Internship Report:

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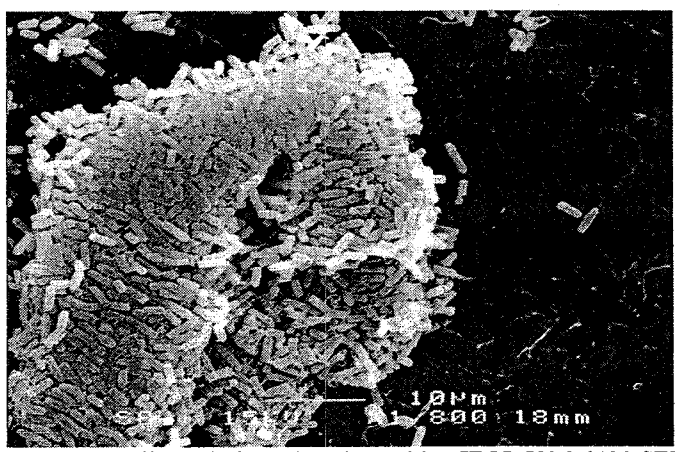
Effect of pH on the Production of Exopolysaccharides by 3 strains of *Bacillus sp.* - Application of Electron Microscopy



Clamp of *Bacillus subtilis* cells, taken with a JEOL JSM-6400 SEM

Biochemical and Food engineering department, INSA – September 2007

Summary

The study consisted in growing 3 *Bacillus* strains (*A*, *B* and *B.subtilis*) at various pH to study the variation in production of exopolysaccharides. The idea underlying the project was to watch these variations under electron microscope and try to relate them to the quantity produced. It appeared that scanning electron microscopy was useful to give a qualitative appreciation of EPS production. It has also been shown that the 3 strains did not grow at acid pH and presented the best EPS production at pH 7 .Furthermore, this internship consisted in learning methods, using apparatus, and acquiring a background for my next project in SUT concerning the study of lactic acid bacteria isolated from Thai fermented vegetables; study regarding food safety and quality.

Résumé

L'étude a consisté à faire pousser 3 souches de *Bacillus* (*A*, *B*, *B.subtilis*) à différents pH pour étudier les variations de production d'exopolysaccharides. L'idée soutenant ce projet était de voir ces variations au microscope électronique et essayer de les relier à la quantité produite. Il est apparu que le microscope électronique à balayage était utile pour donner une appréciation qualitative sur la production d'EPS. Il a aussi été montré que les 3 souches ne poussaient pas à pH acide et qu'elles présentaient la meilleure production d'EPS à pH 7. De plus, ce stage a consisté à apprendre des méthodes, utiliser des appareils et acquérir un bagage pour mon prochain stage à SUT concernant l'étude des bactéries lactiques isolées des végétaux fermentés thaïlandais ; étude à propos de la sécurité et qualité alimentaire.

TABLE OF CONTENT

INTRODUCTION

Exopolysaccharides (EPS) are polymers of sugars naturally secreted by some bacteria strains. Bacteria synthesize a number of polysaccharides which are defined by their location relative to the cell. Some are intracellularly located in the cytosol and used as carbon sources, others are cell wall constituents such as peptidoglycan and teichoic acids and a third group is located outside the cell wall.
Due to their many interesting physico-chemical and rheological properties with novel functionality, the microbial polysaccharides (EPS) act as new biomaterials and find wide range of applications in many industrial fields.

Research on microbial polysaccharides is typically interdisciplinary and normally involves a complex series of stages, starting from the selection and manipulation of cellular lines and ending with the structural physicochemical characterization of the excreted biopolymers as illustrated schematically in Figure 1.

I have been given a project on the production of EPS by 3 Bacillus strains, the aim being to study the influence of pH in the culture medium on the production of EPS. My work dealt mostly with the aspect of EPS production in the field of microorganisms' physiology and started to focus on the primary structure determination using carbohydrate chemistry.

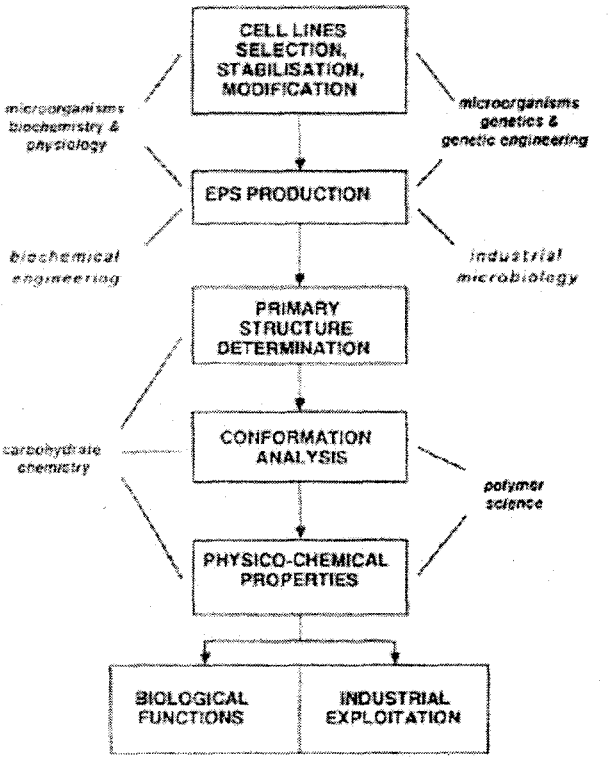


Figure 1 – Series of stages in the research on EPS

The observation of cultures was done under electron microscopy to observe the variations in EPS production. EPS are then to be quantified by a colorimetric assay to relate the results obtained with SEM with a quantitative method. Finally an analysis of EPS has been carried with HPLC in order to determine the monomer composition and quantity.

My work consisted in making my own stocks of Bacillus, reviewing EPS applications, reviewing the use of electron microscopy for the study of EPS, establishing a protocol for the sample preparation to be viewed under electron microscope, performing the experiments. Then I reviewed method for EPS extraction and quantification, performed

ethanol extraction and colorimetric assay for total sugar, and finally tried to determine the monomer composition by HPLC.
In a first part, a review on the different aspects of the project has been done to understand the knowledge state on this particular subject. The second part is presenting methods, protocols, and apparatus used. The third part focuses on the results and their explanation.

