluorescence is plotted against cycle number and a threshold value is set where the amplification is logarithmic. The sample is analyzed by measure the cycle number where the fluorescence crosses the threshold, called the crossing point (Ct) value. This information can be used to estimate the amount of start template and in turn the relative levels of mRNA. Diluted samples will cross at later Ct values, because it takes more cycles before the amplification is detectable.

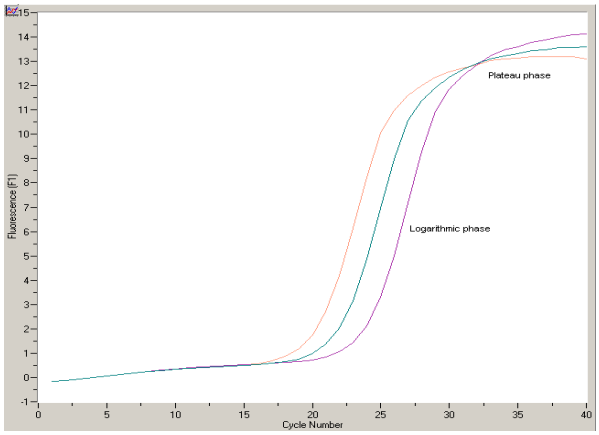


Figure 1.7: An example of a quantification graph. This specific example shows 10^{-2}, $10^{-2,5}$ and 10^{-3} dilutions of Oxde.

1.4.4.4 SYBR Green

SYBR green is a fluorescence dye, binding to the minor groove of dsDNA. SYBR green exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to dsDNA (Morrison, 1998), shown in figure 1.8.

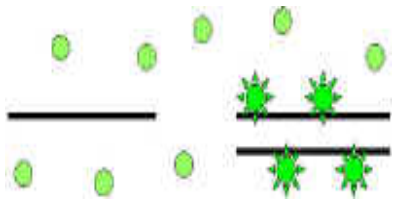


Figure 1.8:
SYBR Green fluorescence upon binding to ds cDNA.

As dsDNA is synthesized during real-time PCR more SYBR Green will bind and the fluorescence will increase. One disadvantage is that SYBR Green will bind to any type of dsDNA in the reaction, including primer dimers artifacts or other non-specific products, which can result in an overestimation of the target concentration.

1.4.4.5 Melting temperature

In a real-time PCR reaction is it only an increase in fluorescence that is measured and therefore you want to be sure, that this is due only to the amplification of a specific cDNA fragment. This is done by making a melting curve of the amplicon, in which the temperature is increased slowly from 50 to 95 °C, and changes in fluorescence are measured. At the melting point, the two strands of DNA will separate and results in a drop in fluorescence. The melting curves are converted to distinct melting peaks by plotting the first negative derivative of the fluorescence unit (RFU) with time (T) (-d(RFU)/dT) on the Y-axis versus temperature on the X-axis (Figure 1.9).

Different DNA molecules have distinct melting point, based on its base composition and length. All PCR products for a particular primer pair should have the same melting temperature and if the melting curve shows something else, this is an indication of contamination, mispriming or primer dimer artifact. Primer dimer artifact would give a peak with lower melting temperature because it is a shorter piece of DNA.

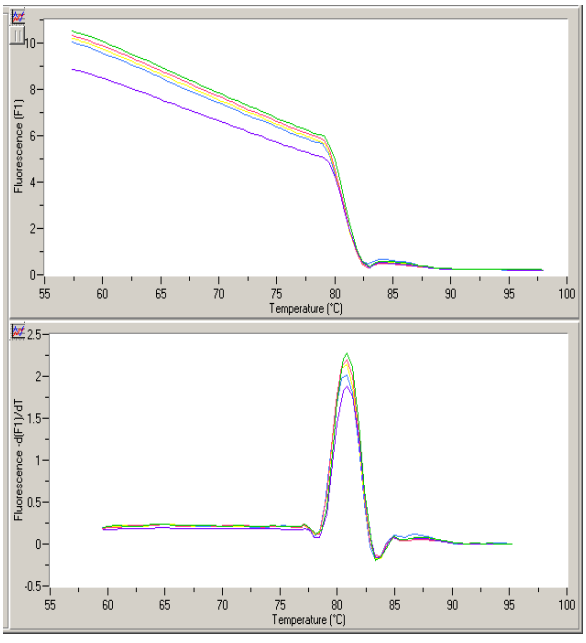


Figure 1.9: Example of melting curves. All the five samples have the same melting point (80.82 °C) indicating that they contain the same amplified fragment.

1.5 Aims of the study

During batch growth bacteria are exposed to different types of stress, such as changes in pH, accumulation of waste product and decrease in nutrients.

The aims of this study are:

1) Grow *B. cereus* strain ATCC 14579 under different growth conditions and in different growth media and measure the following parameters at different times during growth: cell number, pH, oxygen level, glucose level, cell morphology and the proportions of live/dead bacteria.

2) Identify a growth medium that gives satisfactory growth, minimal changes in pH and no changes in cell morphology.

3) Quantify gene expression for selected genes involved in energy metabolism under different growth conditions.

2.MATERIALS

2.1 Chemicals

2.1.1 Inorganic chemicals

All inorganic chemicals were pro-analysis quality.

Chemical compound	Supplier
Ammonium Acetate	Merck
$CaSO_4 * 2H_2O$	FERAK
$CoCl_2 * 6H_2O$	Merck
$CuCl_2 * 2H_2O$	Merck
$FeCl_2 * 4H_2O$	FERAK
H_3BO_3	Merck
K_2HPO_4	Merck
KH_2PO_4	Merck
$MgSO_4 * 7H_2O$	Merck
$MnCl_2 * 4H_2O$	Sigma
$Na_2MoO_4 * 2H_2O$	J.T Baker
$Na_2WO_4 * 2H_2O$	Sigma
NaCl	Merck
NaOH-Pellets	Merck
$NiCl_2 * 6H_2O$	Kebo
Tris base	Sigma
$VOSO_4 * 2H_2O$	Fluka
$ZnCl_2$	Merck

2.1.2 Organic chemicals and reagents

Chemical compound	Supplier
Agar	Oxoid
Agarose I (Biotechnology grade)	Amresco
Bacto-Tryptone	Becton Dickinson
Bacto-Yeast extract	Becton Dickinson
Chloroform	Merck
D (+)-glucose-Monohydrate	Merck
Diethylpyrocarbonate (DEPC)	FLUKA
Ethanol	Arcus A/S
Ethylenediaminetetraacetic acid (EDTA)	Applichem
Etidiumbromid (EtBr)	Merck
Formaldehyd-36%	ProLabo
Formamide	Sigma
Glacial acetic acid	Merck
L-phenylalanine	Sigma
L-tryptophan	Sigma
Methanol	Merck
MOPS	Applichem
Sodium Acetate 3-Hydrat	Merck
Tryptic Soy Broth	Becton Dickinson

2.2 Bacteria strain

Bacillus cereus ATCC 14579 from the American Type Culture Collection, was obtained from Professor A-B Kolstø (School of Pharmacy, University of Oslo).

2.3 Growth media for *B. cereus* ATCC 14579

2.3.1 Liquid media

All liquid media was sterilised by filtration using a Stericup™ (0.22 µm GP express PLUS Membrane) filtration and storage system, Millipore.

Tryptic soy broth(TSB) media:

	25% TSB + 50% Mineral medium	**50% TSB + 50% Mineral medium**	**100% TSB**
Tryptic soy broth	2.5 g	5 g	10 g
Glucose	5 g	5 g	-
5X Mineral medium *	200 ml	200 ml	-
dH_2O	Adjust volume to1 L	Adjust volume to1 L	Adjust volume to 1L

Luria Bertani (LB) media:

	20% LB + 50% Mineral medium	**50% LB + 50% Mineral medium**	**100% LB**
Bacto-Tryptone	2 g	5 g	10 g
Bacto-Yeast extract	1 g	2.5 g	5 g
NaCl	2 g	5 g	10 g
Glucose	5 g	5 g	-
5XMineral medium *	200 ml	200 ml	-
dH_2O	Adjust volume to 1 L	Adjust volume to 1 L	Adjust volume to 1L

In the text below the different LB and TSB media will be referred to as 25% TSB, 50% TSB, 100% TSB, 20% LB, 50% LB and 100% LB, unless other is specified.

B. subtilis defined medium (Steil et al., 2003) :

0.5% Glucose-0.5 g/100 ml dH_2O	1.25 g
50X L-tryptophan stock solution **	5 ml
50X L-phenylalanine stock solution **	5 ml
Trace elements ***	250 ml
5X Mineral medium *	50 ml
dH_2O	Adjust volume to 250 ml

*) 5X Mineral medium:

K_2HPO_4	4 g
KH_2PO_4	1 g
$CaSO_4$* $2H_2O$	0.25 g
$MgSO_4$* $7H_2O$	2.5 g
dH_2O	1 L

**) 50X Amino acid stock solution:

L-tryptophan (100 mg) was added to 80 ml filtered dH_2O. The pH was measured and adjusted to 7.0. The volume was adjusted to 100 ml to give a final concentration of 20 mg/L.

L-phenylalanine (90 mg) was added to 80 ml filtered dH_2O. The pH was measured and adjusted to 7.0. The volume was adjusted to 100 ml to give a final concentration of 18 mg/L.

***) Trace elements:

EDTA	5.2 g
$FeCl_2$*$4H_2O$	1.5 g
$ZnCl_2$	70 mg
$MnCl_2$*$4H_2O$	100 mg
H_3BO_3	6 mg
$CoCl_2$*$6H_2O$	190 mg
$CuCl_2$*$2H_2O$	17 mg
$NiCl_2$*$6H_2O$	25 mg
Na_2MoO_4 *$2H_2O$	188 mg
$VOSO_4$ * $2H_2O$	30 mg
Na_2WO_4*$2H_2O$	1 mg
dH_2O	1 L

2.3.2 Solid medium

100% LB Agar

100% LB medium

Agar 2%(w/v)

The agar suspension in LB medium was autoclaved at 140 ºC for 20 min. cooled to 60 ºC and poured into sterile Petri plates. The Petri plates were placed at RT over night and then stored at 4 ºC.

2.4 Real-time RT-PCR oligonucleotide primers

The gene sequences for selected genes were obtained from the KEGG (Kyoto Encyclopeida of Genes and Genomes) database at http://www.kegg.com. Appropriate primers were designed using primer-3-program at http://www-genome.wi.mit.edu /cgi-bin/primer/primer3_www.cgi and the following parameters were specified: Tm=58-63 °C, primer length=20 nt, GC%= 40-60%, Product size=~200 bp.

In order to check for unspecific priming to other parts of the genome the primer sequences were queried against the genome sequence of *B. cereus* ATCC 14579 and ATCC 10987 using BLAST at http://www.ncbi.nlm.nih.gov/sutils/genome_table.cgi
The primers were synthesised by MWG-Biotech AG and are listed in Table 2.4.

Table 2.4:Oligonucleotide primers used for real-time RT-PCR

Gene	Direction [1)]	Primer sequence- 5'to'3 (length)	Tm [2)]
Fructose-6-phosphatekinase (Fru) BC4600	F	GCTGAAGGTGTTGGAAGTGC (20)	60.84
	R	CCTACACAACGTCCGCCTTT (20)	62.35
Lactate dehydrogenase (Lac1) BC4870. BC1924	F	GAAGGGGAAGCAATGGACTTA (21)	60.44
	R	AACCAGATTCTTTCCAAGTTACG (23)	58.75
Lactate dehydrogenase (Lac2) BC4996	F	CACCAAAACCAGGGCAAAGT (20)	62.20
	R	CCAGTACCGATTACGCGATTTC (22)	62.83
Acetyl-CoA synthetase (Acco1) BC2489	F	ACGCATGCAGGATTTCCTTT (20)	61.89
	R	TCCCATAACCGGTCTGCTTC (20)	62.30
Acetyl-CoA synthetase (Acco2)BC4645	F	TCAGGAACGACAGGAAATCCA (21)	62.88
	R	CCTGTTGCCCCTGATCCTAA (20)	62.28
Acetyl-CoA synthetase (Acco3)BC4659	F	TCAAACGGCGAAGTGGGTAT (20)	62.68
	R	AATGCTGTTGGTGCGCTGTA (20)	62.75
Pyruvate oxidase (Pyro)BC2328	F	GGATTGATTGGCACGAAACC (20)	62.54
	R	TCGCATAACGTTTCCCGATT (20)	62.58
Glyceraldehyde-3-phosphate dehydrogenase (Glyde) BC5140	F	ACGCGGAATGATGACAACAA (20)	62.45
	R	ACAGCGCCACCGTTTAATTT (20)	61.74
Citrate synthase (Cisy1)BC2285	F	GCAGCGGAGACGAAAATCTC (20)	62.33
	R	TCCATGGGATGCGTCTCTTT (20)	62.88
Citrate synthase (Cisy2)BC4594	F	TTGCTGCTTACGCGAGAATC (20)	61.57
	R	AGCTACGCAAACACGTGCAG (20)	62.53
α-ketoglutarate dehydrogenase E1 (Oxde) BC1252	F	GCACGTCCTGAGCGTTTCTT (20)	62.82
	R	CAGGTTGGAAACGTCCTTCG (20)	62.89
Cytochrome C oxidase (Cytc) BC3941	F	CGGTGGCGTATCCAAAAACT (20)	62.13
	R	TCAAATCCACACGATTGTCA (20)	58.46
ATP synthase ε-chain (Atp) BC5305	F	AAGCGGTGGCTTTATCGAAG (20)	61.60
	R	CAAACGGTTCACAGCACGTT (20)	62.11
Glutaminase (Glu1) BC0481	F	ATCCATGCAGGCAACTCACA (20	62.68
	R	AGGGGTTTGGACGGACTTGT (20)	62.98
Glutaminase (Glu2) BC3115	F	CCGCTCTGGGGGAAATAAAT (20)	62.32
	R	GCATCTCCAGTTGGCTCTACG (21)	62.24
DNA polymerase III, delta chain (Poly) BC4321	F	ATGTTGGCGAAGGAAATGGA (20)	62.65
	R	GGCTCTTCCTGCTGCGTAAA (20)	62.83

[1)] Direction: F=Forward or R=Reverse [2)] Melting temperature

Primer working solution (2 µM of each primer):

Stock solutions were prepared by adding dH_2O to the dehydrated primer to give a concentration of 100 pmol/µl (List provided by supplier).

Working primer solution for reverse transcription was prepared by adding (2µl 100 pmol/µl reverse primer) to 98 µl dH_2O to give a final concentration 2 pmol/µl (µM).

Working primer solution for real-time RT-PCR was prepared by adding each primer (2 µl 100 pmol/µl forward primer and 2µl 100 pmol/µl reverse primer) to 96 µl dH_2O to give a final concentration 2 pmol/µl (µM).

2.5 Bacteria DNA used for testing of oligonucleotide primers

Oligonucleotide primers was tested on DNA isolated from *B. cereus* strain ATCC 14579 and ATCC 10987.

2.6 Buffers and solutions

2.6.1 Agarose gel electrophoresis buffers and solutions

1XTAE/L:

The following reagents was dissolved in 1 L dH_2O:

Tris Base	4.84 g	
Glacial acetic acid	1.14 ml	
0.5 M EDTA (pH 8.0)	2.0 ml	(**, Section 2.6.2)

6X Gel loading buffer- MBI Fermentas:

0.09 %	Bromophenol blue
0.09%	Xylene cyanol FF
60%	Glycerol
60 mM	EDTA

Standard –100 bp DNA Ladder-mix:

100 bp ladder	10 µl
6X Gel loading Buffer	10 µl
1XTAE buffer	80 µl

2.6.2 Formaldehyde gel electrophoresis buffers and solutions

All solutions used in RNA work were prepared in DEPC treated water.

0.1 % DEPC-H_2O:

1 ml DEPC was added to 1 L dH_2O and mixed overnight. The solution was then autoclaved at 140 ºC for 20 min. in order to hydrolyse and destroy DEPC, which is highly toxic.

10XMOPS Buffer:

MOPS	41.2 g	
5M sodium acetate	16.0 ml	*
0,5 M EDTA (pH 8.0)	20.0 ml	**

41.2 g MOPS and 16.0 ml 5M sodium acetate was dissolved in 800 ml DEPC-H_2O. The pH was adjusted to 7.0. using NaOH, and 20.0 ml 0.5 M EDTA was added. The volume was adjusted to 1L with DEPC-H_2O and sterilized at 140 ºC for 20 min. 10XMOPS buffer was stored at RT in the dark. Buffers yellows with age if it is exposed to light or autoclaved. A light yellow buffer works well but a dark yellow buffer does not.

*5M Sodium Acetat:

Dissolve 41.521 g sodium acetate in 100 ml DEPC H_2O.

**0.5 M EDTA:

93.06 g EDTA was added to 500 ml DEPC-H_2O and mixed. NaOH was added until everything was solved, at pH=8.0

2X RNA Ladder sample Buffer- Bio Labs:

2X TBE (pH 8.3)
13% Ficoll (w/v)
0.01% Bromophenol blue
7 M Urea

Ethidium bromide (0,5µg/ml):

50 µl 1% EtBr and 7.81 g ammonium acetate was added to 1 L 1XMOPS buffer or

50 ul 1% EtBr was added to 1L 1XTAE buffer.

2.7 DNA and RNA standards

100 bp DNA ladder	Bio Labs
RNA ladder	Bio Labs

2.8 Kits and enzymes

Procedure:	**Kit:**	**Supplier:**
Cell number Determination	**Bacteria Counting Kit(B-7277)** SYTO BC Bacteria Stain (Component A) Micropheres Standard (Component B)	Molecular Probes
Live/Dead Staining	**LIVE/DEAD® BacLight Bacterial™ Viability Kit (L-7007)** Comp.A: 1.67 mM SYTO9 + 1.67 mM Propidium iodide Comp.B: 1.67 mM SYTO9 + 18.3 mM Propidium iodide	Molecular Probes
Oxygen determination	**CHEMets Kit Dissolved Oxygen K-7501**	CHEMetrics
PH determination	pH indicator strips- Neutralit pH 5-10 and Acilit pH 0-6	Merck
RNA Isolation	**FastRNA® Pro Blue Kit** RNApro Solution, Matrix tubes and DEPC-H_2O	Q-Biogene
PCR	**DyNAzyme ™ II DNA polymerase**	Finnzymes
DNase treatment	**Deoxyribonuclease I, Amplification Grade** Dnase I, Amp grade 10X Dnase I reaction Buffer 25 mM EDTA (pH 8.0)	Invitrogen
DNA synthesis	10 mM dNTP-mix.	Finnzymes
Reverse transcription	**SuperScript ™ II Revers Transcriptase** SuperScript II RT 5X First Strand Buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) 0.1 M DTT	Invitrogen
Real time RT-PCR	**DyNAmo ™ Capillary SYBR®Green qPCR (F-420S)** 2X Mastermix: Modified DyNAmo hot start DNA polymerase, SYBR Green I, optimised PCR Buffer, 6 mM $MgCl_2$, dNTP mix including dUTP	Finnzymes

2.9 Laboratory and technical equipment

Standard laboratory equipments and following technical equipment were used.

Procedure:	**Equipments:**	**Supplier:**
Filter sterilization	SteriCup-0.22 µm	Millipore Corporation
Cell number determination	FACSCalibur CellQuest Pro software 5 ml Polystyrene Round-Bottom tube 12x75 mm style	Becton Dickinson
Microscope	AxioPlan 2 Imaging and AxioCam HRC Axiovision 3.1	Carl Zeiss
Cell lysing and RNA isolation	FastPrep FP120	Thermo electron Corporation
Determine RNA concentration	PU8750 UV/VIS Scanning Spectrophotometer	Philips
	Quarts cuvettes	Pharmacia
PCR	Techne Genius	Tamro Lab AS
Gel electrophoresis	Power PAC 300	BioRad
UV-light camera	UVP BioDoc-It system	UVP
Real-time RT-PCR	LightCycler Light cycler Capillaries Roche Molecular Biochemicals LightCycler Software version 3.5	Roche

2.10 Bioinformatics resources on internet

BLAST	http://www.ncbi.nlm.nih.gov/BLAST
KEGG	http://www.kegg.com
Primer 3	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi
Pubmed	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi

3.METHODS

3.1 Sterilization

Standard sterile techniques for micro- and molecular- biologic work were used. Glassware and most solutions were autoclaved at 140 ºC for 20 min., while growth media and water used in flow cytometry were sterilized by filtration using Stericup^{TM} (0.22 µm GP express PLUS Membrane) and the storage system from Millipore.

3.2 Media and growth conditions

B. cereus ATCC 14579 was grown in a *B. subtilis* defined medium and different TSB and LB based media (Section 2.3.1).

3.2.1 Over night culture of *B. cereus* ATCC 14579 (ON-culture)

One colony from a LB agar plate was transferred into 20 ml liquid media in a 100 ml Erlenmeyer flask and incubated at 30 ºC, with shaking at 175 rpm, over night.

3.2.2 Growing *B. cereus* ATCC 14579 - Growth curve

In the determination of the growth curves, 100 ml liquid media was inoculated with 10 µl of an ON-Culture in a 1L Erlenmeyer flask (surface area 113 cm^2) or a box with a large surface area (530 cm^2) and incubated at 30 or 37 ºC, with or without shaking at 175 rpm, for 24 hours (Figure 3.1 a and b).

a) **b)**

Figure 3.1: Pictures shows an example of **a)** A batch culture in a 1 L Erlenmeyer flask (surface area 113 cm^2) and **b)** A large surface area culture (530 cm^2), both containing 100 ml liquid media.

3.3 Determination of cell number, pH and oxygen levels

3.3.1 Determination of cell number using flow cytometer

The cell number was determined using the Bacteria Counting Kit (B-7277) from Molecular probes and provides the data for a detailed growth curve. The bacteria cultures grown without shaking, were mixed for approximately 5 sec. to give a representative sample, before the culture was removed for cell counting.

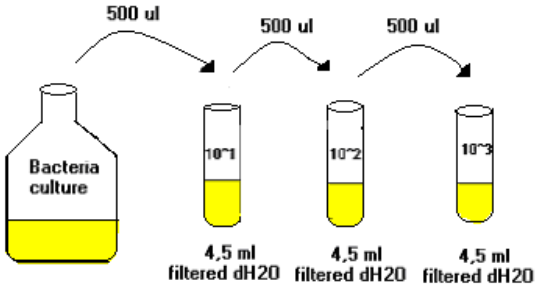


Figure 3.2: Sampling and dilution of bacteria suspension (10^1,10^2 and 10^3 times) for the determination of cell numbers.

Bacteria suspension samples (500µl) were removed at one hourly interval, and diluted 10^1,10^2 and 10^3 times in sterile filtered dH_2O (Figure 3.2). The diluted bacteria suspension (1 ml) was transferred to a 5 ml Polystyrene Round-Bottom tube. SYTO BC Bacteria stain (1 µl) and microsphere standard (10 µl) were added to the diluted bacteria suspension and mixed. 1 ml filtered dH_2O + 1 µl SYTO BC was used as a blank. The sample was analyzed on a FACSCalibur flow cytometer, with CellQuest Pro software (Both Becton Dickinson).

Number of bacteria in 1 ml was calculated in following way:

Time 0: (No.of bacteria (ON-culture)/No.of microsphere)* D.f*10^6 *0.01 /10000

Time 1-24: (No.of Bacteria/No.of Microsphere) * D.f * 10^6

3.3.2 Determination of cell number using viable count

The cell number was also determined using viable counts. Samples of the bacteria suspension (500 µl) were removed every second hour and diluted 10^1-10^8 times in LB media. 100 µl from different dilutions was plated on agar plate and incubation at 30 °C overnight. The numbers of colonies were counted.

Number of bacteria in 1 ml was calculated in following way:

Number of colonies*10*D.f

3.3.3 Generation time

The generation time was calculated during exponential growth for *B. cereus* ATCC 14579 using following formula (Madigan et al.,2003, pp. 143):

$$N=N_0 2^n$$

Whereas N=Final cell number, N_0=Initial cell number and n= Number of generation

Log N= log N_0 + n Log 2

Log N – Log N_0= n log 2

n= log N-log N_0/ log 2

It is possible to calculate n from the above formula and when t is known it is possible to calculate the generation time, g.

$$g= t/n$$

where g= Generation time and t=hours/minutes of exponential growth

3.3.4 pH measurement

The pH of the bacteria suspension was determined using Neutralit® pH-indicator strips from Merck with 0.5 pH unit precision. The bacteria suspension (15 µl) was applied directly to the strips.

3.3.5 Determination of oxygen level

Oxygen levels were determined at given times during growth using Oxygen CHEMets Kit 0-1 ppm (mg/L) with 0.2 unit precision. Bacteria suspension samples (1-2 ml) were removed and applied to the glass tubes and placed on bench for two minutes. The CHEMet ampoule was inserted so that the tip is at the bottom of the sampling tube. The tip was then snapped and the ampoule was filled. Color comparison was performed within 30 sec. to a standard. Best color matches were found and oxygen concentrations were estimated.

3.4 Microscope

3.4.1 Light microscopy

Light microscopy was used to investigate possible contaminations in the bacteria cultures three or four times during each growth experiments. Bacteria suspension (5 µl) was applied to a microscope slide, a cover slip was added on the top and the slide was examined in a phase contrast microscope.

3.4.2 Live/Dead staining

Bacteria suspension were treated with LIVE/DEAD BacLight™ Bacterial Viability Kit and viewed in a fluorescence microscope in order to distinguish live from dead bacteria and see the proportions of live and dead bacteria at different times during growth. AxioPlan 2 Imaging, AxioCam HRC and Axiovision 3.1 software, were used to view and take picture of the preparations.

1. Samples (1 ml) were removed and centrifuged at 13000 rpm for 5 min.
2. The supernatant was discarded, the pellet was resuspended in 1 ml of filtered dH_2O and incubated at room temperature for 1 hour with mixing every 15 min.
3. Centrifuge at 13000 rpm in 5 min.
4. The supernatant was discarded. The pellet was resuspended in 1ml filtered dH_2O and incubated at RT for 30 min.
5. 1.5 µl Component A (SYTO 9 stain: Propidium iodide (1:1)) and 1.5 µl of Component B (SYTO 9 stain: Propidium iodide (1:10)) were added to 1 ml bacteria suspension.
6. Samples were incubated in the dark at RT for 15 min.
7. 5 µl of stained culture was applied to a microscope slide and air dried in dark.
8. 1 drop of Component C (mounting oil) was added to the dried suspension and a cover glass was applied on top.
9. The preparation was excitated at 480 or 490 nm and observed in the fluorescent microscope. Pictures were taken using 40X and 100X enlargement.

3.5. Testing of PCR Primers

3.5.1 Polymerase chain reaction

The specificity of the primers were tested by performing PCR on DNA extracted from *B. cereus* strain ATCC 14579 and ATCC 10987 (PCR program table 3.1).

The following components were mixed in a PCR-tube:

2.5 µl 10X PCR reaction buffer (Finnzymes)

1.25 µl 200 µM dNTP Mix

2.0 µl DNA template (ATCC 10987 or ATCC14579)

2.5 µl Primer working solution

0.5 µl DyNAzyme ^{TM} II DNA Polymerase

16.25 µl Autoclaved dH_2O

Table 3.1:PCR Program

Step	Incubation Temperature	Incubation time
Initial denaturation	94 ºC	2 min.
Denaturation	94 ºC	30 sec.
Annealing	50 ºC	30 sec.
Elongation	72 ºC	30 sec. (Go to step 2, repeats 29 times)
	72 ºC	5 min.
	4 ºC	Forever

3.5.2 Agarose gel electrophoresis

The PCR products were analyzed on a 1.5 % Agarose gel. 1.8 g agarose was suspended in 120 ml 1XTAE buffer and heated in a microwave until the agarose was dissolved. The solution was cooled to approximately 60 ºC before pouring into a gel chamber and the well forming comb was put into place. After cooling the gel was transferred to the electrophoresis chamber containing 1XTAE buffer.

The PCR-Product (5 µl), 6XGel Loading Buffer (2 µl) and 1XTAE-Buffer (3 µl) were mixed and loaded onto the gel. Standard-100 bp DNA ladder mix (10 µl) was used as a size standard (Section 2.6.1). The electrophoresis was carried out in a horizontal chamber at 90 V for 120 min. (Power PAC 300(BioRad)). The gel was stained with ethidium bromide (0.5 µg/ml) for 45 min. and destained overnight in dH_2O. The gel was illuminated with UV-light to visual the ethidium bromide stained PCR-product and a picture was taken (UVP BioDoc-It system (UVP)).

3.6 RNA isolation and formaldehyde gel electrophoresis

3.6.1 Isolation of Total RNA using FastPrep

Total RNA was isolated from the bacteria culture using FastPrep FP120 (Thermo electron Corporation). Samples containing approximately $5.0*10^9$ and $1.0*10^9$ bacteria cells were taken after 5,6,7,8,9,10,12 and 24 hours of cultures grown with and without shaking. The amount of bacteria cells extracted for RNA isolation, was calculated on the basis of earlier growth experiment. Results and are shown in table 3.2.

Table 3.2: Volume of cell suspension extract for RNA isolation

Time	Batch culture grown with shaking			Batch culture grown without shaking		
	# Cells in 1 ml [1)]	ml extract [2)]	# Cell [3)]	# Cells in 1 ml [1)]	ml extract [2)]	# Cell [3)]
5	4.66E+08	10.00	5.0E+09	8.12E+07	12.35	1.0E+09
6	1.79E+09	2.90	5.0E+09	1.43E+08	7.14	1.0E+09
7	2.41E+09	2.00	5.0E+09	2.08E+08	5.00	1.0E+09
8	3.54E+09	1.67	5.0E+09	2.24E+08	4.54	1.0E+09
9	3.30E+09	1.67	5.0E+09	2.88E+08	3.57	1.0E+09
10	3.95E+09	1.67	5.0E+09	5.69E+08	1.75	1.0E+09
12	3.48E+09	1.67	5.0E+09	3.75E+08	1.75	1.0E+09
24	3.68E+09	1.67	5.0E+09	7.77E+08	1.75	1.0E+09

1) Approximately cell number determined in earlier experiments

2) X ml extract = Required #cell /# Cells in 1 ml

3) Required # cells

RNA isolation was performed according to manufactures instruction step (Detailed protocol in Appendix 1) with the following modifications: Step 1-5 was performed in the following way:

1) An equal volume of the bacteria suspension and 100% ice cold methanol was mixed in order to kill the bacteria and avoid change in mRNA level. Tubes were inverted a couple of times and placed at RT for 5 min.
2) Samples were centrifuged at 4000 rpm at 4 ºC for 5 min., the supernatant was discarded and the pellet was quickly cooled on ice and stored at –70 ºC overnight.
3) RNA was extracted by resupending the pellet in 1 ml RNApro solution and transfer to a blue-cap tube containing Lysing Matrix B provided in the kit.

Step 7 was performed in RT and in step 17 RNA was stored at –70 ºC.

3.6.2 Spectrophotometric determination of RNA concentration

RNA concentration was determined and purity estimated by measuring the absorbance at 260 nm and doing a λ-scan between 230-600 nm, using a PU8750 UV/VIS Scanning Spectrophotometer (Philips) and 500 µl quartz cuvette (Pharmacia). The quartz cuvette was washed with DEPC-H_2O twice before use, to remove Rnases. The purified RNA (5 µl) was diluted in DEPC-H_2O (495 µl). 500 µl DEPC-H_2O was used as a blank.

The RNA concentration was calculated using the following formula:

$$\mu g \text{ RNA per ml} = 100 * OD_{260} * 40 \ \mu g/ml$$

Where an absorbance of 1 is equivalent to an RNA concentration of 40 µg/ml for single stranded RNA. The most accurate readings are obtained when the absorbance is between 0.15 and 1.0. If the absorbance was above 1.0 the sample was diluted using DEPC-H_2O.

3.6.3 Formaldehyde gel electrophoresis of isolating RNA

Purified RNA was analyzed by formaldehyde gel electrophoresis.

1) 1.2% Agarose formaldehyde gels:

Small gel- 50 ml (10 cm)	**Large gel-120 ml (20 cm)**	
0.6 g	1.44 g	Agarose
30 ml	72 ml	DEPC-H_2O
9.2 ml	22 ml	36% Formaldehyde
5 ml	12 ml	10XMOPS Buffer

Agarose was suspended in DEPC-H_2O and heated in a microwave until the agarose was dissolved. The solution was cooled to approximately 60 °C and a given amount of 36% formaldehyde and 10XMOPS buffer (Section 2.6.2) were added. The volume was adjusted to 50 ml or 120 ml using DEPC-H_2O and poured into a gel chamber. The solidified gel was transferred to the electrophoresis chamber containing 1XMOPS buffer.

The RNA samples were prepared by mixing following component in a PCR-tube:

5 µl	RNA or RNA Ladder
2 µl	10XMOPS Buffer
2 µl	36% Formaldehyde
10 µl	Formamide
1 µl	DEPC-H_2O

- Samples were incubated for 15 min. at 70 ˚C and quickly chilled on ice.
- 8 µl RNA sample was mixed with 2 µl 2XRNA Sample Buffer.
- The gel was pre-run for 5 min. at 5V/cm before loading the samples.
- The RNA samples were loaded onto the gel and run in 1XMOPS Buffer for 75 min. at 4V/cm.
- The gel was stained with ethidium bromide (0.5 µg/ml) in 0.15 M ammonia acetate for 45 min. and destained overnight in dH_2O.
- The gel was removed from the chamber and illuminated by UV-light to visual RNA bands and a picture was taken (UVP BioDoc-It system (UVP)).

3.7 Real-time RT-PCR

3.7.1 DNase treatment of isolated RNA

Genomic DNA contamination can lead to false positive RT-PCR results. DNase treatment is used to eliminate contaminating DNA from the RNA samples prior to RT-PCR. Deoxyribonuclease I, Amplification Grade from Invitrogen was used.

- Following components was added to a PCR-tube on ice.
  1 µg RNA
  1 µl 10X DNase reaction buffer
  1 µl DNase 1, Amp grade, 1U/µl
  Adjust Volume to 10 µl with DEPC-H_2O.
- The mixture was incubating at room temperature for 30 min.
- 1 µl 25 mM EDTA was added and a new incubation at 65 ºC for 10 min. was performed to inactivate DNase.

3.7.2 Reverse transcriptase

Reverse transcription of RNA were performed using the SuperScript TM II Reverse Transcriptase Kit from Invitrogen.

The following components are added to a nuclease free centrifuge tube:

1 µl gene specific primer (reverse) (2pmol/ul)

11 µl of the DNase treated RNA-mix.

1 µl 10 mM dNTP-mix

- The mixture was incubated at 65 ºC for 5 min. and was quickly chilled on ice.
- The following components was added and gently mixed:

  4 µl 5X first-strand buffer

  2 µl 0.1 M DTT

- The mixture was incubating at 42 ºC for 2 min.
- 1 µl (200 U) SuperScript II RT was added and mixed thoroughly
- Incubation was carried out at 42 ºC for 50 min., and the enzyme was inactivated by heating at 70 ºC for 15 min.

3.7.3 Real-time PCR using SYBR Green

Real time PCR was performed using a LightCycler, Roche Molecular Biochemicals. The cDNA was diluted by a factor of 10^1, $10^{1,5}$,10^2, $10^{2,5}$, 10^3 prior to real-time PCR (Figure 3.3).

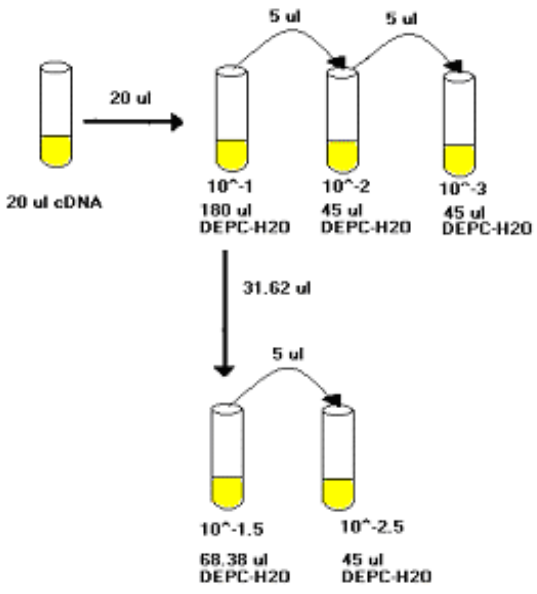


Figure 3.3: Dilution of cDNA samples prior to real-time PCR.

The following components were mixed and transferred to LightCycler capillary tubes:

- 10 µl 2X Master Mix (DyNAmo^{TM}Capillary SYBR$^{®}$Green qPCR.)
- 5 µl gene specific primers (Forward and Reverse, 2µM each)
- 5 µl cDNA template

To check for DNA contamination two negative controls were performed for each experiment. One with DEPC-H_2O instead of cDNA template and one with RNA treated with DNase but not reverse transcribed. The latter was diluted 10 times using DEPC-H_2O. The capillary tubes were placed in the Light Cycler carousel and centrifuged. The carousel was then placed in the thermal cycler and run using the cycling program described in Table 3.3. Analyses of Real-time PCR data were performed using Roche Molecular Biochemicals LightCycler Software version 3.5.

Table 3.3-Light cycle program

Step	Temp. [1)]	Time (min:sec)	Temperature Transistion rate (˚C /sec)	Acquisition mode	Analysis mode
Initial Denaturation	95 ºC	10:00	20.00	None	None
Amplification x 40					
Denaturation	95 ºC	00:10	20.00	None	Quantitiative
Annealing	53 ºC	00:20	20.00	None	Quantitiative
Extension	72 ºC	00:10	20.00	Single	Quantitiative
Melting curve					
Denaturation	95 ºC	00:00	20.00	None	Melting curve
Re-annealing	57 ºC	00:15	20.00	None	Melting curve
Denaturation	98 ºC	00:00	0.10	Cont. [2)]	Melting curve
Cooling	40 ºC	00:10	20.00	None	None

1) Temp. =Temperature , 2) CONT.= Continuous

3.8 Mathematical and statistical analysis

Relative quantification is used to determined changes in mRNA expression levels. This method measures the changes in mRNA expression level of a target gene relative to a reference gene for which the expression levels do not change.

3.8.1 Amplification efficiency (amp.eff)

Amplification efficiency is a measurement of the efficiency of amplification of a particular sequence. Amp.eff is determined by plotting a regression line for each gene,

in which $\log_{10}$ of the dilution factors are plotted against Ct-values (Lunde et al. 2003). An example of a regression line is shown in figure 3.4.

The formula describes PCR in the exponential phase:

$$X_n = X_0 * (1 + E_x)^n \qquad (1)$$

X_n is the number of target molecules in cycle n, X_0 is the number of target molecules before amplification, E_x is the amp.eff of the target sequence and n is the number of cycles. This formula can be reorganized and used to calculate amp.eff:

$$E_x = (X_n / X_0)^{1/n} - 1 \qquad (2)$$

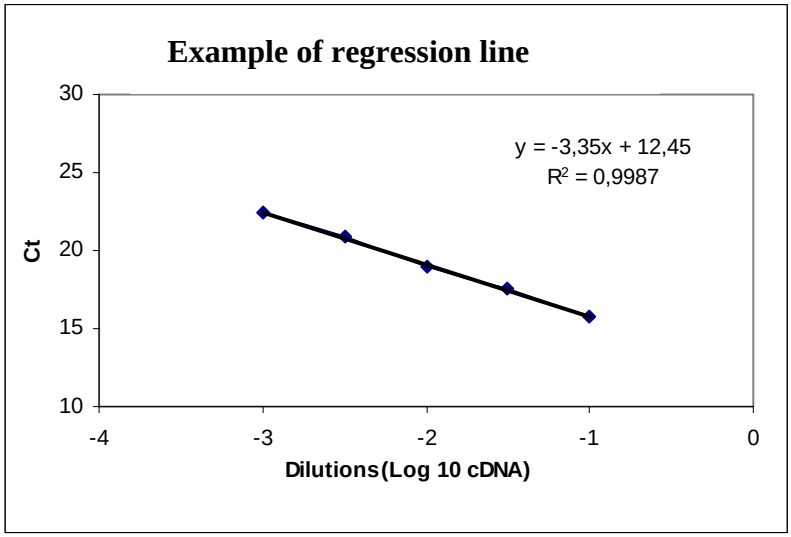


Figure 3.4: Illustrating a regression line used to calculate amp.eff. Log_{10} of the dilution factors are plotted against C_T-values.

The amount of cDNA increases by a factor of ten each unit from left to right along the x-axis. This means that X_n / X_0 can be set to 10. The absolute value of the slope of the regression line therefore gives the number of cycle needed to multiply the amount of cDNA by 10. The amp.eff (E_x) of a sequence is calculated from the slope, according to formula 3, where n is the absolute value of the slope.

$$\mathbf{E_x = 10^{1/n} - 1} \qquad (3)$$

3.8.2 Relative quantification in Real-time PCR: the Pfaffl Method

Pfaffl presented a new mathematical model to determine relative quantification of a target gene in real-time RT-PCR (Pfaffl, 2001). This method uses amplification efficiencies and crossing point (Ct) to determine the relative expression ratio of a target gene versus a reference gene.

A reference gene is used to normalize sample data with respect to tube-to-tube differences caused by variation in RNA quality, PCR reaction, amplification efficiencies or sample loading. Glyceraldehyd-3-phosphate dehydrogenase was used as a reference gene in this project. The mRNA synthesis of a reference gene is considered to be stable and constant in all samples, even under experimental treatments (Marten et al. 1994; Foss et al. 1998; Thellin et al. 1999). However, numerous studies have shown that the housekeeping genes are regulated and vary under experimental conditions (Zhang & Snyder 1992; Bhatia et al. 1994; Bereta 1995; Chang et al. 1998).

Sample isolated 5 hours after inoculation are used as a control and the amplification of the target sequences is calculated according to the results from time 5. Time 5 is defined to have an expression of 1 and the other samples are calculated as expression fold changes compared to the results from time 5. Calculation of relative expression ratio for selected genes is shown in the equation 4:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta Ct\ \text{target (control-sample)}}}{(E_{\text{ref}})^{\Delta Ct\ \text{ref (control-sample)}}} \qquad (4)$$

Where E_{target} is the amp.eff of the target gene + 1, ΔCt_{target} is the Ct-value of the control minus Ct value of the target sequence. E_{ref} is the amp.eff of the reference gene +1. ΔCt ref is the Ct value of the control minus Ct value of the target sequences.

4.RESULTS

A series of experiments and calculation were carried out in order to find a suitable inoculum volume. 10 µl inoculum of an overnight culture was found to be acceptable for growth of *B. cereus* ATCC 14579 in 100 ml medium, and was used as inoculum volume in all experiments.

4.1 Comparison of bacteria counting methods

In this project flow cytometry was the main method used to determine the number of bacteria cells (Section 3.3.1). Viable counts were performed in order to compare the results from flow cytometry to this often used method. *B. cereus* were grown in 1L Erlenmeyer flask (surface area 113 cm^2) in 20%, 50% and 100% LB media and growth curves determined by different counting methods are shown in figure 4.2 a, b and c.

$OD_{600\ nm}$ measurements were also measured for the bacteria grown in 100% LB culture, by Karoline Fægri from "Kolstø group"(School of Pharmacy, University of Oslo). The results were plotted into the graph, shown in figure 4.1.