I. REVIEW

1. Exopolysaccharides (EPS)

a. Definition of EPS

Bacterial polysaccharides can be found as cytolasmic (storage of starch, glycogen) or wall (structural) components. It may also be totally dissociated of the cell and observed as a loose slime, which is then called extracellular polysaccharides (EPS).
These polymers may be assembled as capsular (CPS) polysaccharides that are tightly associated with the cell surface, or they may be liberated into the growth medium (i.e., "ropy" polysaccharide). The term exopolysaccharide (EPS) may be used to describe either type of extracellular polysaccharide [Sutherland, 1972].
Microbial EPS are produced by various genera of bacteria and yeasts. EPS present, thus, an infinity of forms.

b. EPS components

Bacterial EPS can be composed of one type of sugar monomer (homopolysaccharide, e.g., dextran by *Leuconostoc mesenteroides*) or consist of several types of monomers (heteropolysaccharide, e.g., xanthan by *Xanthomonas campestris*).

Polysaccharides are defined in terms of composition, primary structure, average relative molecular mass, and type and arrangements of substituents.
For example, xanthan described according to these 4 parameters:

- *Composition:* glucose, glucuronic acid, mannose, ratio 2:1:2
- *Structure:* poly-β(1→4)D-gluco-pyranose backbone linked with a trimer of mannose, glucuronic acid, and mannose every second glucose
- *Mr*: (2-15) x 10^6
- *Substituents:* acetate and pyruvate groups on mannose

These parameters determine the functional properties of the polysaccharides, such as water solubility, relative and apparent viscosity, and Newtonian or non-Newtonian behavior [Morin 1998].

More than 100 different monosaccharides components have been identified in bacterial polysaccharides, compared to about 20 different monosaccharides found in animals and plants. In addition, 50 nonsugar components, linked to different positions of the sugar residues, have been observed.
Bacterial polysaccharides can be made of aldoses (pentoses, hexoses, heptoses, branched-chain aldoses), glyculoses, amino sugars (mono- or di-), acidic sugars (glycuronic acids, glyculosonics acids), ether substituents (nonsugar aglycons, methyl ethers, 1-carboxyethyl ethers), acetals, acyl groups (derivatives of fatty acids, hydroxy acids, amino acids), amides of glycuronic acids, sulfuric esters and phosphate esters. [Lindberg, 1990]

c. Physiological role of EPS

EPS production has different applications for cells. The polymer matrix of EPS directly surrounding cell wall is called capsular EPS. It helps protecting the bacterium from its environment. EPS great water-binding properties and thus prevent cell from dessication in low-moisture environment [Ophir & Gutnick, 1993]. EPS have also a protective nature against viruses, predation by protozoans, and evasion of phagocytosis. In addition, EPS may alter the diffusion properties both into and out of the cell and have a protective effects against antimicrobial agents or toxic compounds such as furfurals or affect the penetration of toxic metal ions. Because EPS are a slimy substance, they also allow the bacterium to attach itself to inert surfaces. The adhesive properties of EPS are of considerable interest because of the key role EPS plays in biofilm formation and dental caries pathogenesis [Cerning, 1990].
Biofilms can be described as organic material that includes microorganisms embedded in a polymer matrix of their own making. The matrix consists largely of exopolysaccharides and is a tough, elastic, mucoidal material that adheres strongly to soil particles.

d. Various applications of EPS

EPS can be used as thickeners, emulsifiers, stabilizers, gel-forming agents, selective adsorbents, and additive for rheological control. They can find wide range of applications in various industries such as textiles, detergents, adhesives, microbial enhanced oil recovery (MEOR), wastewater treatment, dredging, brewing, downstream processing, cosmetology, pharmacology, and as food additives. They can also be used as a biofilm in geologic applications, and as a plasma extender in blood substitutes [Turner & Bulla, 2007].
Examples of microbial EPS of industrial importance are xanthan produced by *Xanthomonas campestris*, dextran produced by *Leuconostoc mesenteroides*, and gellan produced by *Aureomonas elodea*.

2. Properties of EPS produced by LAB

Lactic acid bacteria are food-grade microorganisms used for dairy products. The ability of some LAB to produce EPS is extensively studied as it would be a way to enhance the viscosity of dairy products without the use of external additives (i.e., xanthan, gellan). Food-grade lactic acid bacteria are particularly studied in order to give a specific rheology to milk or milk-derived products, and to improve the texture of certain meat products [Morin 1998]. Incorporation of EPS may contribute positively to the mouth-feel, texture, and taste perception of fermented dairy products. EPS produced by LAB have themselves no taste. But since fermented milk product becomes more viscous, its residence time in the mouth and time of contact with the palate and taste receptors is increased. As a result, taste perception is increased through an improved volatilization of the intrinsic yoghurt flavors. The benefits of EPS are detectable at extremely low concentrations. The aim is to obtain an appealing visual appearance (gloss) of a product,

to prevent syneresis, to have a creamy and firm texture, and to give a pleasant mouth-feel. [Duboc & Mollet 2001]

In addition to technological benefits, certain EPS produced by LAB are also claimed to have beneficial physiological effects on the consumer. It is speculated that the increased viscosity of EPS containing foods may increase the residence time of ingested fermented milk in the gastrointestinal tract and therefore be beneficial to a transient colonization by probiotic bacteria [German et al., 1999]. A further example of a suggested health benefit of some EPS is the generation of short-chain fatty acids (SCFAs) upon degradation in the gut by the colonic microflora. SCFAs provide energy to epithelial cells and some have been claimed to play a role in the prevention of colon cancer [Cummings & Englyst, 1995; Harris & Ferguson, 1993].
Further health beneficial effects of EPS were postulated in the literature: e.g. an anti-tumor effect of EPS produced by *Lactobacillus* [Oda et al., 1983], a cholesterol-lowering effect by fermented milk viili [Nakajima et al., 1992], immune-modulatory effects from *Bifidobacterium adolescentis* M101-4 [Hosono et al., 1997]. [Duboc & Mollet, 2001]

The negative attributes of EPS synthesis are then associated with their spoilage properties. The synthesis of EPS by LAB during wine and cider production leads to products having undesirable rheological properties. The formation of dental plaques is related to EPS synthesis by LAB. The EPSs from LAB are responsible for biofilm formation that can lead to biofouling. The most notable examples of biofouling are associated with biofilm formation in the equipment used for the processing of dairy products. Biofilms cause a significant number of technical and hygiene problems for the dairy industry. [Laws, 2001]

3. Properties of EPS produced by *Bacillus sp.*

EPS from Bacillus have been relatively little studied as compared to researches on LAB. Some have focused on the negative attributes of EPS, as a way to prevent ropiness in cider for example [Larpin et al., 2002]. Others are trying to isolate EPS from bacillus growing in extreme conditions (i.e. halophilic and thermophilic bacteria) as a source of thermostable biomolecules for new applications [Ganesh Kumar, 2004; Manca et al., 1996; Maugeri et al., 2002]. Researches have also been lead on B.polymyxa which can produce Biopolymer PS 87 that can have many domestic and industrial uses as a suspending agent or thickener. Researches were directing toward the increase in productivity to meet economical needs [Lee et al., 1997].