4.1.1 Comparing flow cytometry vs. OD_{600nm} measurement

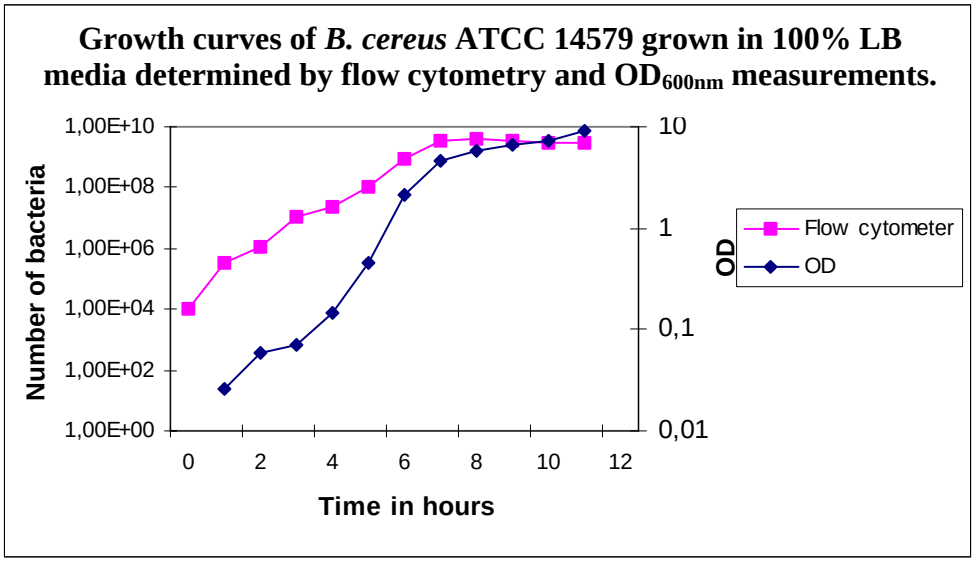
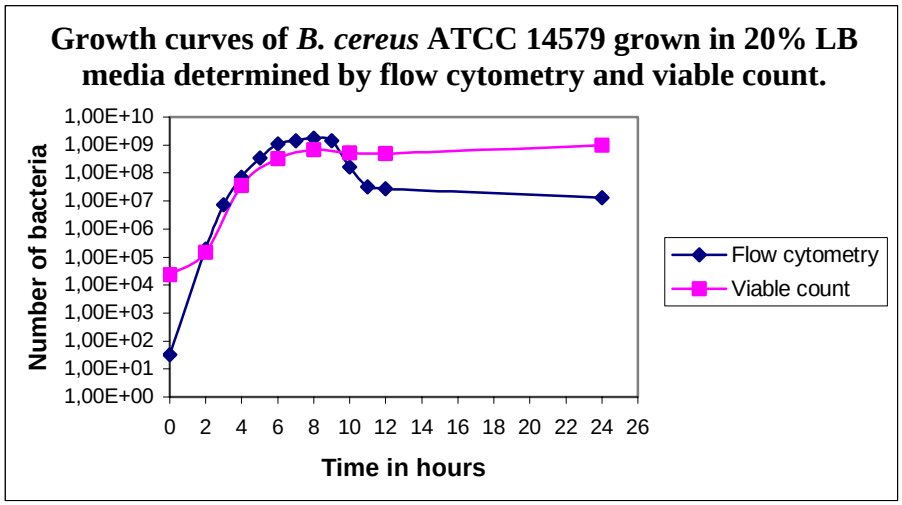


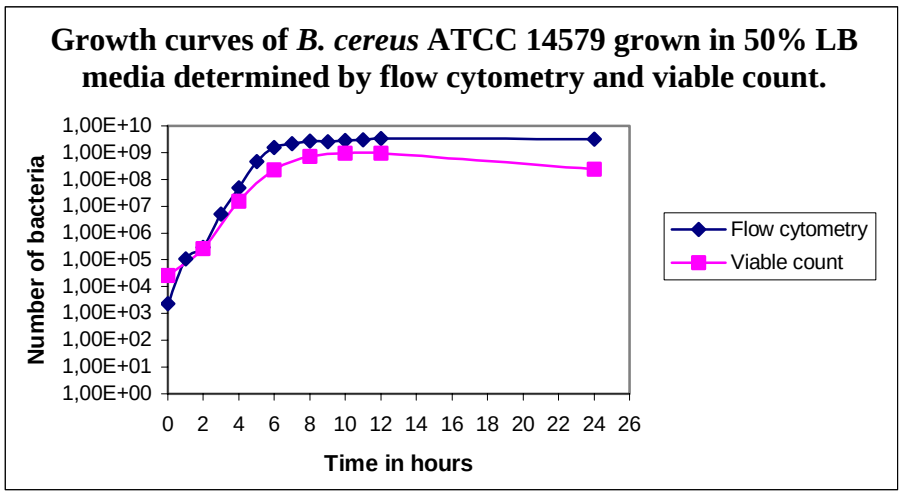
Figure 4.1: Comparison of cell numbers determined by flow cytometry and cell density determined by $OD_{600\ nm}$, for *B. cereus* ATCC 14579 grown in 1L Erlenmeyer flask in 100% LB medium at 30 ºC with shaking (175 rpm). Both Y-axis are logarithmic. Data can be found in table 5.1 and 5.4 (Appendix 5). Insert shows the identity of different curves.

4.1.2 Comparing flow cytometry vs. viable count

a)



b)



c)

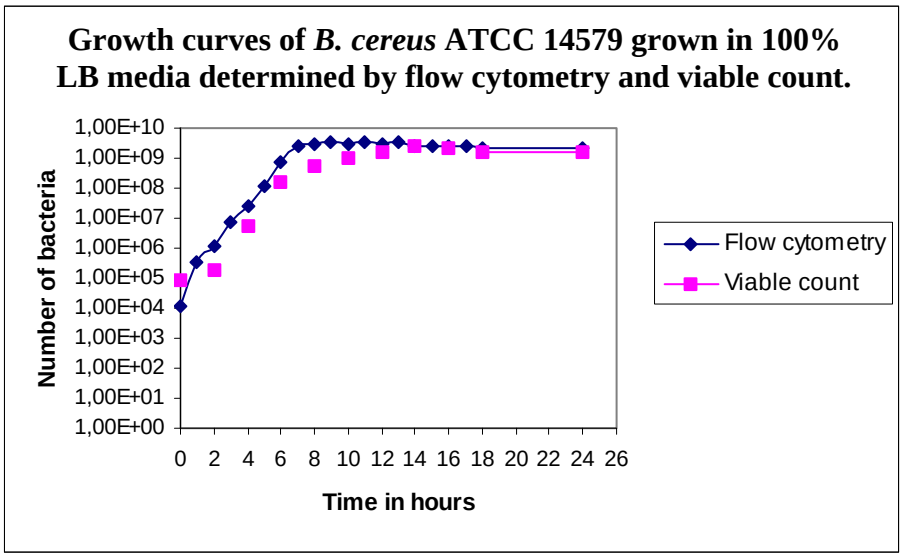
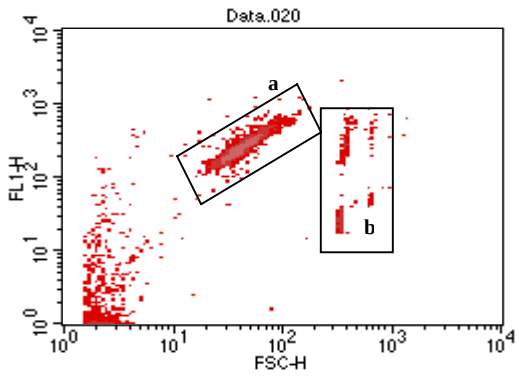


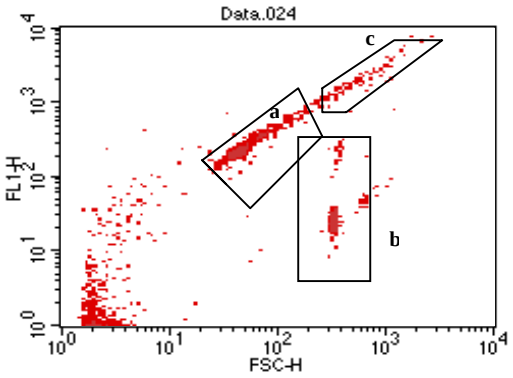
Figure 4.2: Growth curves of *B. cereus* ATCC 14579 grown in: **a)** 20% LB medium **b)** 50% LB medium and **c)** 100% LB medium. Cell numbers were determined by flow cytometry and viable counts. All experiments were performed at 30 ºC with shaking at 175 rpm. Data and calculation of cell number/ml can be found in table 3.1 and 3.2 (Appendix 3), 4.1 and 4.2 (Appendix 4) and 5.1 and 5.2 (Appendix 5) for bacteria grown in 20%, 50% and 100% LB media, respectively. Insert shows the identity of the different curves.

An example of the flow cytometry results:

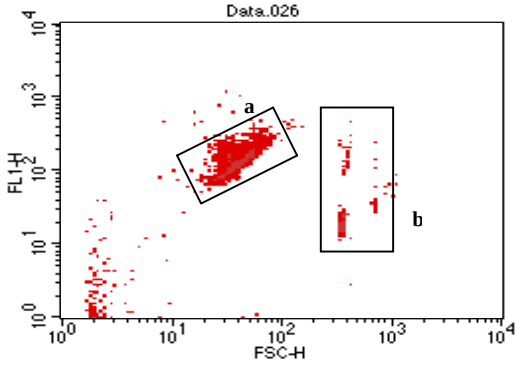
The figures below show examples of graphical plots of the primary flow cytometry data. Forward scatter is plotted against green fluorescence. Growth media and time are indicated below. Bacteria normally appear as points in the upper left hand frame (a), microsphere particles appear in the lower right hand frame (b), which were both used to estimate the number of bacteria cells. Points appear in another area (c) in the graphical plot for bacteria culture with a relatively large extent of aggregation.

20% LB medium (time 8 and 10 hours)

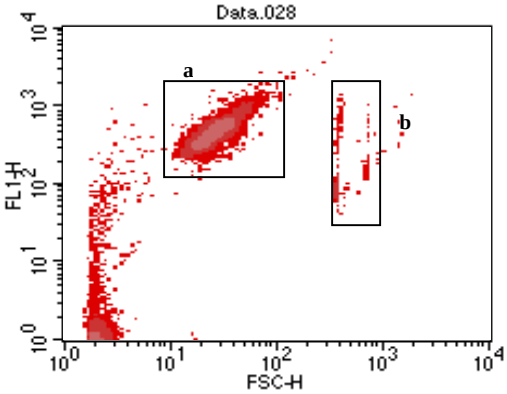




50% LB medium (time 10 hours)



100% LB medium (time 10 hours)



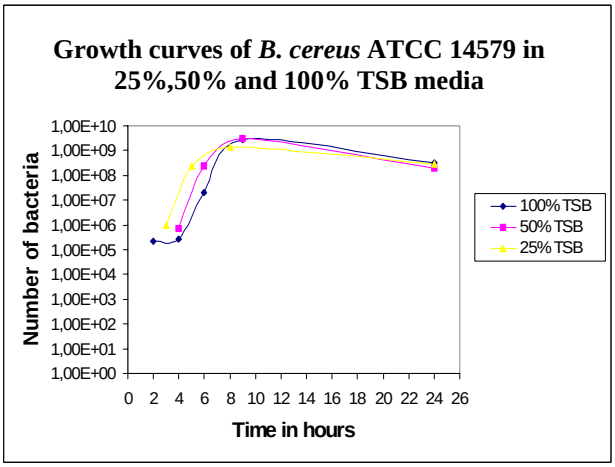
4.2 Comparing growth and pH in seven different media

B. cereus strain ATCC 14579 was grown in seven different media: 1) *B. subtilis* defined medium, 2) 25% TSB, 3) 50% TSB, 4) 100% TSB,5) 20% LB, 6) 50% LB and 7)100% LB media (Section 2.3.1). All experiments were carried out in 1 L Erlenmeyer flasks with shaking at 175 rpm. Growth in TSB media was carried out at 37 °C, whereas the growth experiments in different LB media were performed at 30 °C. Earlier growth experiments carried out at the Biotechnology center indicated that plasmid loss was more likely to occur at 37 °C than 30 °C.

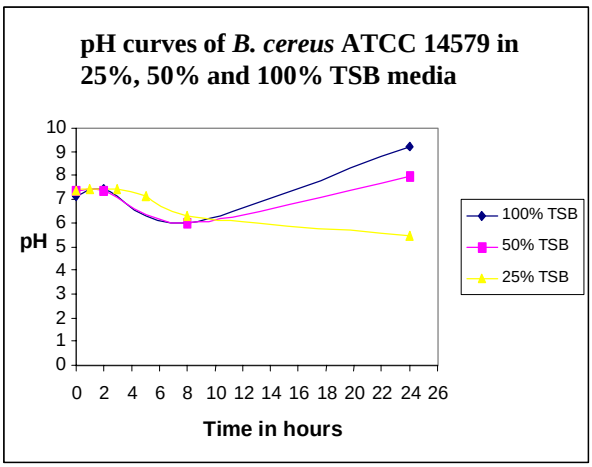
Samples were taken at times indicated in figure 4.3. The number of bacteria cells was determined by flow cytometry and pH strips was used to determined pH (Section 3.3.1 and 3.3.4). The pH measurements were also performed on a negative control, consisting of 100 ml 50% LB medium with no inoculum, in 1 L Erlenmeyer flask. No changes in pH were observed in the control.

B. cereus ATCC 14579 grown in the *B. subtilis* defined medium gave a 1000 times lower bacteria number (10^6 vs. 10^9 bacteria cells/ml), after 24 hours of growth, than bacteria grown in LB or TSB media (Data not shown).

a)



b)



c) d)

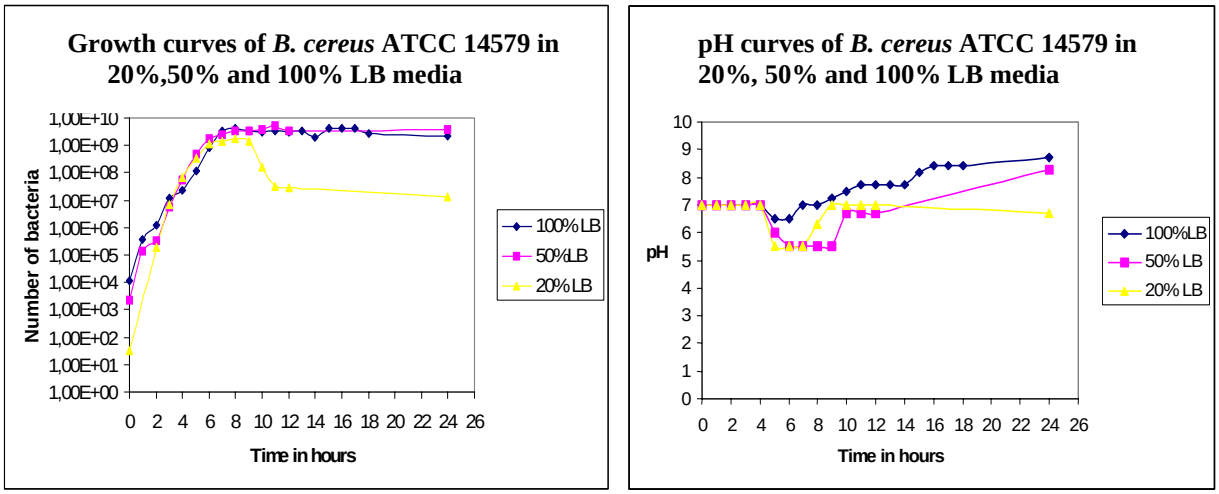


Figure 4.3:a) and **b)** Shows comparison of growth and pH curves of *B. cereus* ATCC 14579 grown in 25% TSB, 50% TSB and 100% TSB media, at 37 ºC with shaking (175 rpm). **c)** and **d)** Shows comparison of growth and pH for *B .cereus* grown in 20%,50% and 100% LB media, at 30 ºC with shaking (175 rpm.) Calculation of cell number/ml and pH can be found in table 2.1, 2.2, 2.3 and 2.4 (Appendix 2) for the TSB cultures, and in table 3.1, 3.3 4.1,4.3, 5.1 and 5.3 (Appendix 3,4 and 5) for the LB cultures. Color codes in the insert show the identity of different curves.

4.3 Glucose level

The glucose concentration was determined in 50% LB medium culture at 37 °C, by Solveig Sirnes and Simen Kristoffersen, using Glucose (GO) assay kit from Sigma. The number of bacteria cells and pH data was plotted into the same graphical view for cultures grown at 37 °C and 30 °C, figure 4.4.

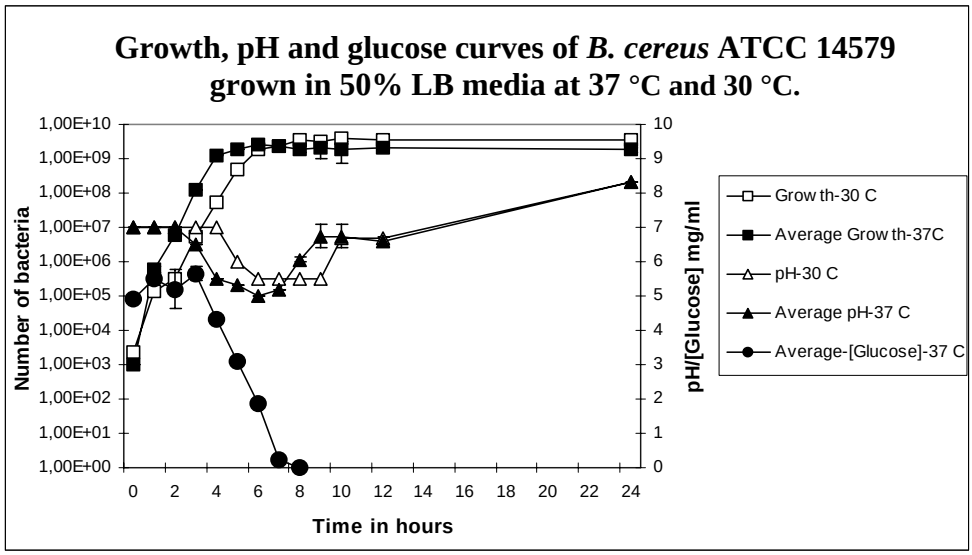


Figure 4.4: Growth, pH and glucose curves of *B. cereus* ATCC 14579 grown in 1L Erlenmeyer flask with shaking. Experiments were carried out in 50% LB medium, at 30 °C or 37 °C. Average cell number for bacteria grown at 37 °C was calculated and the error bars show the standard deviation. The cell number/ml, pH and glucose level can be found in table 12.1 and 4.1(Appendix 12 and 4), for bacteria grown at 37 °C and 30 °C, respectively.

Examples of microscopic images of *B. cereus* ATCC 14579, taken at different times during growth, for bacteria grown in 1L Erlenmeyer flask in 20%, 50% or 100% LB media. The numbers on the pictures indicates time of sampling in hours after inoculation. No contaminations were observed in these cultures (Figure 4.5).

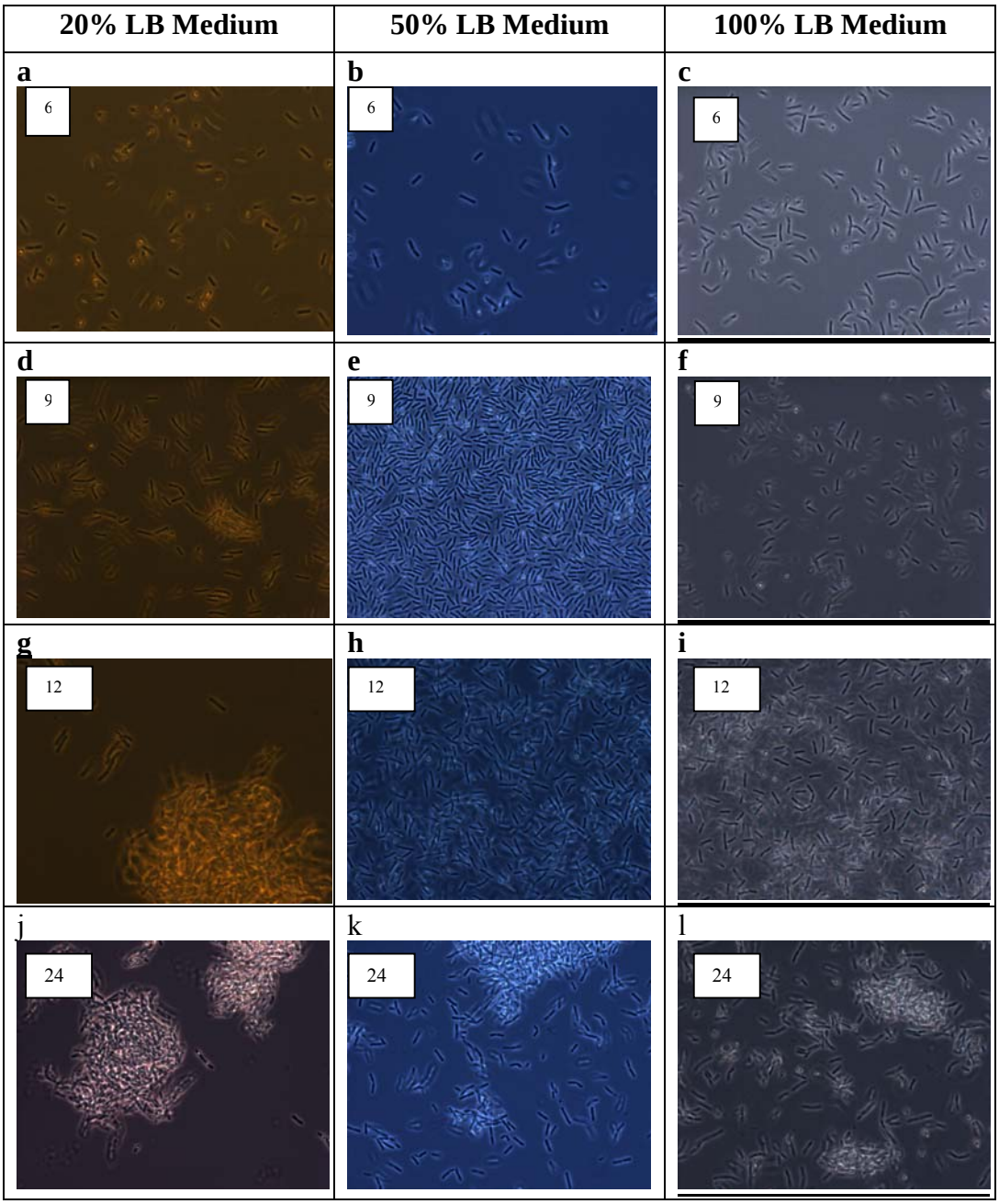


Figure 4.5: Microscopic images of *B. cereus* ATCC 14579 (size~5 µm). The numbers on the pictures indicates time of sampling in hours after inoculation. Picture **a-l** showing bacteria grown in 1 L Erlenmeyer flask with 20%, 50% or 100% LB media, at 30 ºC, with shaking at 175 rpm. Pictures were taken using 100X enlargement.

Proportions of live and dead bacteria cells were also determined in 20%, 50% and 100% LB media cultures, using live/dead staining. Samples were removed at the time indicated in figure 4.6, stained and observed in the microscope.

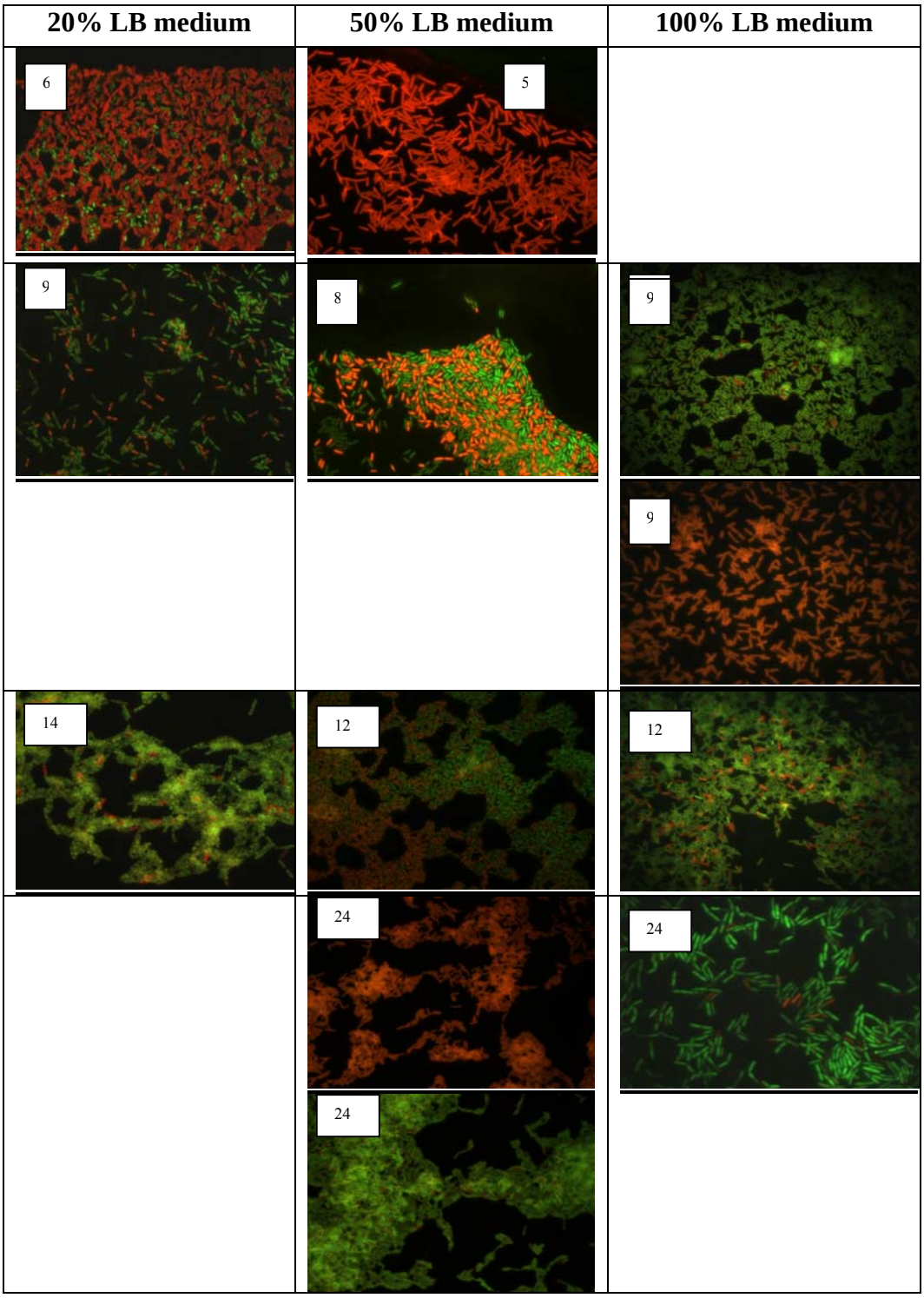


Figure 4.6: Live/dead staining of *B. cereus* ATCC 14579.The bacteria were stained with fluorescent dyes, SYTO9 and Propidium iodide to be able to distinguish live (green) and dead (red) bacteria cells. Pictures were taken using 40X or 100X enlargement. The number on the pictures gives the time of sampling. Sometimes the preparations were so diverse for the same samples that two pictures are shown to give a more representative view of the results.

4.4 Oxygen level

Oxygen level was determined at 0, 3, 5, 8, 12 and 24 hours in all TSB and LB cultures grown with shaking, using Oxygen CHEMets (Section 3.3.5). All shows maximum oxygen levels (1.0 ppm) for all times, even after 24 hours after inoculation (Data not shown).

4.5 Effect of growing *B. cereus* with and without shaking

Bacteria grown in 20% LB medium showed a higher level of aggregation than bacteria grown in 50% or 100% LB media. Bacteria grown in 100% and 50% LB media gave approximately the same number of cells, while the latter showed less change in pH, during growth. Therefore, only the 50% LB medium was used in further growth experiments.

The effect of shaking on bacteria growth was investigated by comparing growth and pH for cultures grown with and without shaking.

4.5.1 Growth in 1 L Erlenmeyer flasks with and without shaking

B. cereus ATCC 14579 were grown in 50% LB medium in 1L Erlenmeyer flasks (surface area 113 cm^2) with and without shaking, at 30 ºC. The results from three independent experiments grown with and without shaking are shown in figure 4.7.

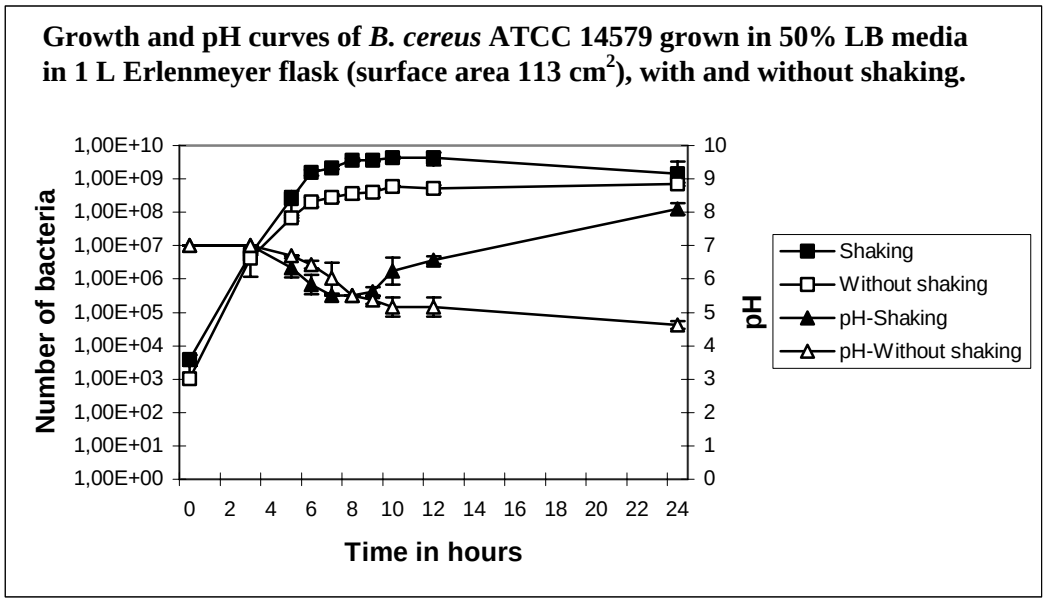


Figure 4.7: Growth and pH curves of *B. cereus* ATCC 14579 grown in 1L Erlenmeyer flask with and without shaking (175 rpm). Experiments were carried out in 50% B medium at 30 ºC. The average cell number was calculated for the three experiments and the error bars show the standard deviation. Cell number/ml can be found in table 4.1 and 6.3 for the cultures grown with shaking and table 6.1 and 6.4 for cultures grown without shaking. pH data can be found in table 4.3, 6.2 and 6.5 (Appendix 4 and 6).

4.5.2 Growth in a large surface area culture, with and without shaking

B. cereus ATCC 14579 was grown in 50% LB medium in a large surface area culture (surface area 530 cm^2) with and without shaking at 30 ºC. Experiments were repeated once and all data was plotted into the same graphical view shown in figure 4.8.

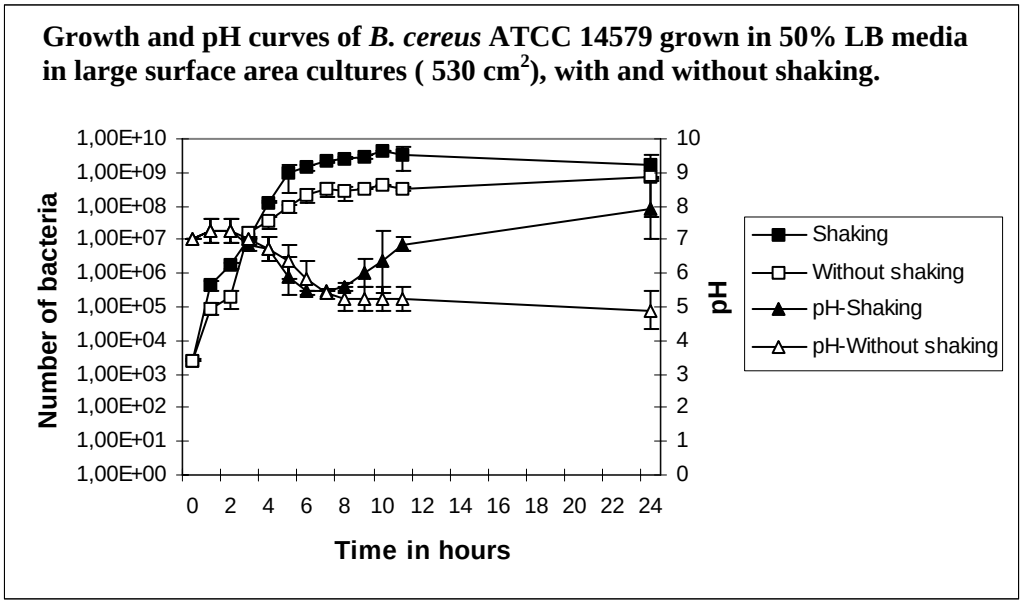


Figure 4.8: Growth and pH curves of *B. cereus* ATCC 14579 grown in a large surface area culture (530 cm^2). Experiments were carried out in 50% LB medium with and without shaking at 175 rpm, at 30 ºC. The average cell number was calculated for the duplicate experiments and the error bars show the standard deviation. Calculation of cell number/ml can be found in table 7.1 and 7.4 for bacteria cultures grown with shaking and table 7.2 and 7.5 for bacteria cultures grown without shaking. pH data can be found in table 7.3 and 7.6 (Appendix 7).

4.6 Effect of surface area

The effect of surface area on bacterial growth was investigated by comparing growth and pH in cultures with different surface area, in 1 L Erlenmeyer flask (surface area 113 cm^2) and with large surface area (530 cm^2), both with and without shaking.

4.6.1 Growth in 1 L Erlenmeyer flasks vs. large surface area cultures, both with shaking

B. cereus ATCC 14579 were grown in 50% LB medium at 30 ºC. All data was plotted into the same graphical view to be able to compare consequence of surface volume (Figure 4.9).

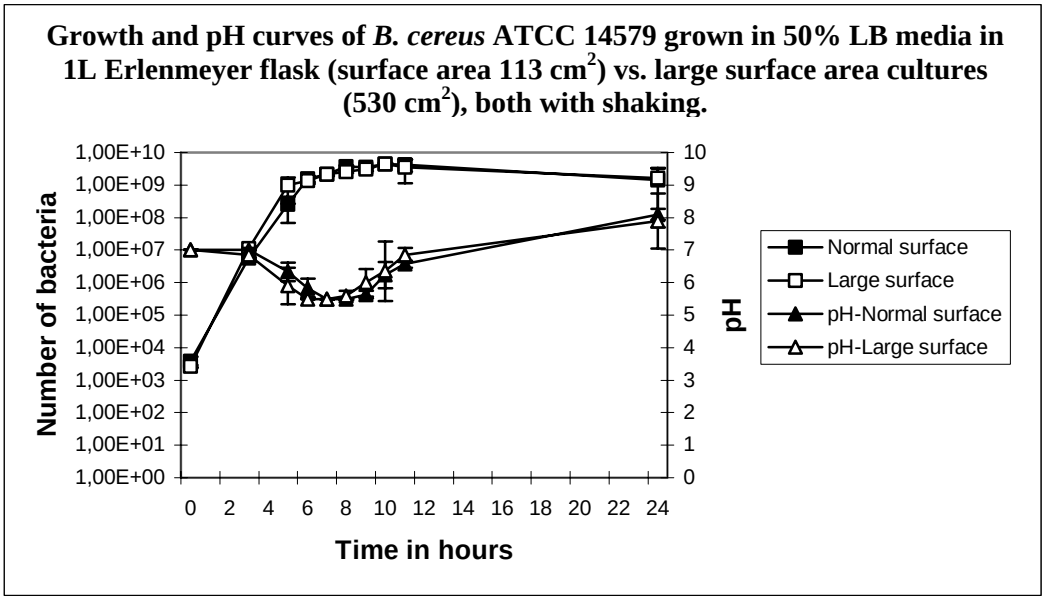


Figure 4.9: Growth and pH curves of *B. cereus* ATCC 14579 grown in 1L Erlenmeyer flasks (surface area 113 cm^2) vs. large surface area cultures (530 cm^2), both with shaking (175 rpm). The experiments were performed in 50% LB medium, at 30 ºC. The average cell number was calculated and the error bars show the standard deviation. Cell number/ml can be found in table 4.1 and 6.3 for bacteria grown in 1 L Erlenmeyer flask and table 7.1 and 7.4 for large surface area culture. pH data can be found in table 4.3, 6.5, 7.3 and 7.6 (Appendix 4,6 and 7).

4.6.2 Growth in 1 L Erlenmeyer flask vs. large surface area cultures, both without shaking

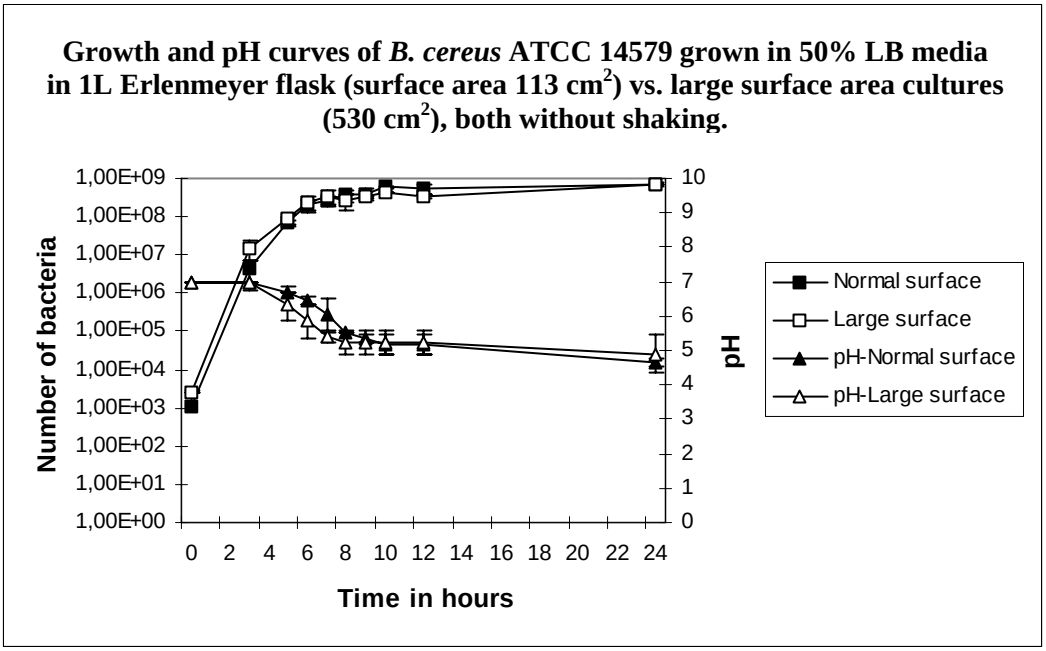


Figure 4.10: Growth and pH curves of *B. cereus* ATCC 14579 grown in 1L Erlenmeyer flasks (surface area 113 cm^2) vs. large surface area cultures (surface area 530 cm^2), both without shaking. The experiments were performed in 50%LB medium, at 30 ºC. The average cell number was calculated and the error bars show the standard deviation. Cell number/ml can be found in table 6.1 and 6.4 for bacteria grown in 1L Erlenmeyer flask and table 7.2 and 7.5 for large surface area culture. pH data could be seen in table 6.2, 6.5, 7.3 and 7.6 (Appendix 6 and 7).

Microscopic images of *B. cereus* ATCC 14579 were also taken during different stage of growth for bacteria grown in 50% LB medium in 1 L Erlenmeyer flask (surface area 113 cm^2) without shaking or in large surface area cultures (surface area 530 cm^2) with and without shaking. No contaminations were observed in these cultures (Figure 4.11).

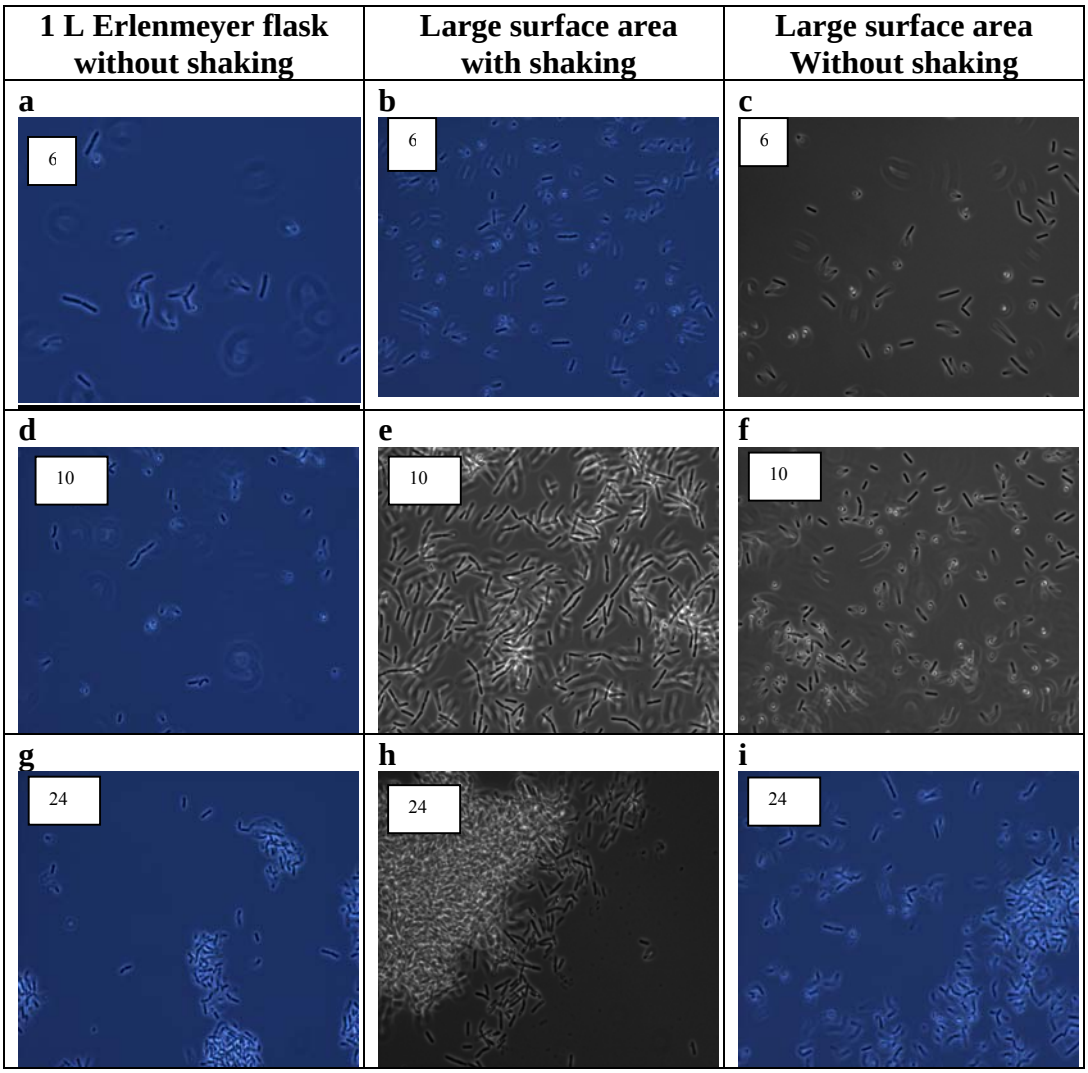


Figure 4.11: Microscopic images of *B. cereus* ATCC 14579 (size~5 µm). The numbers on the pictures gives the time of sampling in hours after inoculation. Pictures shows bacteria grown in 50% LB media in a 1 L Erlenmeyer flask (surface area 113 cm^2) without shaking, and in a large surface area culture (surface area 530 cm^2) with and without shaking. All experiments was performed at 30 ºC. Pictures were taken using 40X and 100X enlargement.