4. Electron microscopy

a. Definition

It is a type of microscopy that uses electrons instead of light to illuminate and create an image of a specimen. Because electrons have a much shorter wavelength, images can be

obtained with a much higher magnification and resolution, up to 10 million times instead of 10,000. Details up to 0.1nm can be seen, instead of 0.1μm. Unlike a light microscope, which uses glass lenses to focus light, the electron microscope uses electrostatic and electromagnetic lenses to control the illumination and imaging of the specimen.

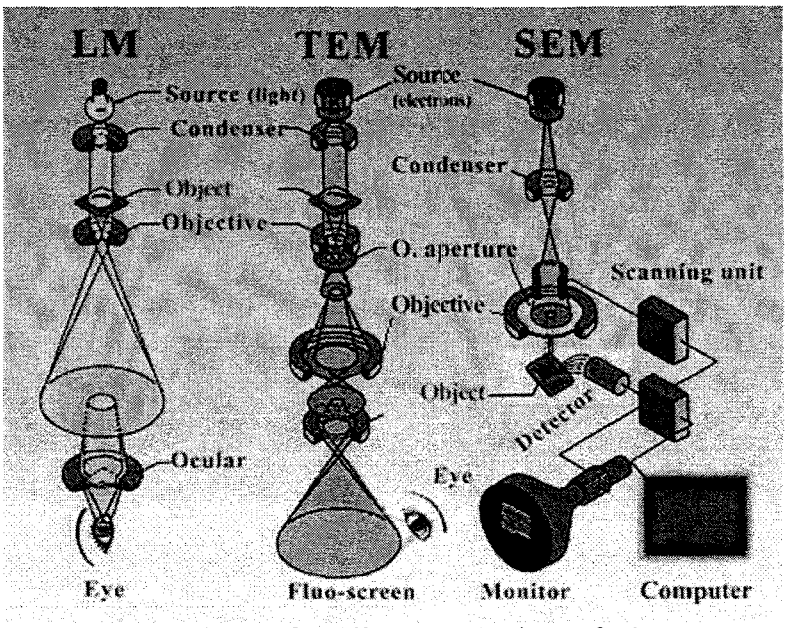


Figure 2 – scheme of 3 different mechanisms of microscopy

EM includes 2 types of conventional instruments that permit to see 2 different types of images, as seen in figures 2, 3 and 4:

- Transmission EM projects a beam of electrons through a very thin slice of specimen and produces a 2D image.

- Scanning EM scans the surface of the specimen with a spot of electrons to generate secondary electrons from the specimen then detected by a sensor. Viewing the surface gives an impression of 3D.

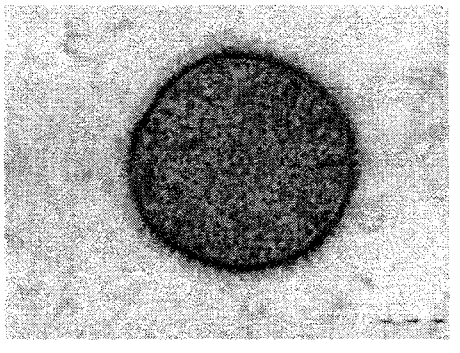


Figure 3 - Section of a cell of *Bacillus subtilis*, taken with a Tecnai T-12 TEM. The scale bar is 200nm.

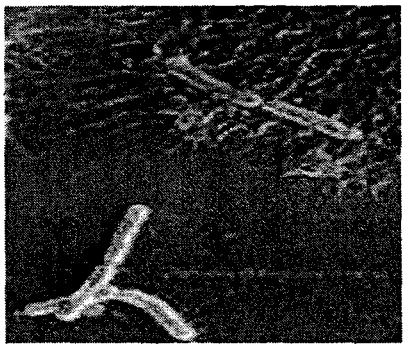


Figure 4 - *Bacillus subtilis hly* entering Int407 cell (J. Wiśniewski, J. Bielecki and M. Sobolewska, SEM)

Light microscope does not fit the study of EPS for 2 main reasons. First of all, medium is largely washed away during sample preparation. Second of all conventional stains do not stain polysaccharides. EPS could be viewed with epifluorescence microscopy, but it is a costly technique. TEM would be of use if we wanted to study polysaccharides synthesis, to know the location of polysaccharides within the cells and at the periphery of cell wall. SEM seems like the best way to closely watch cells' environment.

b. Viewing biological samples and EPS under EM

Conventional EMs are working at high vacuum (less than 1 ten millionth of an atmosphere) and high energy beam (resulting in a considerable rise in temperature). These conditions are totally alien to a biological specimen and would destroy it if the sample were not previously modified.
Living specimens must undergo a number of modifications before it can be seen under electron microscope. Samples are imaged under vaccum, which means that samples that would produce a significant amount of vapor (biological samples) need to be dried. This results in alterations of preservation.
Carbohydrates and mucosubstances are materials with which there are difficulties of preservation. Up to 50% of glycogen is lost with gluteraldehyde fixed tissues and even greater amounts with formaldehyde.

c. Ways to enhance preservation

→ It has been found that additives such as Ruthenium red, when added to the first fixation step, can achieve non-specific preservation and demonstration of cell surface glycolcalyx [Hopwood, 1991].

→ Ultra-rapid freezing achieves fixation and embedment within milliseconds by immobilizing water. It presents several advantages, such as:
- maintaining the original composition of the specimen
- stopping transient morphological changes
- avoiding denaturation of proteins
- allowing cells to be studied in their natural environment
- providing hard specimen that can be sectioned without the introduction of extraneous materials

→ Environmental (or else called "low vacuum") Electron Microscope (ESEM) was specifically designed to study wet, fatty, or insulating materials such as polymers, living cells, food materials, and liquid suspensions, without fixation, dehydration or metal coating, under very low vacuum. In 'wet mode' water is the imaging gas and a separate vacuum pump permits fine control of its vapor pressure (up to 10 Torr) in the specimen chamber. A system of pressure limiting apertures with differential pumping permits the gun to be maintained at high vacuum despite the gas in the sample chamber, which is isolated from the rest of the vacuum system.