The proportions of live and dead bacteria cells were also determined using live/dead staining. Samples were removed at times indicated in figure 4.12, stained and viewed in microscope.

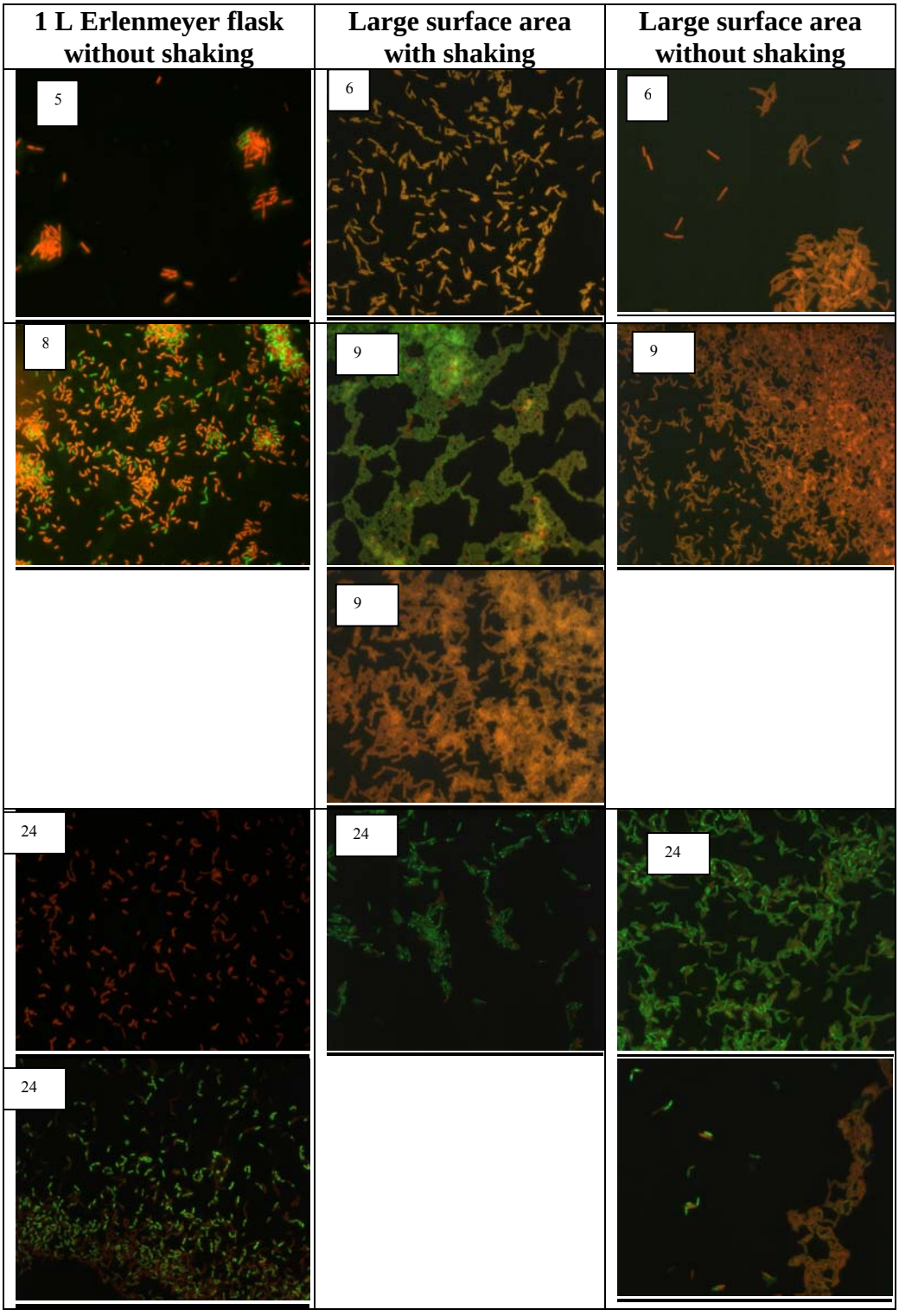


Figure 4.12: Live/dead staining of *B. cereus* ATCC 14579. The bacteria were stained with fluorescent dyes, SYTO9 and Propidium iodide to be able to distinguish live (green) and dead (red) bacteria cells. Pictures were taken using 40X and 100X enlargement. The number on the pictures gives the time of sampling. Some of the preparations were heterogenous and two pictures are shown in order to give a more representative view of the bacteria.

4.7 Generation time

In the laboratory, under optimal growth conditions *Bacillus* species are found to exhibit a generation time of about 25 minutes. The generation time was calculated during exponential growth for *B. cereus* ATCC 14579 (Section 3.3.3).

Table 4.1 Generation time for *B. cereus* ATCC 14579	
Culture	**Generation time**
100% LB-Flow cytometry	25.24 min.
100% LB-Viable count	24.91 min.
50% LB-Flow cytometry	21.53 min.
50% LB-Viable count	23.89 min.
20% LB-Flow cytometry	19.25 min.
20% LB-Viable count	21.73 min.
50% LB-1L Erlenmeyer flask- with Shaking	21.82 min.
50% LB-1 L Erlenmeyer flask-without Shaking	26.86 min.
50% LB-Large surface area culture-with shaking	24.74 min.
50% LB-Large surface area culture with shaking	24.98 min.
50% LB-Large surface area culture without shaking	30.25 min.
50% LB-Large surface area culture without shaking	22.12 min.

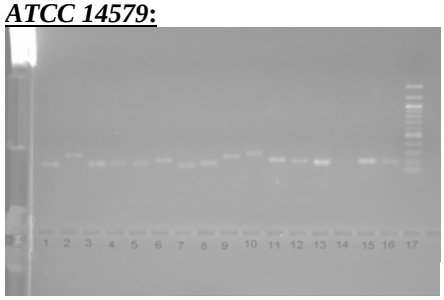
Detail data in appendix 9.

4.8 Primers

Sixteen genes were selected for further investigation. Primers for real-time RT-PCR were tested for specificity, by amplifying DNA from two different *B. cereus* strains, ATCC 14579 and ATCC 10987. The products were analyzed by electrophoresis on a 1.5 % agarose gel and detected by ethidium bromide staining (Figure 4.13).

Lane	Primer name	Product size
1	Fru	194 bp
2	Lac1	283 bp
3	Lac 2	190 bp
4	Acco1	200 bp
5	Acco2	188 bp
6	Acco3	216 bp
7	Pyro	157 bp
8	Glyde	183 bp
9	Cisy1	245 bp
10	Cisy2	200 bp
11	Oxde	220 bp
12	Cytc	203 bp
13	Atp	193 bp
14	Glu1	182 bp
15	Glu2	199 bp
16	Poly	194 bp
17	DNA Ladder	100-1000 bp

***ATCC 14579*:**



ATCC10987:

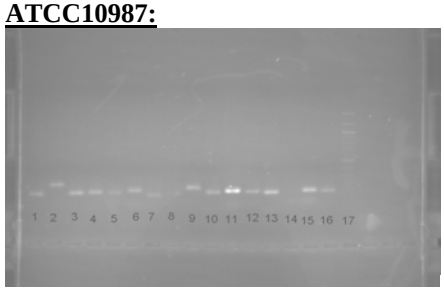


Figure 4.13: Shows the PCR product separated by electrophoresis on a 1.5% agarose gel, for 120 min. at 90V.The table shows the lane number and the corresponding primer names for the selected genes. Lane 17 contains the DNA standard of fragments with know size: 1.517, 1.200,1.000, 900, 800, 700, 600, 500, 400, 300, 200,100 bp. Further details on these primers (Section 2.4).

Due to limited time some restrictive choice was made. Only seven of the sixteen genes above were selected for quantification of gene expression, at time 5 and 12 hours after inoculation. It was decided to focus on the following metabolic genes: Fructose-6-phosphatekinase (Fru), Lactate dehydrogenase (Lac1), Acetyl-CoA synthetase (Acco1), Citrate synthase (Cisy1), α-ketoglutarate dehydrogenase E1(Oxde), Glutaminase (Glu2) and ATP synthase ε-chain(Atp). Glyceraldehyde-3-phosphate dehydrogenase (Glyde) was used as reference gene.

4.9 RNA isolation and determination of RNA concentrations

The surface area does not seem to have any significant influence on cell number and pH, and it was decided to focus on gene expression in bacteria grown in 1L Erlenmeyer flask, with and without shaking.

B. cereus were grown in 1L Erlenmeyer flask in 50% LB medium, with and without shaking at 30 ºC. Bacteria cells were lysed and the total RNA was isolated from both cultures, using the FastPrep method (Section 3.6.1). This was performed in duplicate at the times indicated in table 4.2. The concentration and quality of RNA was estimated by spectrometry. Table 4.2 shows the amount of RNA in µg/ml isolated from each sample and the calculated amount of RNA per cell (Calculation-Appendix 10). The amount of RNA per cell is shown graphically in figure 4.14. The samples were run on formaldehyde gels, and detected by ethidium bromide staining. Results are shown in figure 4.15.

Table 4.2: Total RNA concentration and amount RNA per cell

	Growth with shaking			Growth without shaking		
Sample	**[RNA] µg/ml**	**#cells /ml**	**µg/cells**	**[RNA] µg/ml**	**# cells /ml**	**µg/cell**
5.1	2072	1.79E+09	1.16E-06	724	7.01E+08	1.03E-06
5.2	1956	1.19E+09	1.64E-06	900	8.12E+08	1.11E-06
6.1	1536	5.05E+09	3.04E-07	776	1.46E+09	5.32E-07
6.2	2728	3.45E+09	7.91E-07	776	1.97E+09	3.94E-07
7.1	1808	4.18E+09	4.33E-07	896	1.77E+09	5.06E-07
7.2	1976	4.04E+09	4.89E-07	1012	1.41E+09	7.18E-07
8.1	1392	6.95E+09	2.00E-07	860	2.07E+09	4.15E-07
8.2	1532	5.64E+09	2.72E-07	1332	1.91E+09	6.97E-07
9.1	3412	5.83E+09	5.85E-07	880	1.90E+09	4.63E-07
9.2	2472	6.43E+09	3.84E-07	792	1.38E+09	5.74E-07
10.1	3500	7.62E+09	4.59E-07			
10.2	5152	7.72E+09	6.67E-07			
12.1	2376	1.06E+10	2.24E-07	440	9.43E+08	4.67E-07
12.2	2196	5.23E+09	4.20E-07	492	1.14E+09	4.32E-07
24.1	1136	4.88E+09	2.33E-07			
24.2	1264	4.56E+09	2.77E-07			

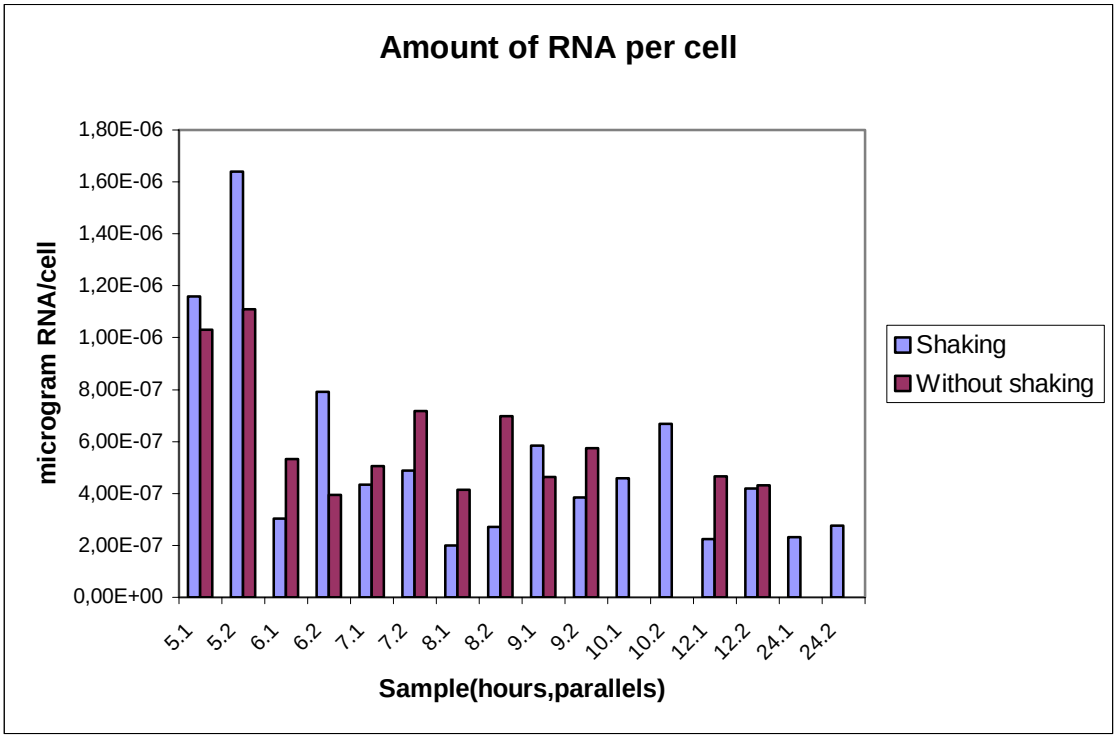
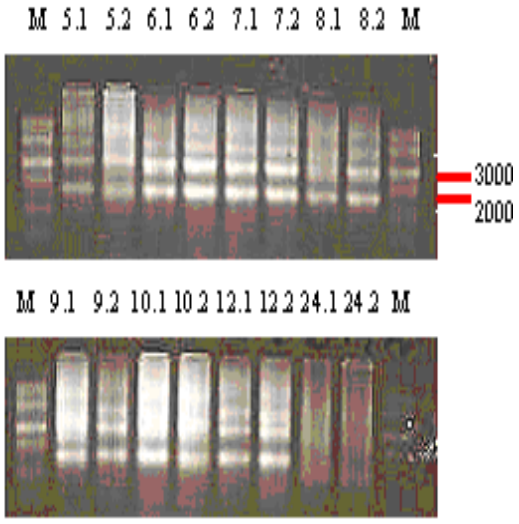


Figure 4.14: The amount of RNA per cell at different times from cultures grown with and without shaking, parallels 1 and 2. The cells were lysed and the RNA was isolated using the FastPrep method. RNA was not isolated from time points 10 and 24 h in the cultures grown without shaking.

a) RNA isolated from cultures grown with shaking



b) RNA isolated from cultures grown without shaking

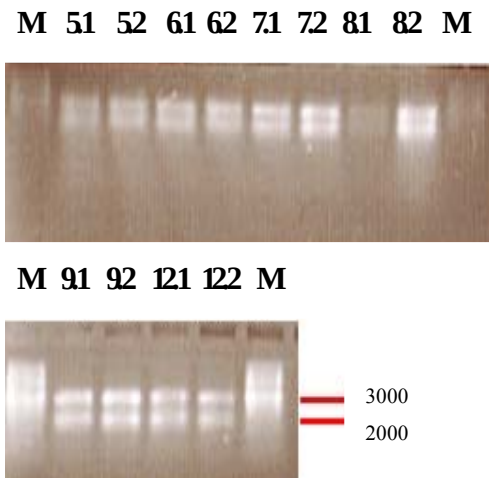


Figure 4.15: Ethidium bromide stained agarose-formaldehyd gel with RNA samples. The times and duplicates are indicated above the samples. The size marker M is RNA with band sizes of 9000, 7000, 5000, 3000, 2000, 1000 and 500 bases. **a)** RNA isolated from cultures grown with shaking. **b)** RNA isolated from cultures grown without shaking. Gel electrophoresis was performed on a 1.2% formaldehyde agarose gel at 4V/cm for 75 min.

4.10 Real-time RT-PCR

Real time RT-PCR was carried out on RNA isolated from bacteria grown in 1L Erlenmeyer flask with and without shaking, in order to determine the gene expression levels. Total RNA was reverse transcribed and subsequently amplifying using real-time PCR.

4.10.1 The primary cDNA product inhibits RT-PCR

Undiluted and diluted cDNA were tested to see eventual inhibition of SYBR green excitation and the results are shown in figure 4.16. This result indicates that the cDNA must be diluted prior to real time RT-PCR.

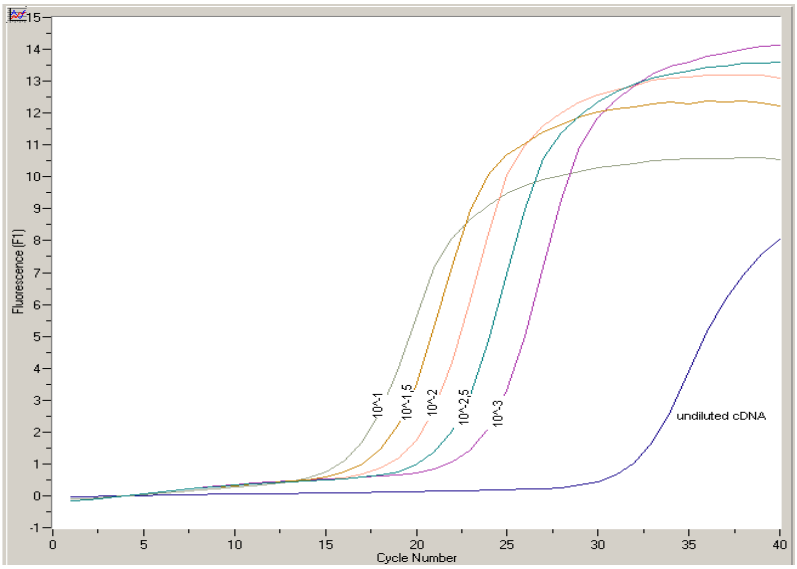


Figure 4.16: Example of a quantification curve for diluted and undiluted cDNA (Oxde) Samples were diluted 10^{-1},$10^{-1,5}$,10^{-2},$10^{-2,5}$,10^{-3}. Undiluted cDNA gives a higher Ct value compare to diluted samples.

4.10.2 Amplification efficiences (Amp.eff)

Amplification efficiencies were determined for the selected genes, 5 and 12 hours after inoculation. A regression line was made by plotting the Ct values of the sequences versus log10 of the dilution and the amp.eff was calculated based on the slope of the regression lines (Section 3.8.1). Table 4.3 a and b shows the calculated amp.eff. Figure 4.17 shows some examples of the regression lines. The remaining regression lines can be found in appendix 11.