II. MATERIALS AND METHODS

1. *Bacillus* culture

a. Bacillus strains

3 strains of Bacillus sp. have been used: 2 unknown strains (which will be called *A* and *B*) isolated by Awanwee, a Ph.D. student, and one strain of *Bacillus subtilis* (named S in these experiments) from the food department laboratory.

b. Media and culture conditions

Liquid medium was a nutrient broth made of beef extract (3 g/l) and peptone (5 g/l). Erlenmeyer flasks 250 ml or 125 ml were incubated for 24h, at 37°C, and shaken at 120 rpm

Solid media was made of beef extract (3 g/l), peptone (5 g/l), and agar (15 g/l). 20ml of this broth was poured into sterilized Petri-dish made of glass (sterilization is made in a dry-air oven at 180°C for 3h). Plates were incubated aerobically at 37°C for 24h.

pH was rectified with HCl and NaOH and checked with pHmeter

Cultures were analyzed for EPS after 22H incubation (approximately at the end of log phase).

c. Preparation of bacteria stock

From frozen samples, I had to prepare my own stock of bacteria for my project.
For each strain:
1 – Pour 200 µl into 100ml of sterilized nutrient broth contained into a 125 ml Erlenmeyer with screw cap.
2 - After 24h incubation at 37°C, measure the optical density (OD) at 660nm, which should be between 1.5 and 2.
3 - Transfer Vi = (Cf * Vf) / Ci into new sterilized nutrient broth. (Vi = volume of preculture to transfer; Cf = OD of the new nutrient broth (around 0.1); Vf = volume of new NB; Ci = OD of preculture)
4 - Incubate at 37°C for 24h. OD should be again 1.5 – 2.
5 - Culture is ready for conservation:
- keep in freezer, 10 eppendorf containing 1 ml culture and 1 ml glycerol 50%
- keep in refrigerator, 5 agar slants inseminated with a loop and incubated for 24h at 37°C

d. Gram Staining

1 - Smear bacteria culture on a slide
2 - Heat the slide a few seconds to fix the sample

3 - Cover with crystal violet (primary stain) for 1 min
4 - Rinse with distilled water
5 - Cover with iodine solution (mordant) for 1 min
6 - Rinse with distilled water
7 - Wash with 95% ethanol (decolorizer) for 15s
8 - Rinse with distilled water
9 - Cover with safranin (counterstain) for 1 min
10 - Rinse with distilled water and dry
11 - Watch under light microscope

2. SEM

a. Protocol for sample preparation

Step	Activity	Time involved
Fixation	Smear sample on pieces of coverslip and let it semi-dry at room temperature	30 min
	Primary fixation: coverslips put into vials with 2-4% gluteraldehyde in buffer	2 hr
	Washing: Cacodylate buffer (2 changes)	2 x 30 min
	Secondary fixation: osmium tetroxide (1-2%)	2 hr
Dehydration	30% ethanol	5 min
	50% ethanol	10 min
	70% ethanol	10 min
	95% ethanol (2 changes)	2 x 10 min
	absolute ethanol (2 changes)	2 x 20 min
Critical point drying	transitional fluid: ethanol is displaced by liquid CO2	45 min
	Critical point is achieved at 31.1°C and 1,073 PSI	
	vapor slowly released until atmospheric pressure	
Coating	mount specimen on stub	45 min
	vacuum sputter coating chamber until 0.1 Pa	
	gold coating of 15-40 nm	

b. Apparatus

On figures 5, 6 and 7 can be seen the specific apparatus used for preparing and imaging culture samples with electron microscopy

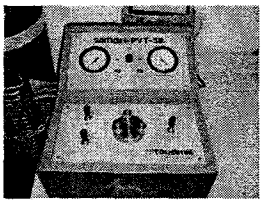


Figure 5- Critical point drier: TOUSIMIS model Samdri-PVT-3B

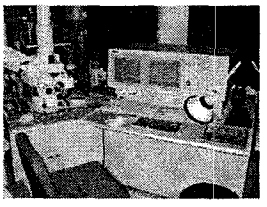


Figure 6- Ion sputtering device: JEOL model JFC-1100E

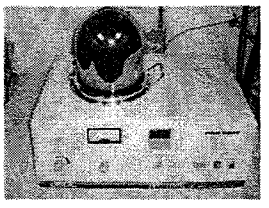


Figure 7- SEM: JEOL model JSM-6400

3. EPS isolation

Rimada and Abraham (2003) have performed a comparative study on methodologies to extract EPS from the supernatant. On the 8 methods tested, I chose the simplest one, though reliable. The protocol was first described by Van Geel-Schutten et al. (1998).

1 – Put sample in a boiling water bath for 10 min in order to remove eps from cells
2 – Centrifuge at 10,000 rpm for 10 min to remove cells
3 – Precipitate EPS by the addition of 2 volumes of cold ethanol to supernatant
4 – Maintain at 4°C for 24h
5 – Centrifuge at 10,000 rpm for 15 min at 4°C
6 – Resuspend pellets in hot water
7 – Repeat steps 3 to 6

4. EPS quantification

Colorimetric assay
Phenol – sulfuric acid method for total sugars [Dubois et al., 1956]

1 – Add 2 ml 5% phenol to 1ml sample
2 – Add 5 ml 96% sulfuric acid
3 – Let it stand for 10 min
4 – Shake and analyze after 20 min with spectrophotometer at 485 nm (490 nm for hexoses and 480 nm for pentoses and uronic acids)

Results are compared to standard curve for glucose. Results are then given in grams equivalent glucose.

5. HPLC

Pellets of EPS isolated from 40 ml culture were dried in a freeze-dryer and kept at -20°C until further processing. Freeze-dryer model was a Lyovac GT 2. Hydrolysis of lyophilized EPSs was performed with 1ml of 0.1 N H_2SO_4 at 100°C for 3h. The solution was then neutralized with 0.1 N NaOH.
Sugar components were identified by high-pressure liquid chromatography (HPLC). HPX-87H ion exchange column with a refractive index detector BioRad model 1755 was eluted with 0.8 ml/min of 5mM H_2SO_4 as a mobile phase. Analysis was performed by injecting 20 µl sample into the solvent dispenser with a syringe. Syringe was rinsed 5 times with sample solution before injection and with 5mM H_2SO_4 solution after injection.

III. RESULTS AND DISCUSSION

1. Bacillus culture

As I was going to use the 3 strains of Bacillus for 3 month, I would need to make a stock. The plan is described in § II.1.c
But, with these culture conditions, OD has never reached 1.5. It was always below 0.8. Some reasons were thought of:

- Because the culture was made from frozen samples, growth of bacteria might have been low in the first days.
- Though bacillus is anaerobic facultative, oxygen transfer was not sufficient

Conditions were slightly changed to try to improve growth of bacteria:

- incubate for several days instead of 24h
- change shaker from 120 to 140 rpm
- close Erlenmeyer flasks with cotton instead of a screw cap

Ultimately, what was gained was contamination.
Cultures were then grown on agar plates to individualize bacillus colonies from contaminants. I went on culturing in flasks, made a stock in agar slants, but did not make a frozen stock.