Table 4.3 a): Relative Quantification-Bacteria grown with shaking									
Gene	**Time**	**Ct-10^{-1}**	**Average Ct-10^{-1}**	**St.dev-Ct**	**Slope [1)]**	**Amp.eff [2)]**	**Average Amp.eff**	**St.dev. Amp.eff**	**Corr.coef (R^2)**
	5	14.44			3.472	0.9410			0.9983
	5	14.61	14.53	0.1202	2.870	1.2307	1.0858	0.2048	0.9967
Glyde	12	18.43	18.43		3.634	0.8844	0.8844		0.9993
	5	21.33			3.624	0.8877			0.9900
	5	19.80	20.57	1.0819	3.783	0.8380	0.8629	0.0351	0.9971
	12	22.88			3.498	0.9314			0.9972
Lac1	12	21.78	22.33	0.7778	3.218	1.0453	0.9884	0.0805	0.9939
	5	25.56			2.990	1.1600			0.9881 *
	5	23.80	24.68	1.2445	3.262	1.0256	1.0928	0.0950	0.9963
	12	20.61			3.928	0.7971			0.9974
Acco1	12	20.35	20.48	0.1838	3.734	0.8527	0.8249	0.0393	0.9936
	5	19.31			3.198	1.0544			0.9974
	5	19.29	19.30	0.0141	2.878	1.2257	1.1401	0.1211	0.9856 *
Oxde	12	21.49	21.49		3.414	0.9630	0.9630		0.9955
	5	15.09	15.09		3.490	0.9340			0.9986
Fru	12	19.79	19.79		3.968	0.7865			0.9756 *
	5	15.19	15.19		3.160	1.0723	1.0723		0.9956
	12	18.88			3.228	1.0407			0.9977
Atp	12	18.27	18.58	0.4313	3.342	0.9917	1.0162	0.0346	0.9975
	5	23.20	23.20		3.344	0.9909	0.9909		0.9957
Cisy	12	18.59	18.59		3.258	1.0274	1.0274		0.9979
	5	26.73			2.808	1.2705			0.9959
	5	26.25	26.49	0.3394	3.018	1.1446	1.2076	0.0890	0.9948
Glu2	12	26.75	26.75		3.318	1.0016	1.0016		0.9726 *

Table 4.3 b): Relative Quantification- Bacteria grown without shaking					
Gene	**Time**	**Ct [1)]**	**Slope [2)]**	**Amp.eff [3)]**	**Corr.coef (R^2) [4)]**
	5	13.54	3.334	0.995	0.9982
Glyde	12	15.51	2.81	1.2692	0.9981
	5	19.28	3.224	1.0426	0.9946
Lac1	12	20.73	1.594	3.2399	0.9290 *
	5	25.74	3.53	0.9199	0.9979
Acco1	12	29.32	3.292	1.0126	0.9994
	5	14.82	3.0491	1.1280	0.9998
Atp	12	17.52	2.768	1.2976	0.9893
	5	15.03	2.464	1.5459	0.9897
Fru	12	18.72	2.830	1.2561	0.9684 *
	5	19.61	3.262	1.0256	0.9978
Oxde	12	18.53	3.7709	0.8416	0.9932
	5	23.51	3.676	0.8708	0.995
Cisy	12	22.77	3.098	1.1028	0.9915
	5	26.25	2.976	1.1678	0.9913
Glu2	12	30.06	1.2034	5.7761	0.9298

1) Ct value for cDNA diluted 10^{-1},
2) Absolute value of the slope
3) Amplification efficiences
4) Correlation coefficient, * Poor R^2

Regression lines:

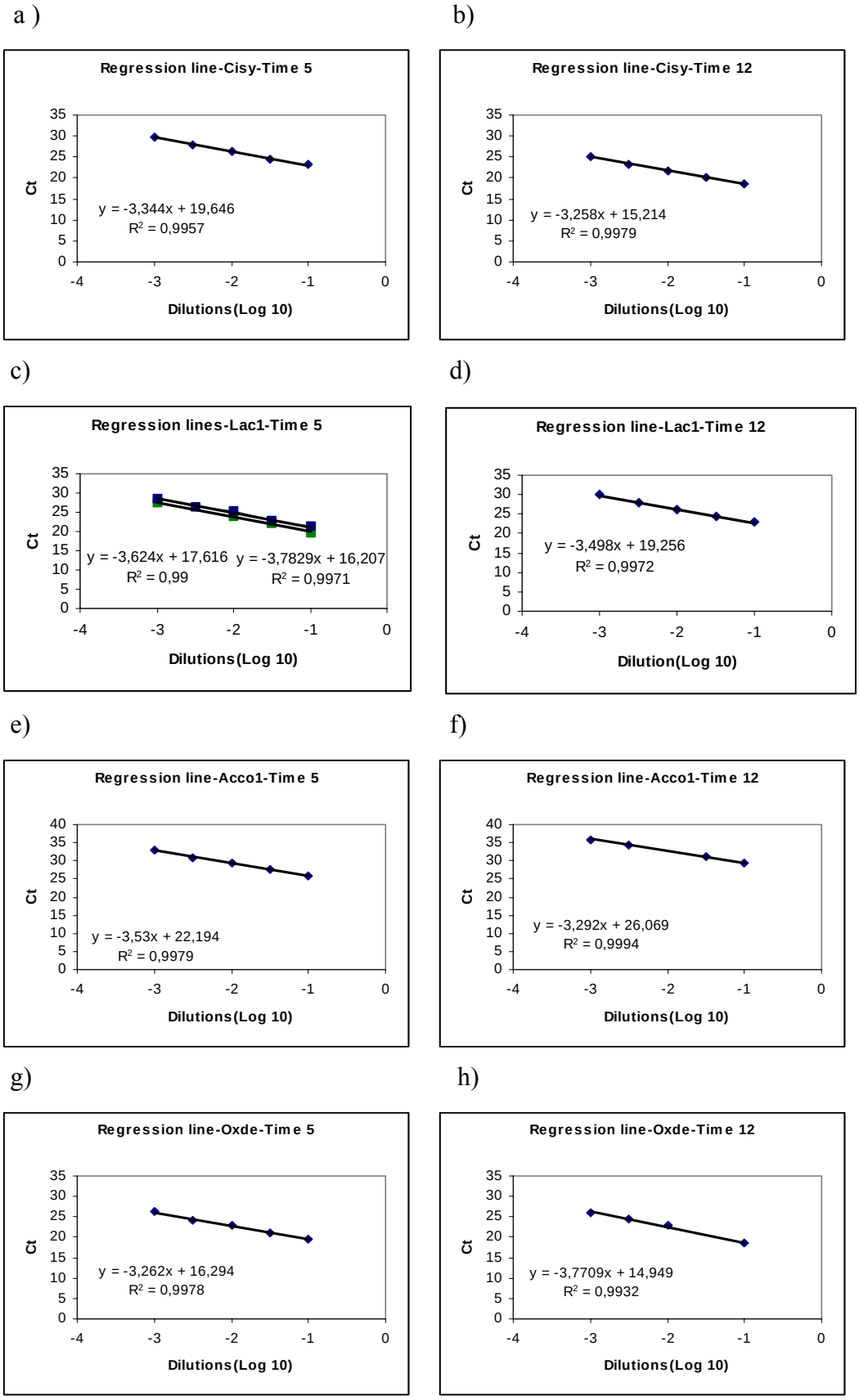


Figure 4.17: Regression lines for calculation of the amplification efficiencies. Sequence and time are indicated on the graphs. A correlation coefficient > 0.990 indicate regression lines that are acceptable. a-d) RNA from cultures grown with shaking and e-h) RNA from cultures grown without shaking. Data can be found in appendix 11.

4.10.3 Relative quantification of gene expression

The Pfaffl method (Section 3.8.2) was used to calculate the gene expression of selected genes at time 5 and 12 hours. All samples are expressed as fold changes relative to time 5 (expressed as 1), using Glyde as a reference gene. The calculation of the relative expression can be seen in appendix 11. The complete experiment, from converting RNA to cDNA and real-time RT-PCR was performed twice in the bacteria culture grown with shaking, for the following genes: Glyde, Lac1, Acco1, Oxde, Fru, Glu2 (at time 5 hours) and Lac1, Acco1, Fru, Atp (at time 12 hours). The remaining genes were only tested once. For the duplicated experiments the average amp.eff were calculated and used to determined the expression level. Figure 4.18 shows the expression of the selected metabolic genes.

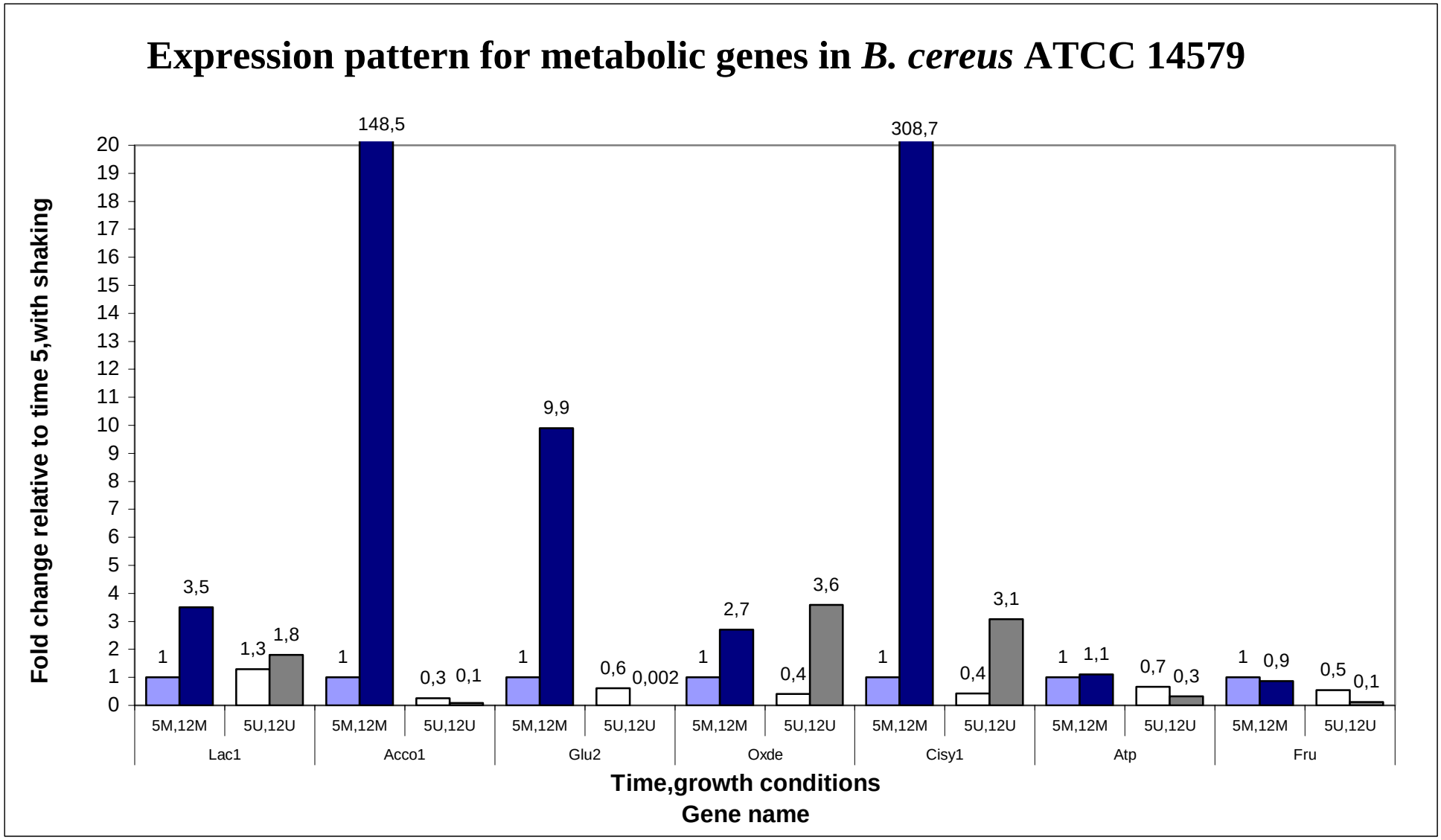


Figure 4.18: Fold changes in gene expression of metabolic genes relative to time 5, with shaking. For the duplicated experiments, average Ct, dilution 10^{-1} and average amp.eff was used to calculate gene expression. Time, growth conditions and gene name are indicated on the x-axis. 5= 5 hours after inoculation, 12 = 12 hours after inoculation. M=With shaking and U = Without shaking. Gene name: Fructose-6-phosphatekinase (Fru), Lactate dehydrogenase (Lac1), Acetyl-CoA synthetase (Acco1), Citrate synthase (Cisy1), α-ketoglutarate dehydrogenase E1 (Oxde), Glutaminase (Glu2) and ATP synthase ε-chain (Atp). Calculation of fold changes can be found in Appendix 11.

5. DISCUSSION

5.1 Comparison of bacteria counting methods

There are several methods for counting cell numbers. In this work flow cytometry was the main method used to determine the number of bacteria. Viable counts and OD_{600nm} measurements were performed to compare the results from flow cytometry to these well established methods. The cell numbers determined by flow cytometry and viable count gave very similar results with a slightly higher number of bacteria estimate by flow cytometer (Figure 4.2 b and c) for bacteria grown in 50% and 100% LB media. This could be due to the fact that flow cytometry estimates both live and dead bacteria, while viable counts estimate only viable bacteria cells. The cell numbers estimated in the 20% LB culture by flow cytometry after 10 hours or more of growth, are significantly lower than the numbers estimates by viable counts (Figure 4.2 a). Microscopic examination shows that bacteria cells grown in 20% LB medium aggregate earlier (time 9 hours) and to a greater extent, compared to bacteria cells grown in 50% or 100% LB media (Figure 4.5 g-l). Flow cytometry should be able to separate aggregated cells due to the high pressure used in generation of the flowing saline stream (Section 1.4.1). However, if the aggregation is large, as in the 20% LB culture, the flow cytometer appeared to be unable to separate the bacteria and count the bacteria cells one at a time. Cell size is one of the factors that the flow cytometer uses to estimate the number of bacteria cells. Cells of different sizes will appear in different areas in the graphical plot in which forward scatter is plotted against green fluorescence. Therefore, cell aggregates could appear in other area than single bacteria cells, and not be taken into account when the numbers of bacteria are determined. Examples of graphical plots in which forward scatter is plotted against green fluorescence are shown on page 36. Bacteria normally appear as points in the upper left hand frame (a), microsphere particles appear in the lower right hand frame (b), which are both used to estimate the number of bacteria cells. In a bacteria culture with a relatively large extent of aggregation (20% LB medium, time 10 hours) points appear in another area (c) in the graphical plot and are not taken into account when the numbers of bacteria are determined.

Optical density measures the decrease in intensity of light of a specific wavelength that is lost to scattering, as it passes through a bacteria suspension. The decrease in intensity is proportional to the number of particles in the suspension and hence the number of bacteria cells. OD measurements estimates both live and dead bacteria. The cell density estimated by OD measurements results in a lower cell density than the number of bacteria cells estimated by flow cytometry, except for samples after 12 hours growth in which the cell density is higher (Figure 4.1). A possible explanation is that optical density measurements cannot distinguish between live and dead cells and other particles that scatter light. Therefore, OD measurement can not be used to accurately measure the decline of growth during the death phase.

Based on these observations, flow cytometry appears to be a fast and accurate way of estimating the number of bacteria cells at different stages of growth, except for cultures with large aggregates of cell.

5.2 Cell growth, pH and oxygen level

When bacteria are grown in a batch culture, it is reasonable to expect that they undergo different forms of stress as the growth proceeds, such as accumulation of waste products, changes in pH, decrease in nutrient and oxygen level.

5.2.1 Comparing growth and pH in seven different growth media

B. cereus was grown in seven different growth media. Since the pH is one of the factors that can affect bacterial growth, the aim of the growth experiments was to investigate the effect of growth medium composition and identify a growth medium for *B. cereus* ATCC 14579, which gives satisfactory growth with minimal change in pH during growth. This was achieved by adding glucose and reducing the amount of organic nitrogen to different levels in the 20%, 25%, 50% and 100% LB or TSB growth media (Section 2.3.1).

B. cereus did not give any significant growth in the *B. subtilis* defined medium (Media composition-Steil et al., 2003). *B. cereus* grown in 25%, 50% and 100% TSB media with shaking gave three almost identical growth curve, which are shown in

figure 4.3 a. The number of cells was not determined at time 0 and the growth curves for these bacteria cultures are incomplete, starting 1 hour after inoculation**.**

The growth curves for *B. cereus* grown in 20%, 50% and 100% LB media with shaking are shown in figure 4.3 c. Bacteria grown in 50% and 100 %LB media gave almost identical growth curves, starting at a cell density of $10^3/10^4$ bacteria per ml and reaching a final cell density of $10^8/10^9$ bacteria per ml. After 10 hours, the growth curve for the 20% LB culture started to diverge from the growth curves for the 50% and 100% LB cultures. This could be due to an underestimation of cell numbers because of the large extent of cell aggregation in the 20% LB culture. None of the growth curves show a lag phase. This can be attributed to the fact that the ON-culture used for inoculation has adapted to the media and growth conditions, and starts to grow exponential almost immediately.

The pH curves, obtained for cultures grown in different TSB and LB media, followed the same general pattern (Figure 4.3 b and d). In all cultures the pH decreased from approximately 7.0 to approximately 5.5 in the middle of the exponential phase (5 hours after inoculation) and further growth gave an increase in pH. The pH of the negative control (no inoculum) remained constant. This indicates that the pH change during growth is due to the fact that *B. cereus* secretes or utilizes certain acidic or basic components.

The increase in pH observed in the cultures was proportional to the amount of organic nitrogen in the media. For example, growth in 100% LB medium gave the greatest increase in pH with a final value of approximately 9.0, while growth in 20% LB medium gave the smallest increase in pH with a final value of approximately 7.0 (Detailed pH values in Appendix 3 and 5). One explanation for the highest increase in pH could be that the 100% growth media, in contrast to the 20%, 25% and 50% media, did not contain any potassium phosphate buffer (KH_2PO_4 and K_2HPO_4). The potassium phosphate buffer should keep the pH relatively constant (near neutral pH ranges (6-7.5)) in the bacteria culture. However, even if the 20%, 25% and 50% media contained the potassium phosphate buffer, there was still a significant increase in the pH in the bacteria cultures grown in 50% compared to the 25% or 20% growth media.

Earlier growth studies of *B. cereus* (Nakata & Halvorson, 1960) showed that growth media with glucose show significant changes in pH during growth. Initially the pH decreased and increased during the sporulation phase. The same study reported no change in pH in media containing no glucose. The authors concluded that glucose is dissimilated to some acidic components, causing a decrease in pH. The rise in pH was attributed to a decrease in the concentration of pyruvate and acetate used as precursors for spore material.

In this work, the bacteria cultures grown in the 100% LB or TSB growth media (no glucose) initially showed a decrease in pH and an increase to approximately pH 9.0 during the stationary phase. On the other hand the pH did not decrease as much as in cultures grown with 50% and 20% LB media.

The glucose level for *B. cereus* ATCC 14579 grown in 1L Erlenmeyer flask in 50% LB medium at 37 °C was measured and showed a depletion of glucose after approximately 8-9 hours followed by the increase in pH (Figure 4.4). Figure 4.4 also shows growth and pH curves for *B. cereus* grown at 37 and 30 °C. The delay in growth and pH curves for the bacteria culture grown at 30 °C compared to bacteria grown at 37 °C are probably due to the fact that enzymatic reactions proceed more rapidly at the higher temperature.

Microscopic examination of bacteria from the exponential phase indicates that the cells occur singly or in short chains and are motile (Figure 4.5 a, b, c and figure 4.11 a, b, c). In the stationary phase, motility is lost and the cells start to form aggregates. The bacteria started to aggregate after 9 hours in the 20% LB culture, while those grown in 50% and 100% LB media remained as single vegetative cells and started to aggregate first after 12 hours (Figure 4.5 d-i). Cell aggregation in *B. cereus* has been reported to be a specific event (Wise & Fraser, 1972) and may play a role in the transfer of genetic material (Vries et al., 2004). Bacteria grown in 20% LB medium showed spore formation after 24 hours of growth (Figure 4.5 j), while bacteria grown in 50% and 100% LB media did not show any sign of spores (Figure 4.5 k and l). Nutrient exhaustion triggers sporulation therefore, the 50% and 100% LB media probably contains sufficient nutrient to maintain vegetative growth. As previously mentioned, the pH was reported to increase in the stationary phase due to removal and

utilization of acidic components in the sporulation process (Nakata & Halvorson, 1960). The pH increases even though the bacteria grown in 50% and 100% LB media did not sporulate. This indicates that the increase in pH could be due to other events, such as accumulation of basic nitrogen compounds (e.g. ammonia) or that acidic products are utilized in metabolic processes.

In conclusion, the composition of the growth media has a significant effect on the pH of the media and cell morphology. The 50% LB media gave satisfactory growth with minimal changes in pH and cell morphology, and was decided to use in all further experiments.

5.2.2 Oxygen level

Atmospheric oxygen diffuses from the surface of the growth media and is distributed throughout the bacteria culture by shaking. The amounts of dissolved oxygen is measured in parts per million (ppm (mg/L)). The dissolved oxygen level in pure water, at equilibrium with saturated air at one atmosphere at 30 ºC is 7.56 ppm (Cole, 1994, pp. 243). The oxygen level was determined in cultures grown with 25%, 50% and 100% TSB media and 20%, 50% and 100% LB media, with shaking. All samples from all the time points showed oxygen levels over 1.0 ppm. The main factors contributing to a decrease in the dissolved oxygen level in growth media are respiration, increased temperature (Cole, 1994, pp. 243 and 247) and accumulation of waste products. Therefore, there are reasons to believe that the oxygen level decrease during respiratoric growth, even though this was not detected using Oxygen CHEMets kit (0.05-1.0 ppm). Earlier reports suggested that vegetative cells of *B. cereus* have a non-functional oxidative mechanism due to the accumulation of acetic acid as an end product in glucose oxidation (Nakata & Halvorson, 1960). Later, Vries et al. (2004) suggested that vegetative *B. cereus* cells are unable to absorb sufficient oxygen to support glucose respiration, even though the oxygen level should be high enough to perform complete oxidation. In addition and as previously mentioned, the decrease in pH indicates that bacteria perform fermentation, which could explain why the oxygen level did not show any significant decrease.

5.2.3 Effect of growing *B. cereus* with or without shaking

The growth curves of *B. cereus* ATCC 14579 grown in cultures with and without shaking are very similar (Figure 4.7 and 4.8). The cultures grown with shaking reach approximately 5-10 folds higher cell number (10^9 versus $10^8/5*10^8$ cells per ml) in the stationary phase.

The pH decreased after approximately 5 hours in the bacteria cultures, grown with and without shaking (Figure 4.7 and 4.8). This happens more slowly in the cultures grown without shaking, probably due to a higher generation time. The pH started to increase 10 hours after inoculation in the bacteria cultures grown with shaking, while the pH remained low (pH 4.5-5.5) in the bacteria cultures grown without shaking. This could indicate that acids are not utilized and removed from the growth media or nitrogenic compounds are not secreted into the growth media giving a corresponding increase in pH.

Earlier study reports that *Vibrio cholera* grown without shaking shown an increase in the cholera toxin expression as the volume to surface ratio was reduced (Sánchez et al., 2003), and this was the reason for growing cultures with and without shaking in cultures vessels with different surface area. Later reports shown that *Pseudomonas fluorescens* cultures grown without shaking adapt to different growth patterns depending on the growth conditions that become non-uniform as the growth progresses. This resulted in a heterogenic bacteria culture (after 5 days), which is reflected in different colony morphologies when the cells are grown on agar plates (Rainey, 2004). Based on this knowledge a possible explanation for the decrease in the number of bacteria cells in the culture grown without shaking could be due to the non-uniform distribution of nutrients and growth conditions that appears in the cultures grown without shaking. The same experiment was carried out with *B. cereus* ATCC 14579, but no differences were observed between the colonies after 5 days of growth (Data not shown). Microscopic images of the bacteria grown without shaking (Figure 4.11 d and f) seem to have a greater variation in cell size compared to the bacteria cultures grown with shaking (Figure 4.11 e and figure 4.5 e).