By the end of the internship, I finally understood how to obtain the bacteria concentration desired. The volume of culture in the flasks had simply to be reduced so as to allow greater oxygen transfer. For example, 25O ml Erlenmeyer flask had to contain 100ml culture instead of 200ml.

2. Cell Concentration

Cells were counted using the pouring plate method:
Bacillus A: approximately 37 colonies were counted on plates inseminated with 0.1 ml of dilution 10^{-6} of the original culture 0.743 OD. Bacteria concentration is 3.7. 10^{8} cells/ml.
Bacillus B: approximately 40 colonies were counted on plates inseminated with 0.1 ml of dilution 10^{-6} of the original culture 0.79 OD. Bacteria concentration is 4. 10^{8} cells/ml.
Bacillus subtilis: approximately 26 colonies were counted on plates inseminated with 0.1 ml of dilution 10^{-6} of the original culture 0.795 OD. Bacteria concentration is 2.6. 10^{8} cells/ml.
The following graph (figure 8) gives the relation between numbers of bacteria per ml with OD

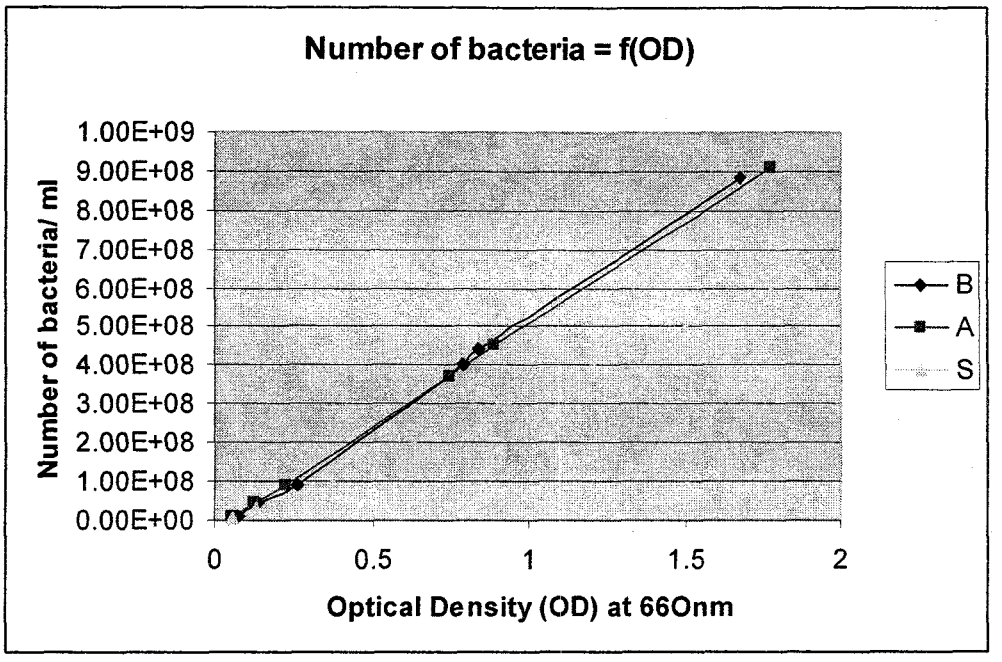


Figure 8- Relation between numbers of bacteria per ml with OD for each strain

3. Gram staining

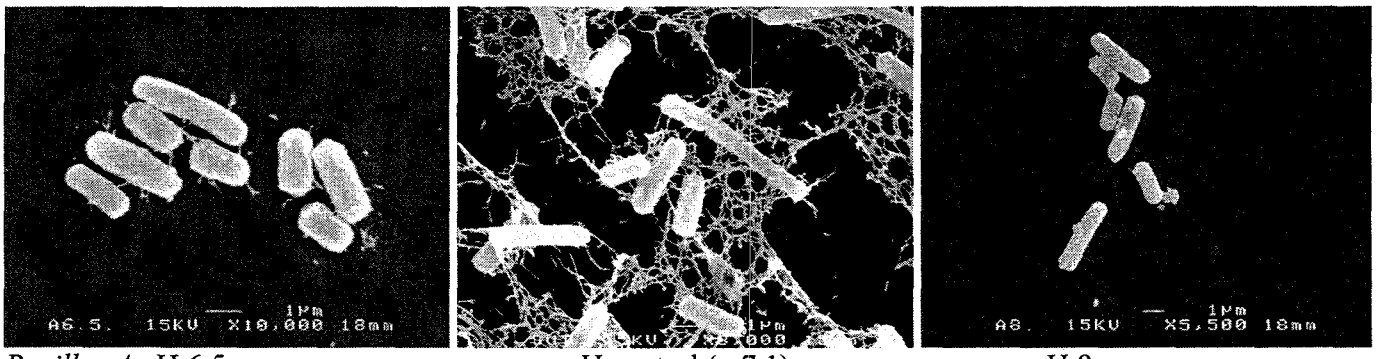
Bacillus sp. is Gram positive. Under the microscope purple rods can be observed, some of them containing spores.

4. Effect of pH on bacillus growth

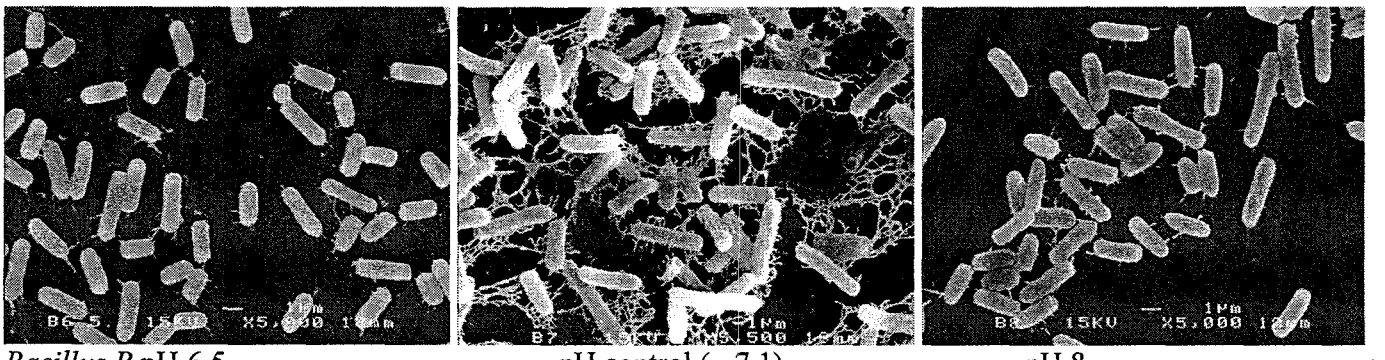
Effects of pH 3.5, 5, 6.5, 8 and control (~7.1) were tested. The 3 strains do not grow at pH 3.5 and 5, but they do at pH 6.5, 8 and control. It confirms the fact that *Bacillus subtilis* has its optimum pH at 7-8. OD has not been checked for cultures at pH 6.5 and 8; assumptions upon SEM pictures could give an indication on growth, but no conclusions can be made.