5.2.4 Effect of surface area

The growth and pH curves for the cultures grown in 1L Erlenmeyer flasks (surface area 113 cm^2) and in a large surface area culture (surface area 530 cm^2) are almost identical (Figure 4.9 and 4.10). All cultures reach a cell density 10^9 or $5*10^8$ cells per ml, and the pH started to decrease after approximately 5 hours. For the cultures grown with shaking, the pH started to increase 10 hours after inoculation, while the pH in the cultures grown without shaking remained low. The surface area does not have any significant influence on growth and changes in pH. The cultures grown with a large surface area in 50% LB media with shaking showed some indication of sporulation after 24 hours (Figure 4.11 h), while neither of the cultures grown without shaking showed signs of sporulation (Figure 4.11 g and i).

5.2.5 Generation time

The generation times for *B. cereus* ATCC 14579 grown in different LB media, were calculated (Table 4.1). Bacteria grown in 1 L Erlenmeyer flask in 20% LB media has a generation time of approximately 20 min. compared to 22 min. and 25 min. for the bacteria grown in 50% and 100% LB media, respectively. The bacteria grown in 50% LB media without shaking have a higher generation time than bacteria grown with shaking (27 min. vs. 22 min.), while the bacteria grown with large a surface area had a higher generation time than bacteria grown in 1 L Erlenmeyer flask. This indicates that the generation time is influence by the media composition, changes in pH and growth conditions. This is in agreement with a previous study in *B. cereus,* which indicated that growth rate of un-buffered cultures decrease as a result of changes in pH (Nakata, 1963).

5.3 Live/dead staining and microscopy

The live/dead staining of the bacteria should indicate the proportions of live and dead bacteria cells in the cultures. Live/Dead staining was performed on bacteria grown in: 1 L Erlenmeyer flask in 20%, 50% and 100% LB media with shaking, as well as 1 L Erlenmeyer flask in 50% LB medium without shaking and in a large surface area culture in 50% LB medium, with and without shaking (Figure 4.6 and 4.12). After 5-6 hours, the cell density is in the range 10^7 or 10^8 bacteria/ml and the majority of the bacteria from the different cultures were stained red, which implies dead bacteria.

In contrast to this result, determination of viable counts at 4 and 6 hours indicated that the cells are viable. There were also large variations in the staining results for the other samples and there appeared to be no general trends regarding the proportion of live and dead bacteria cells in a given culture. This indicates that the live/dead kit is not reliable for *B. cereus* grown under the conditions specified. Another explanation could be that repeated centrifugation and re-suspensions during the staining process cause lysis of the bacteria cells, results in red staining. Viable counts performed on the bacteria culture grown in 50% LB medium, showed a decrease in the number of bacteria cells in the stationary phase. Therefore, it is reasonable to assume that there should be an increase in the numbers of dead bacteria cells (red) as the cultures enter into the stationary phase.

5.4 Primers

Different metabolic genes were selected on the basis of the growth experiments. Based on the decrease and increase in pH we select genes that are involved in glycolysis (fermentation); (Fruktose-6-phosphatekinase (Fru), Lactate dehydrogenase (Lac1), Acetyl-CoA synthetase (Acco1)), TCA cycle; (Citrate synthase(Cisy1), α-ketoglutarate dehydrogenase E1(Oxde)), amino acid metabolism; (Glutaminase (Glu2)), respiration and ATP generation; ATP synthase ε-chain(Atp).

The observed size of the PCR products estimated by agarose gel-electrophoresis is approximately 200 bp, which correlates well with the calculated size of the PCR products (Figure 4.13). Almost all of the primers functioned satisfactory on DNA template from *B. cereus* strain ATCC 14579 and ATCC 10987, giving a single band of the correct size. “Glu1” primers did not give a product with template DNA from neither of the *B. cereus* strain, whereas “Glyde” only gave a product with template DNA from *B. cereus* ATCC 14579.

5.5 RNA isolation and determination of RNA concentration

During the first half of the exponential phase, the cell density was too low for efficient RNA isolation. Therefore, the RNA isolation was first performed 5 hours after inoculation. The amount of RNA isolated per cell decreased by a factor of approximately 2.5 from time 5 hours to time 6 hours in both cultures (Figure 4.14).

After 6 hours of growth there were no large changes in RNA per cell. An explanation for the decrease in the amount of RNA per cell may be that the cells are simply producing less RNA when the cultures are in the stationary phase, where oxygen and nutrient concentration become limited. Approximately 85% of a cell's total RNA is ribosomal. It is possible that cells in the stationary phase produce fewer ribosomes as there is no net growth, which would in turn influence the amount of RNA in a bacteria cell. One other possibility is that the bacteria are structurally changed making it more difficult to isolate the total RNA. The differences in RNA yield from the parallel cultures are probably due to technical factors.

Samples of isolated RNA were denatured for 15 minutes at 70 °C before being analyzed by formaldehyd gel electrophoresis in order to check the quality of the RNA. Ribosomal RNA (rRNA) is used as a marker to assess sample RNA quality. The ethidium bromide stained gel (Figure 4.15 a and b) showed two distinct ribosomal RNA bands with approximately equal fluorescent intensity, at 2.9 kb and 1.5 kb in the majority of the samples. These distinct bands represent 23S and 16S ribosomal RNA. During the first 12 hours of growth, RNA isolated from cultures grown without shaking shows two clear distinct bands for all samples in contrast to RNA isolated from cultures with shaking, which showed smearing above the two ribosomal bands. If the RNA sample were degraded a smear would be visible below the rRNA bands. Smearing above or between the RNA bands could be caused by contamination e.g. DNA. RNA isolated from culture with shaking after 24 hours growth shows no distinct bands, probably due to loading error or poor RNA quality.

5.6 Real-time RT-PCR

Real-time RT-PCR was used to measure changes in gene expression. In real-time RT-PCR the amount of product is estimated after each amplification cycle by measuring the fluorescence intensity, caused by SYBR green binding to DNA. A threshold value is set where the amplification is logarithmic. The point at which the fluorescence crosses the threshold is called the Ct-value, which is proportional to the initial concentration of the cDNA template and in turn proportional to the concentration of the mRNA and gene expression.

The primary cDNA product of reverse transcription inhibits the real-time PCR reaction. This is probably due of to the fact that too much cDNA template inhibit the amount of light that manages to pass through the samples and excite the SYBR green (Figure 4.16). This results in lower Ct value and an incorrect determination of gene expression levels. Dilution of the primary cDNA product by a factor of ten removes this problem.

Use of the LightCycler to carry out PCR amplifications permits a quantitative analysis. The quantitative graph is used to derive the Ct value. As SYBR green binds non-specifically to dsDNA, a melting curve analysis of the amplification product must be performed to ensure that the increase in fluorescence shown in the quantification graph is due to the amplification of a specific target only.

5.6.1 Melting curves

Most of the samples gave a melting curve with a single peak indicating that the PCR amplification product was homogenous. The samples with “Fru” primers, gave two peaks. Formation of more than one melting peak indicates that the product is heterogenous and could consist of primer dimers or unspecific product. This can suppress amplification of the real target and lead to inaccurate quantification. The annealing temperature was increased from 53 °C to 57 °C resulted in a single melting peak.

5.6.2 Relative quantification of gene expression

The Pfaffl method (Section 3.8.2) was used to calculate the gene expression of seven metabolic genes at time 5 and 12 hours after inoculation. The complete experiments, from converting RNA to cDNA and real-time RT-PCR should have been repeated at least three times to verify the expression level for the selected genes.

There are several parameters that could affect the quantification of the gene expression e.g. amplification efficiencies and DNA contamination. A regression line was made for each sample and the amplification efficiencies were calculated, based on the slope of the regression line. Some of the Ct values diverged too much from the regression line and were not taken into consideration when the regression lines were calculated. For the samples performed in duplicate, the regression lines for a given

gene resulted in approximately parallel regression lines (same slope). Different slopes would imply an inhibition of the reaction, which could lead to reduced amplification efficiencies. Most of the regression lines (Appendix 11, some examples shown in figure 4.17) had a slope with an absolute value of approximately 3.0 or higher and a correlation coefficient above 0.990 (Table 4.3 a and b). The regression lines for Glu2 and Lac1 at time 12 hours, for bacteria grown without shaking had a slope with an absolute value below 2.0 resulted in remarkably high amplification efficiency (far above 1). Therefore, it is difficult to get a correct quantification of the gene expression for these two genes, at this time. The experiment should have been performed once more to get better regression lines for these two genes at time 12 hours.

The calculated amplification efficiencies differed between 85–100%. Small differences in amplification efficiency could result in different amounts of the final amplification product, which have a major effect on the Ct values. The Pfaffl methods allow for differences in amplification efficiencies between the reference gene and the target gene, but not for differences in amplification efficiencies at different times. The latter may influence the calculation of the gene expression pattern. However, since expression is calculated relative to time 5 hours, the general trend will still be correct even if the accurate fold changes are not exactly correct.

The purpose of the DNase treatment was to remove genomic DNA from the RNA samples. Negative controls containing RNA treated with DNase but not reverse transcribed were performed for each primer set and each sample and gave a higher Ct value compared to the corresponding cDNA sample. The product from the negative controls has the same melting temperature as the amplified target sequence in most cases and will affect the increase in fluorescence. The DNA contamination was calculated to affect the increase in fluorescence by a factor of 1/10 000, 1/100 000 or less. In the Glu2 and Acco1 samples the DNA contamination contributed approximately 1/100 or 1/1000 of the total fluorescence signal (Calculation shown in appendix 11, table 11.2). This indicates an inefficient DNase treatment. The incubation time was increased from 15 to 30 minutes and the DNase treatments were also performed twice, without any improvement. Since the DNA contamination only slightly affected the fluorescence signal in most of the samples, they were disregarded when the expression levels for the selected genes were determined. A negative control

consisting of DEPC-H_2O instead of cDNA template was used to check for possible DNA contamination of primer or SYBR green master mix. Negative controls without cDNA template gave no product or products with a lower melting temperature than the target product. The latter was probably due to primer dimer formation. There are no signs of primer dimer artifacts in the cDNA samples, indicating that the increase in fluorescence are due to the amplification of a specific target only.

5.6.3 Expression pattern

B. cereus is a facultative aerobic bacterium but can also grow well in the absence of oxygen. Glycolysis is utilized in both aerobic respiration and fermentation, in which one glucose molecule is converted into two molecules of pyruvate. During fermentation, pyruvate is broken down to fermentation products and the ATP synthesis occurs only by substrate level phosphorylation, releasing a total gain of two ATP molecules per glucose. In aerobic respiration pyruvate is completely oxidized to CO_2 and reduced NADH or FADH via the TCA cycle. ATP is formed by substrate level phosphorylation and oxidative phosphorylation. This process gives a higher energy yield (38 molecules of ATP per molecule of glucose) under optimal conditions, compared to the fermentation processes.

Figure 4.18 shows an overview of the gene expression of the seven metabolic genes involved in, glycolysis, fermentation, amino acid metabolism, the TCA cycle and ATP synthesis.

“Fru” encodes a kinase that catalyses the third step in glycolysis, which is the conversion of Fructose-6-phosphate to Fructose-1,6-bisphosphate. The expression level of “Fru” is approximately the same at time 5 hour and 12 hour for bacteria grown with shaking, while it is down regulated in bacteria grown without shaking. This indicates that bacteria grown with shaking utilize glycolysis to a greater extent than bacteria grown without shaking. There is no glucose in the media at time 12 hours but “Fru” is still active. One explanation could be that cell wall material contains 6-C sugar, which can be utilized for energy via glycolysis.

The pH started to decrease from pH 7.0 to 5.5 approximately five hours after inoculation, in cultures grown with and without shaking. This was reported to be due

to accumulation of acid products caused by the fermentation of glucose (Nakata& Halvorson, 1960). As previously mentioned, vegetative *B. cereus* cells are unable to take up sufficient oxygen to support glucose respiration, even though the oxygen level should be high enough to perform complete oxidation (Vries et al., 2004). Therefore, it is reasonable to believe that bacteria obtain ATP by a fermentation processes and not by aerobic respiration. The gene expressions of the two enzymes that function in different fermentation processes were investigated: Acetyl-CoA synthetase (Acco1), which converts acetyl-CoA to acetate, and lactate dehydrogenase (Lac1), which converts pyruvate to lactate. Both "Acco1" and "Lac1" are upregulated to different extents at time 12 hours in bacteria grown with shaking. In bacteria grown without shaking the "Acco1" was down regulated, while "Lac1" was upregulated to a lower extent, 5 and 12 hours after inoculation.

In the cultures grown with shaking, the pH started to increase from 5.5 to 8.3 in the stationary phase. Therefore, the upregulation of "Acco1" and "Lac1" at time 12 hours in bacteria grown with shaking could probably be a consequence that these enzymes also work in the reverse reaction, where the acids (acetate and lactate) are utilized to form acetyl-CoA or pyruvate. Acetyl-CoA and pyruvate can be converted to oxaloacetate, which can be metabolized via the gluconeogenesis or to precursor metabolites via the TCA cycle (Figure 5.1). The utilization of the acidic products could explain the increase in pH. This assumption of the utilization of lactate is in agreement with a previous study in *B. cereus*, in which lactate was reported to be metabolized after glucose exhaustion (Vries et al, 2004). The pH did not increase in the stationary phase in the cultures grown without shaking. This could indicate that acids are not utilized and removed from the growth media, which explain the down regulation of "Acco1" and the relatively low upregulation of "Lac1" in bacteria grown without shaking at time 5 and 12 hours. On the other hand the quantified expression for "Lac1" at time 12 hours for bacteria grown without shaking is probably not reliable due to a poor regression line.

The increase in pH in the culture grown with shaking to a higher level than the initial pH (7.0) of the growth media could also be due to other events e.g. accumulation of ammonia (NH_3), in addition to removal and utilization of acidic components. Glutaminase (Glu2) converts glutamine to glutamate and NH_3. "Glu2" is upregulated

at time 12 hours in bacteria grown with shaking, while it is down regulated at time 5 and 12 hours in bacteria grown without shaking. The upregulation of "Glu2" at time 12 hours leads to the formation of more glutamate. Most amino acids can be converted to different citric acid cycle intermediates. The amino acid, glutamate can be oxidized to α-ketoglutarate, which can be metabolized in the TCA cycle or used in the gluconeogenesis to form glucose. NH_3 is a by-product, which could contribute to the increase in pH. The pH did not increase in the cultures grown without shaking and explain the downregulation of "Glu2" in bacteria grown without shaking.

Under anaerobic conditions the TCA cycle is predominantly used to generate precursor metabolites. The expression of two enzymes in the TCA cycle was investigated: Citrate synthase (Cisy1), which converts oxaloacetate and acetyl-CoA to citrate and α-ketoglutarate dehydrogenase E1 (Oxde) which is part of the multienzyme complex that catalyzes the oxidative decarboxylation of α-ketoglutarat to succinyl-CoA. Both "Oxde" and "Cisy1" are upregulated 12 hours after inoculation in both cultures, but "Cisy1" are upregulated to a greater degree in cultures grown with shaking than without shaking

Upregulation of "Lac1", "Acco1" and "Glu2" could lead to the formation of more components that can be converted to different citric acid cycle intermediates, which are in turn metabolized in the TCA cycle or gluocneogenesis. The formation of more citric acid intermediates in turn upregulates genes involved in the TCA cycle ("Oxde" and "Cisy1"). There appears to be a correlation between the glucose exhaustion (8-9 hours after inoculation) and a requirement of alternative carbon and energy sources (acids and aminoacids) and the upregulation of these five genes.

As previously mentioned, the increase in pH during the stationary phase could be due to the removal of acidic components and formation of nitrogenic compounds. Therefore, if the bacteria continue to ferment, the amount of acid produced must be less than acid removed or ammonia produced. Another explanation could be that *B. cereus* switches from fermentation to aerobic respiration, after the glucose has been deplete from the medium. The TCA cycle is then utilized to oxidize acetyl-CoA (from pyruvate and acetate) to CO_2, NADH and FADH, in addition to providing precursor metabolites.

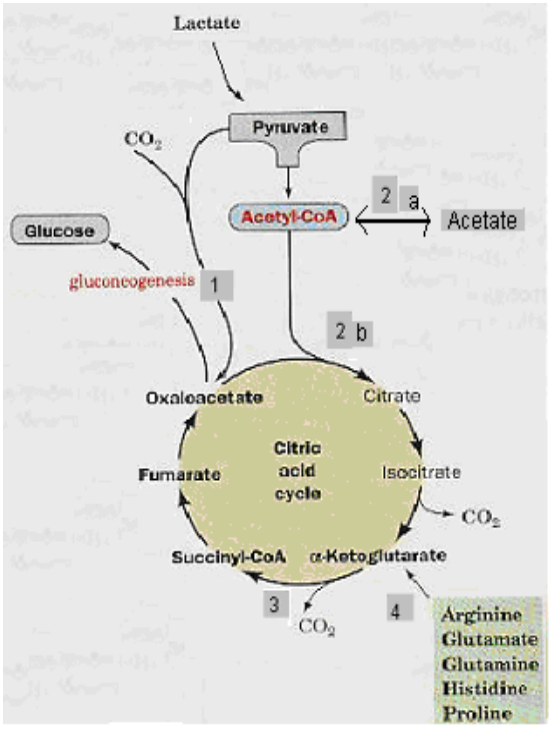


Figure 5.1: Overview of the TCA cycle.
Glutamate, lactate and acetate can be convert to different citric acid intermediates, which can be metabolized in the TCA cycle or used in gluconeogenesis.

1) Lactate and Pyruvate can be converted to Oxaloacetate.

2a) Acetate can be converted to Acetyl-CoA
2b) Oxaloacetate + Acetyl-CoA can be converted to citrate by Citrate synthase (Cisy1)

3) α-ketoglutarate can be converted to succinyl-CoA by the multienzyme complex α - ketoglutarate dehydrogenase (Oxde)

4) Glutaminase (Glu2) converts glutamine to glutamate and NH_3. Glutamate can be converted to α-ketoglutarate.

Lac1, Acco1, Glu2, Cisy1 and Oxde seem to be upregulated when glucose is deplete form the media

In aerobic respiration the electron transport chain is used to form a proton motive force, which in turn is used to synthesize ATP via ATPases. In fermentative bacteria the ATPase complex functions in the reverse direction to maintain a proton gradient, which generate the proton motive force. Proton motive force can be utilized for many purpose in the bacterial cell including: nutrient transport, protein export and maintainance of a favorable intracellular pH. ATP synthase ε-chain (Atp) is part of the ATPases complex. “Atp” was expressed at the same ratio in bacteria grown with shaking, at time 5 and 12 hours, while it is down regulated in bacteria grown without shaking. Bacteria grown with shaking were calculated to have a lower generation time (faster growth rate) than bacteria grown without shaking. A higher growth rate requires faster nutrient transport and metabolic rates, which in turn could explain the down regulation of “Atp” in bacteria grown without shaking. Due to no net increase in cell number there are reasons to believe that the “Atp” should have been down regulated in the stationary phase, at time 12 hours. This is the case for bacteria grown without shaking but not for the bacteria grown with shaking. As previously mention, the decrease in pH indicates that *B. cereus* ATCC 14579 performs fermentation rather than aerobic respiration. Therefore, there is reason to believe that the ATPase complex is utilized to establish a proton gradient rather than forming ATP.

In conclusion, this project shows different levels of gene expression in bacteria grown under different conditions. Studies in *Borrelia burgdoferi* indicate that pH, temperature and dissolved oxygen levels alter gene expression (Seshu et al., 2004). The effect of surface area to volume on expression of toxin genes has been investigated in cultures of *Vibrio cholerae* grown without shaking. No shaking and a high surface to volume ratio induce the expression of cholera toxins (Sánchez et al. 2003). Therefore, it is important to remember that there are many different factors that can influence bacteria growth and gene expression. Real-time PCR is an extremely powerful tool for investigating gene expression patterns, but it is difficult to see how this method will achieve it's true potential unless we can devise laboratory methods, for the bacterial growth, which mimic bacterial growth in natural environments.

The work described in this thesis has been used as a starting point for other projects carried out by Solveig Sirnes (Project thesis 2004;Diploma thesis 2005) and Simen Kristoffersen.

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7. APPENDIXES

APPENDIX 1: Total RNA isolation-Detailed Protocol

APPENDIX 2: Growth and pH in different Tryptic soy broth media

APPENDIX 3: Growth and pH in 20% LB media with shaking

APPENDIX 4: Growth and pH in 50% LB media with shaking

APPENDIX 5: Growth and pH in 100% LB media with shaking

APPENDIX 6: Growth and pH in 1L Erlenmeyer flask with and without shaking

APPENDIX 7: Growth and pH in larger surface culture with and without shaking

APPENDIX 8: Calculation of Average cell number and Standard deviation (St.dev)

APPENDIX 9: Generation time

APPENDIX 10: RNA isolation

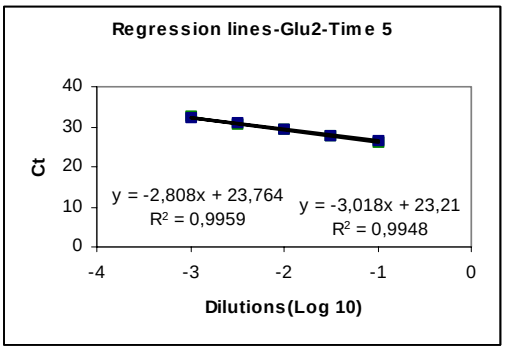
APPENDIX 11: Real time RT-PCR

APPENDIX 12: Comparing Growth, pH and Glucose level

Please find the appendixes on enclosed CD.
Regression lines in Appendix 11 are also shown below.