5. Bacillus EPS under SEM

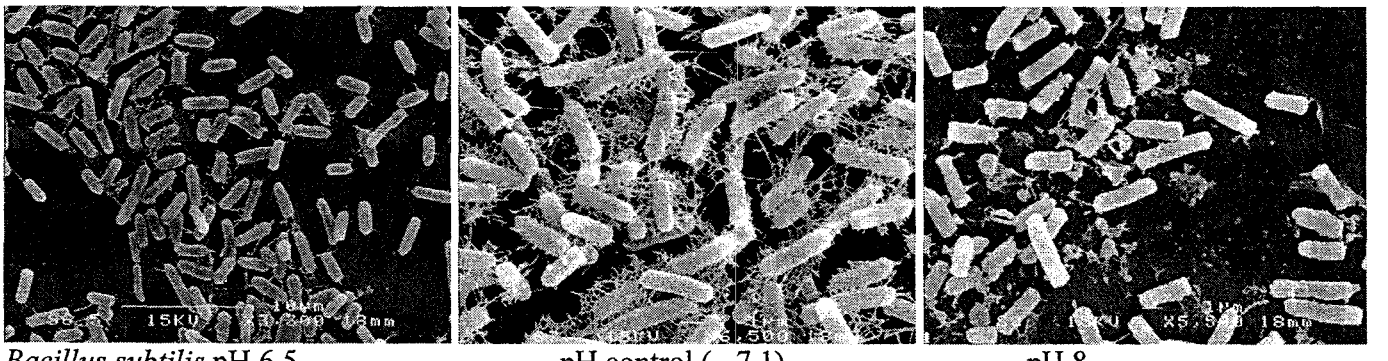
Though comparisons have been made on many more pictures, on figure 9 is presented a selection of SEM pictures trying to be the most representative and comparable of the 3 strains at 3 different pH:



Bacillus A pH 6.5 pH control (~ 7.1) pH 8



Bacillus B pH 6.5 pH control (~ 7.1) pH 8



Bacillus subtilis pH 6.5 pH control (~ 7.1) pH 8

Figure 9– SEM pictures of *Bacillus A*, *B* and *subtilis* at pH 6.5, control (~7.1) and 8

Bacillus can be seen as round-ended straight rods of 1 to 5 μm. EPS appear as lines, net or substance, holding bacteria together, attaching them to the surface.
For each strain, and in these selected pictures, we can observe a lot more EPS surrounding cells at pH 7 than at pH 6.5 and 8. This is because there were 2 batch of culture. Culture of pH control was made using 100 ml medium in 250 ml Erlenmeyer flasks, instead of 100 ml medium in 125 ml Erlenmeyer flasks. Rise in O_2 transfer resulted in much better growth of bacteria (up to 1.8 OD) and apparently, greater EPS production. We can say that EPS production is O_2 dependent.
Though no obvious differences can be made, it seems that there are more EPS at pH 6.5 than at pH 8, especially for *Bacillus A*. There would be a better EPS production around neutral pH.
All conclusions have to be read carefully, because SEM gives a qualitative point of view. For example, on the basis of these selected pictures, it could seem like growth of *Bacillus A* was less than *B* and *subtilis*, but regarding OD results, it is not true. At control pH, OD of Bacillus A was 1.774, whereas OD of B was 1.678.
It must be kept in mind that conventional SEM modifies greatly the specimen by a succession of steps which causes the loss of at least 50% of EPS. Results may not be reproducible if conditions slightly change.
SEM can be seen as a way of global appreciation of EPS production, but not as a method to quantify EPS.

6. EPS quantification

Colorimetric method as described by Dubois et al. (1956) was used to calculate the total sugar content in the supernatant. A first batch of assay was done for pH 3.5, 6.5 and 8. Then a second batch was done for pH control (~7.1) and for the medium.

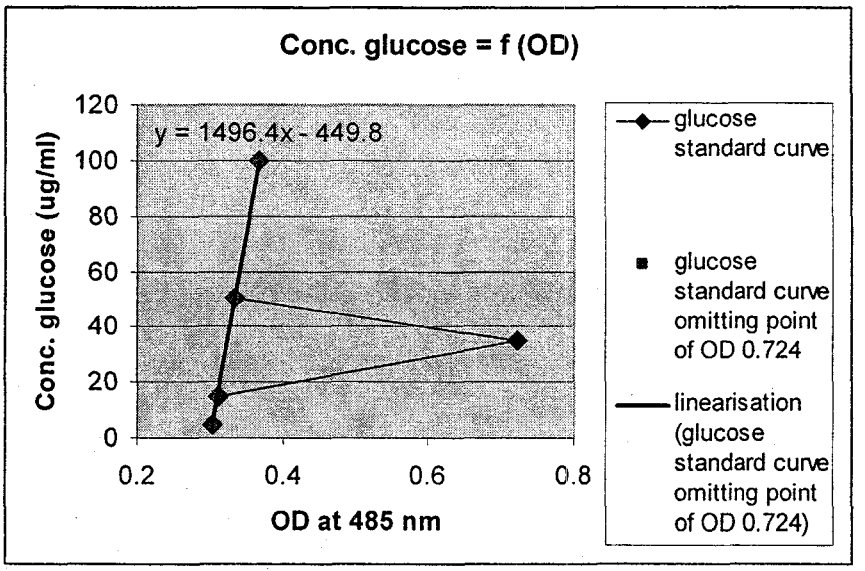


Figure 10– 1st Standard curve used in the colorimetric method for determination of sugars

Table 1 - Concentration of EPS for each strain at pH 3.5, 6.5 and 8.