Regression lines- Bacteria culture grown with shaking

a)



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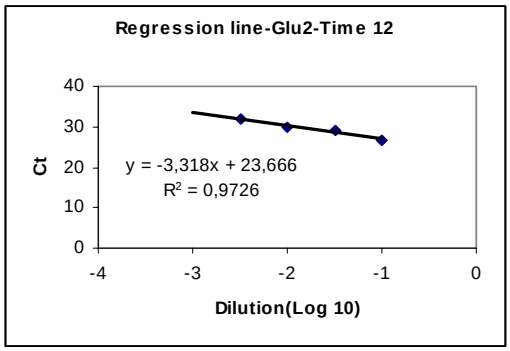

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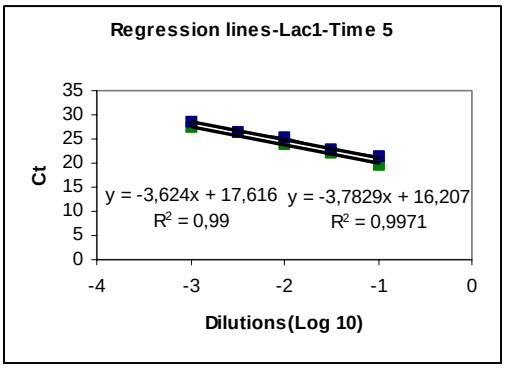

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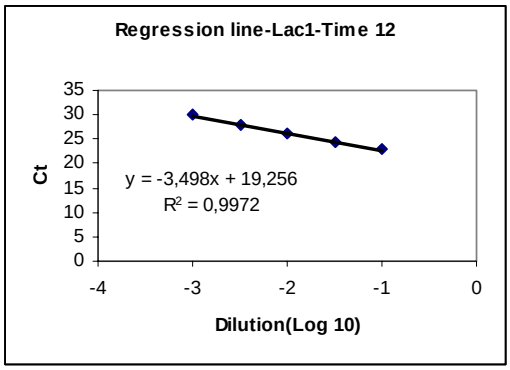

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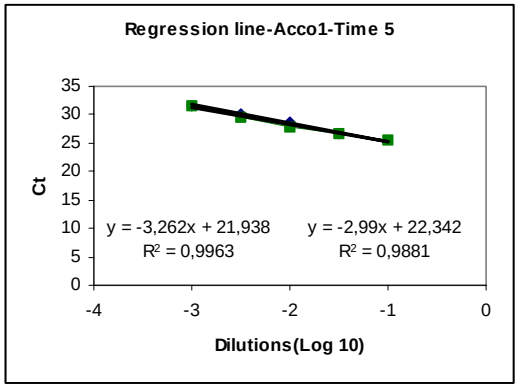

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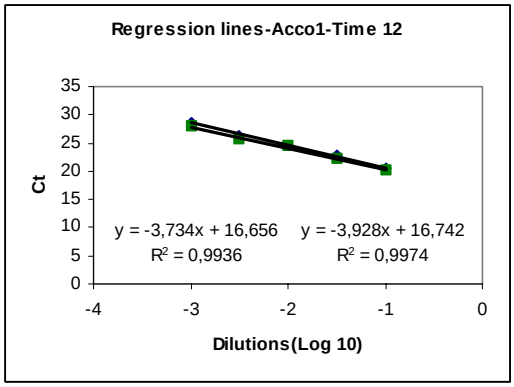
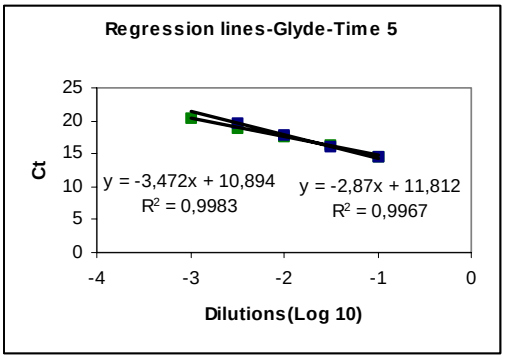
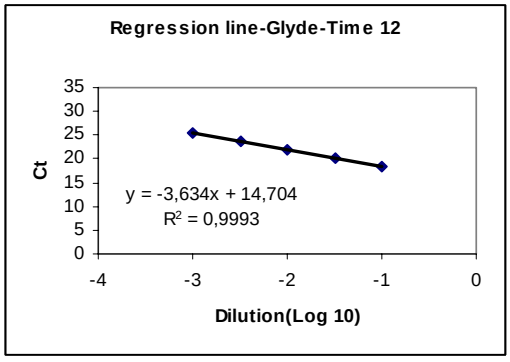


g)



h)



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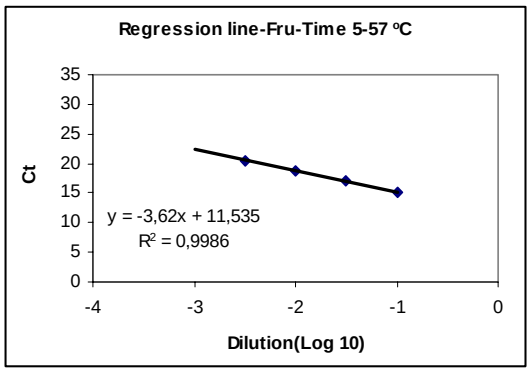
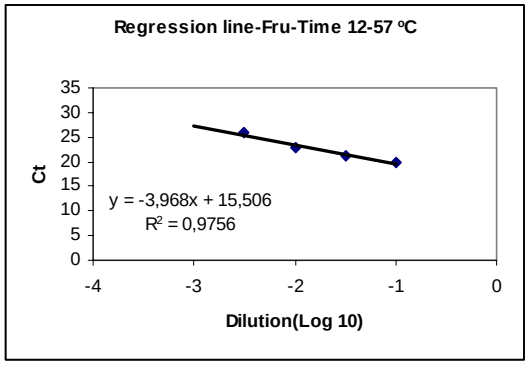
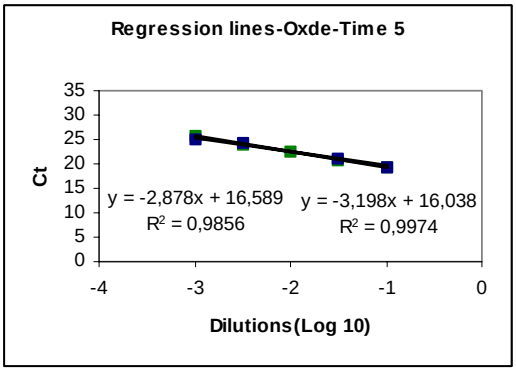

Regression line-Fru-Time 5-57 ºC
Ct
Dilution(Log 10)
y = -3,62x + 11,535
R² = 0,9986

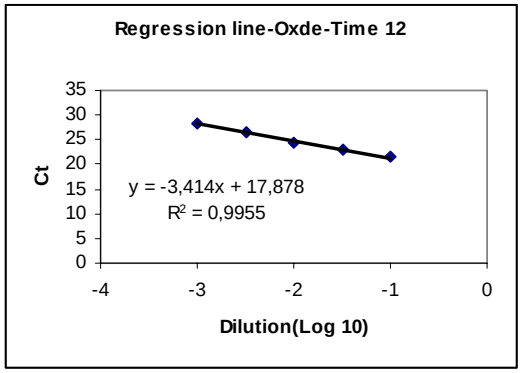
j)


Regression line-Fru-Time 12-57 ºC
Ct
Dilution(Log 10)
y = -3,968x + 15,506
R² = 0,9756

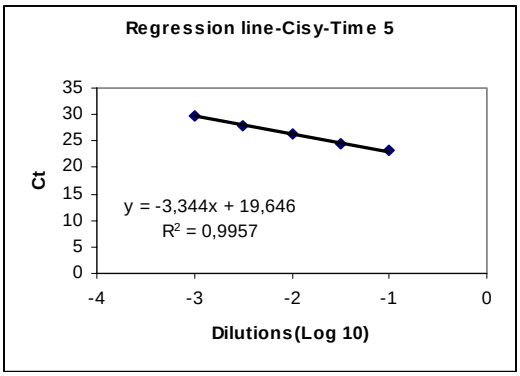
k)


Regression lines-Oxde-Time 5
Ct
Dilutions(Log 10)
y = -2,878x + 16,589
R² = 0,9856
y = -3,198x + 16,038
R² = 0,9974

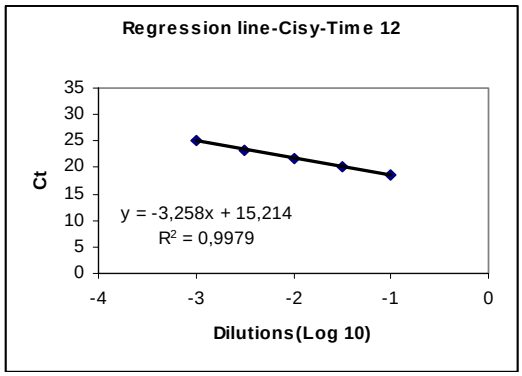
l)


Regression line-Oxde-Time 12
Ct
Dilution(Log 10)
y = -3,414x + 17,878
R² = 0,9955

m)

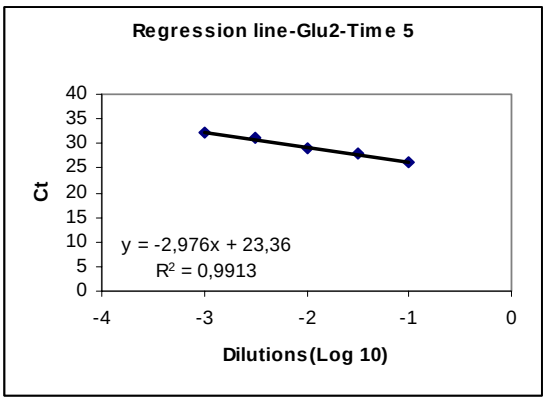

Regression line-Cisy-Time 5
Ct
Dilutions(Log 10)
y = -3,344x + 19,646
R² = 0,9957

n)


Regression line-Cisy-Time 12
Ct
Dilutions(Log 10)
y = -3,258x + 15,214
R² = 0,9979

Regression lines- Bacteria culture grown without shaking

a)



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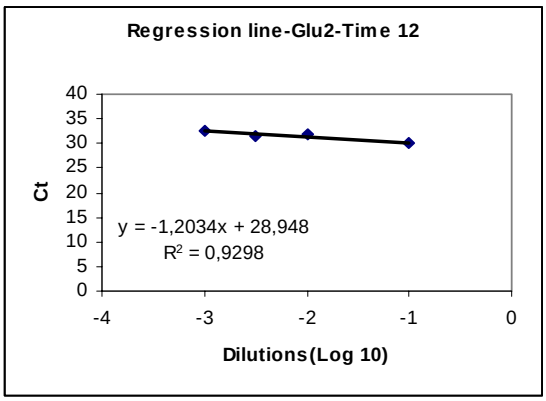

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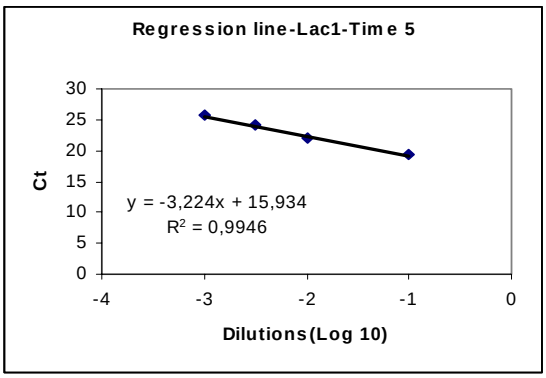

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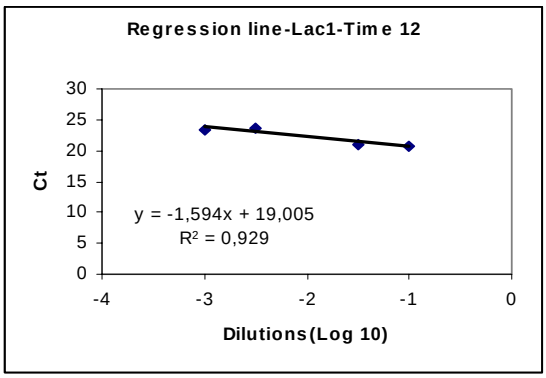

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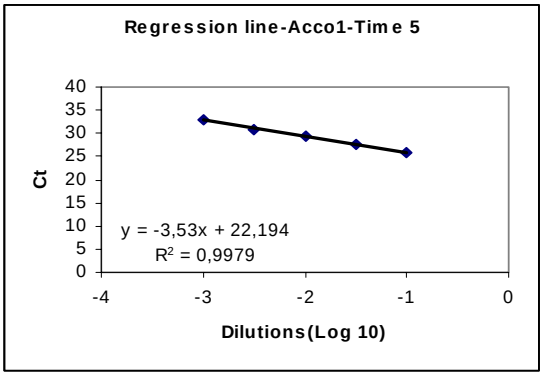

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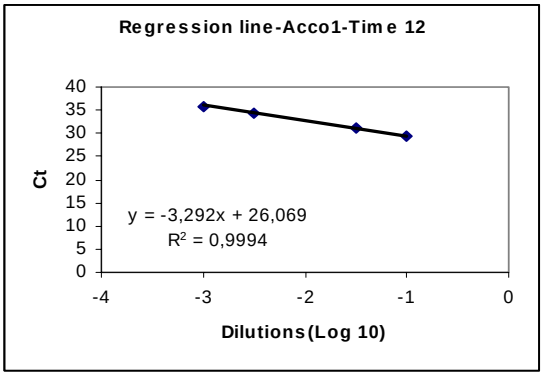
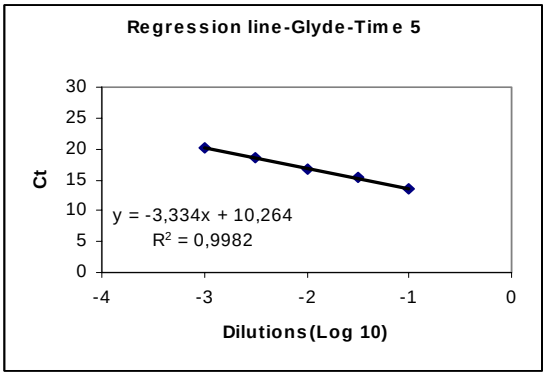


g)



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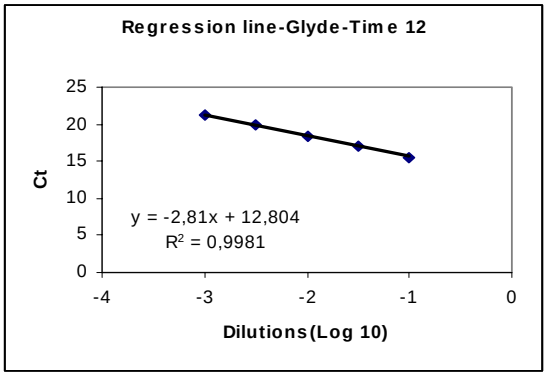

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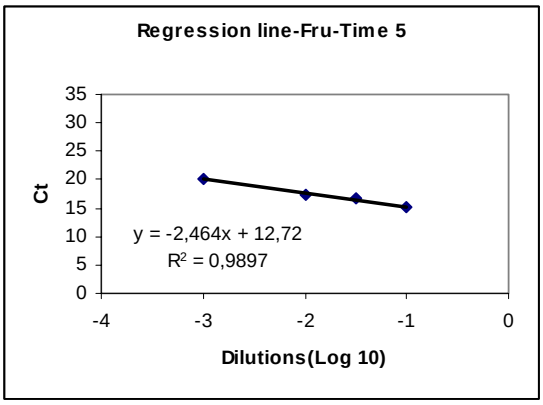
Regression line-Fru-Time 5
Ct
y = -2,464x + 12,72
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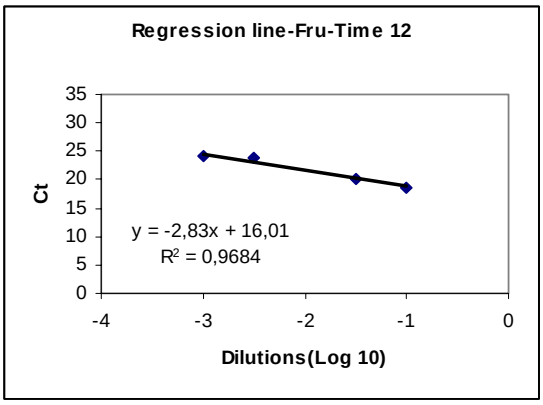
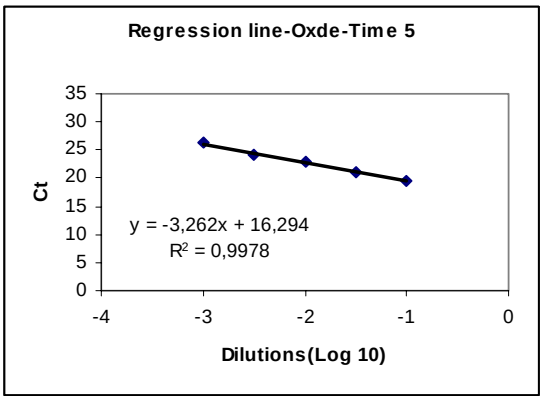
Dilutions(Log 10)

j)


Regression line-Fru-Time 12
Ct
y = -2,83x + 16,01
R² = 0,9684
Dilutions(Log 10)

k)


Regression line-Oxde-Time 5
Ct
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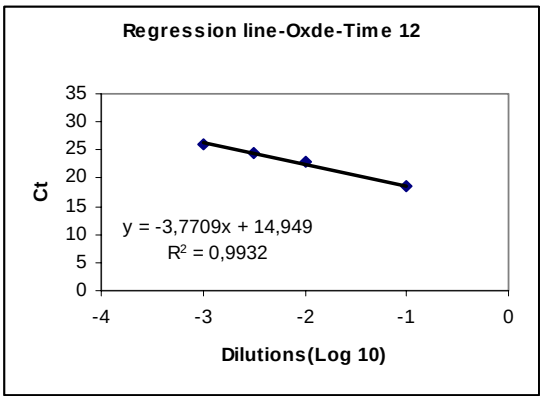
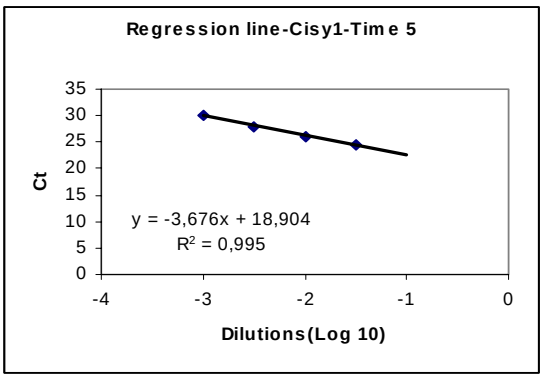
R² = 0,9978
Dilutions(Log 10)

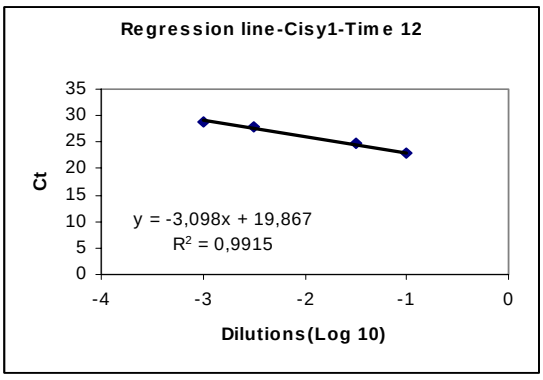
l)


Regression line-Oxde-Time 12
Ct
y = -3,7709x + 14,949
R² = 0,9932
Dilutions(Log 10)

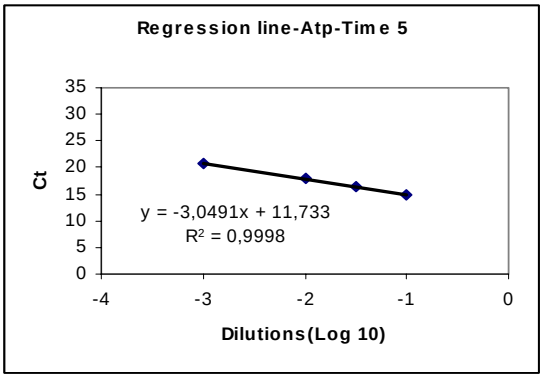
m)


Regression line-Cisy1-Time 5
Ct
y = -3,676x + 18,904
R² = 0,995
Dilutions(Log 10)

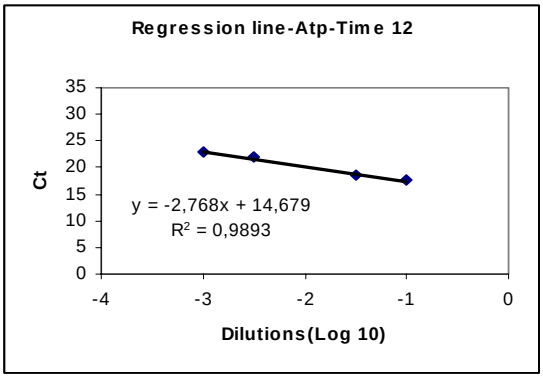
n)


Regression line-Cisy1-Time 12
Ct
y = -3,098x + 19,867
R² = 0,9915
Dilutions(Log 10)

o)


Regression line-Atp-Time 5
Ct
y = -3,0491x + 11,733
R² = 0,9998
Dilutions(Log 10)

p)


Regression line-Atp-Time 12
Ct
y = -2,768x + 14,679
R² = 0,9893
Dilutions(Log 10)