	0D at 485 nm	conc eq. gluc (µg/ml)
A 3.5	0.419	177.1916
A 6.5	1.546	1863.6344
A 8	0.707	608.1548
B 3.5	0.668	549.7952
B 6.5	1.102	1199.2328
B 8	0.446	217.5944
S 3.5	0.459	237.0476
S 6.5	0.496	292.4144
S 8	0.417	174.1988

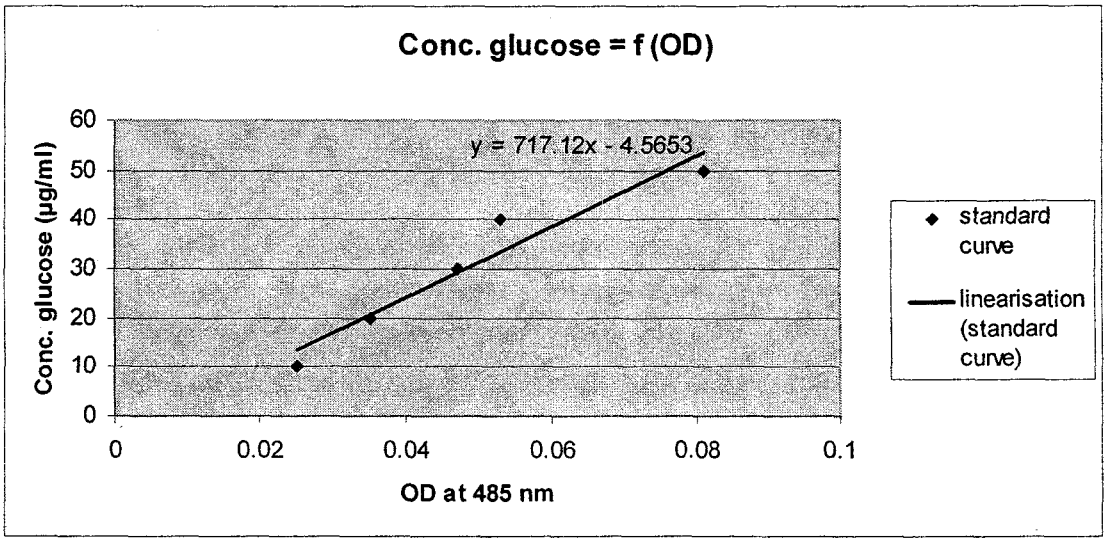


Figure 11 – 2nd Standard curve used in the colorimetric method for determination of sugars

Table 2 - Concentration of EPS for each strain and in the medium at pH control

	OD	Conc. eq. Glucose (µg/ml)
A7	0.033	19.09966
B7	0.04	24.1195
S7	0.04	24.1195
Medium	0.064	41.33038

These experiments were performed to try to relate what is seen under SEM to the actual quantity.

The first batch of results is unusable since the standard curve does not make sense, thus we cannot rely on the results obtained for pH 3.5, 6.5, 8. Since there was no growth at pH 3.5, these results should give the original concentration of carbohydrates in the medium. But results at pH 3.5 greatly differ, from 177 to 549 µg/ml.

Bacillus subtilis seems to produce much less EPS, which is in contradiction with what is seen under SEM and the second batch of results.

The second standard makes more sense, but compared to the data given in Dubois et al. 1956, numbers are not good. The important thing would be the reproducibility of the results, but it has not been done on this experiment
Second batch of results gives an amount of EPS greater in the original medium than after fermentation. It is normal, because bacteria are using medium for their growth. As the medium is not chemically defined, sources of carbon are unknown. Some sugars from the original medium are still present after fermentation, depending on quantity used for bacteria's growth. It is not possible to determine real EPS production in a nutrient broth. Fermentation process should be done in, at least, a semi-chemically fixed medium, and EPS isolation should contain a step removing the original source of carbon.

7. Monomer composition

A mixture of known sugars was analyzed to have standard retention time. For each sugar, 2 concentrations were analyzed to make the standard quantification curves.

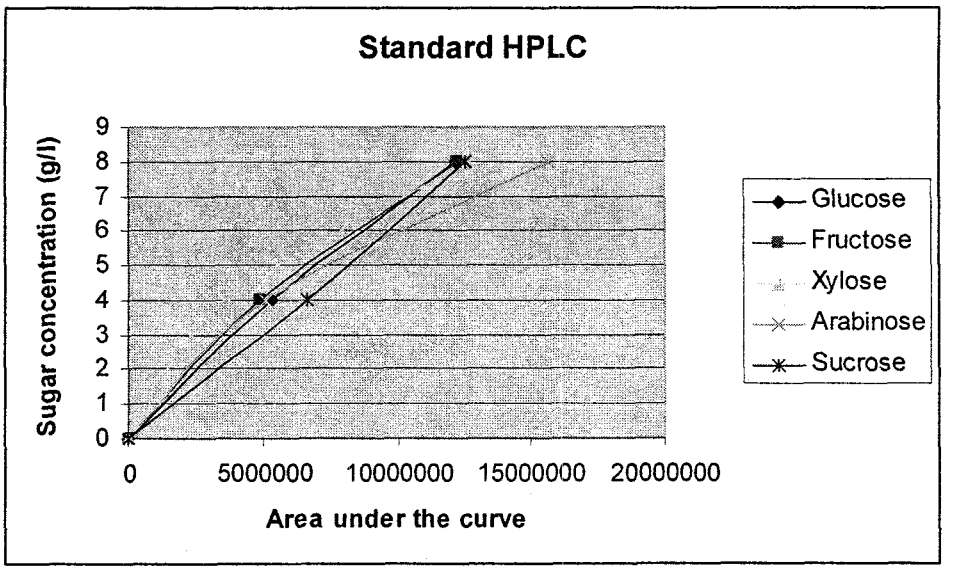


Figure 12 – Standard curve for quantification of sugars with HPLC

Table 3 – Retention times in HPLC column of 5 standard sugars tested

Sugar	Retention time (min)
Sucrose	6.092
Glucose	7.305
Xylose	7.875
Fructose	8.163
Arabinose	8.812

Bacillus A, B, subtilis and medium at pH control were analyzed with HPLC. The 4 results showed a peak at 4.98 min of area ranging from 6,584,853 to 8,444,206. This retention time is way inferior to the sugars tested. Plus, area under the curve gives a concentration

of sugar approximately equals to 4 g/l, whereas sugar concentration in the samples should not be more than 1g/l. Then we can wonder what in the process of EPS extraction could give this peak. It is known that some proteins might get extracted as well during the process, but it would not give the same big peak for all 4 samples. It seems more probable that this peak is due to ethanol, since the solvent is present in large quantities throughout the process.
Few smaller peaks are also present. But their height must be superior to 3 standard deviations (= 3 times higher than noise) to be taken in account. For Bacillus A, one peak is big enough and corresponds to a sugar tested. Its retention time is 7.35 and its area is 1721. We can conclude that Bacillus A supernatant contains 0.2 g/l monomers of glucose. No other significant peaks can be noticed.

CONCLUSION

Discovering new microbial strains producing high amounts of EPS is of an industrial importance. Methods to study EPS production are the first steps for researches.
It has been shown that SEM can give a good appreciation on EPS production. From a homogeneous solution, pictures should be taken as representative of the total sample as possible. In this manner, comparison of 2 different samples can be done accurately, especially on important variations and only from a qualitative point of view.
Of course, quantitative methods are more reliable if performed in the right manner. In my case, practice and reproducibility would have been necessary to obtain good results. Since these experiments were only for my practical training, it was only important for me to understand the process and draw conclusions. The HPLC was carried out properly displaying an acceptable standard curve. But concentration of sugar in samples was not high enough to be accurately detected.
From a microbial point of view, what was found was that The 3 strains of bacillus did not grow at acid pH (pH 3.5 and 5). The best growth was found to be at neutral pH. It has also been shown that, even though Bacillus sp. is anaerobic facultative, large oxygen transfer is necessary for the cells to grow and obtain a larger production of EPS. Concerning their EPS production, Bacillus B and B.subtilis had the better production (24 µg eq. glucose/ml) compared to Bacillus A (19 µg eq. glucose/ml), results that demand confirmation by using a medium from which residual carbon source would not be counted into this colorimetric assay and by repeating this method a number of times. HPLC showed that EPS from Bacillus A displayed monomers of glucose at a concentration of 0.2 g/l. Other common sugars like mannose, rhamnose, glucuronic acids, or else, should be tested as standard to know their elution time and identify new peaks in samples.

Even though, the laboratory work did not involved handling lactic acid bacteria, it allowed me to see, through the publication study, the industrial importance of EPS for dairy products and other applications. The quantity of researches outgoing on this particular subject shows the financial supports for food quality.
For my next project in SUT, I will study the parameters of lactic acid bacteria (LAB) isolated from Thai fermented vegetables in regard to their antagonistic activity against *Staphylococcus aureus*. The basis of this project concerns food security as well as food quality.
I can say that working on EPS production this year gave me a first approach and highlighted one of the many parameters about researches on fermented food. I improved my knowledge on microbial culture, dealt with very specific equipment, learned the operation of the laboratory, plan the experiments, establish protocols according to the material available, face difficulties with insufficient results and try to make the most of them. At a human level I learned that communication and integration are an essential part of the laboratory life.

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U.S. patent n°. 10682044

ANNEXES

Annex 1:

Dubois et al.'s standard curves in the colorimetric assay for determination of sugars

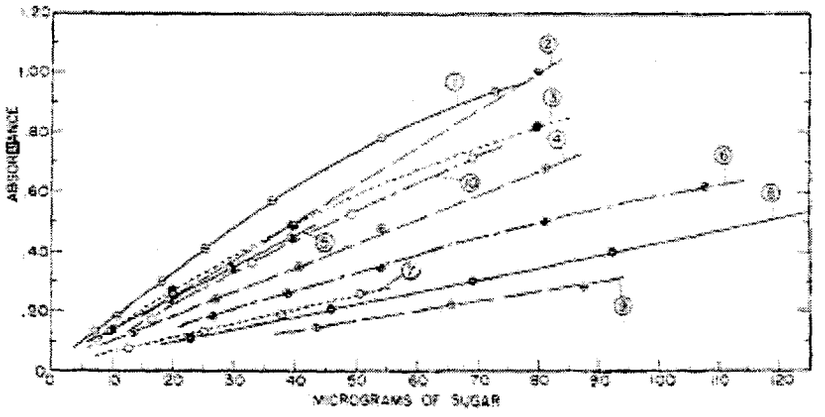


Figure 1. Standard curves

1. D-Xylose, Coleman Jr., 480 mμ, 17 mg. of phenol
2. D-Mannose, Beckman Model DU, 490 mμ, 40 mg. of phenol
3. D-Mannose, Evelyn, filter No. 490, 40 mg. of phenol
4. D-Galactose, Coleman Jr., 490 mμ, 33 mg. of phenol
5. L-Arabinose, Coleman Jr., 480 mμ, 17 mg. of phenol
6. D-Galacturonic acid, Coleman Jr., 483 mμ, 17 mg. of phenol
7. L-Fucose, Coleman Jr., 480 mμ, 40 mg. of phenol
8. D-Glucurone, Coleman Jr., 485 mμ, 17 mg. of phenol
9. 2,3,4,6-Tetra-O-methyl-D-glucose, Coleman Jr., 485 mμ, 17 mg. of phenol
10. D-Glucose, Beckman Model DU, 490 mμ, 100 mg. of phenol

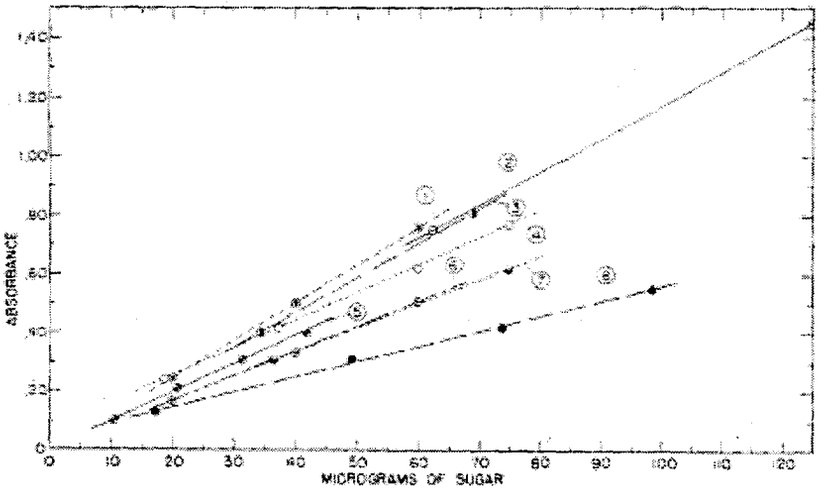
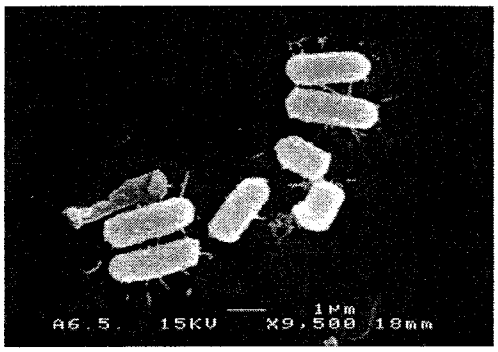


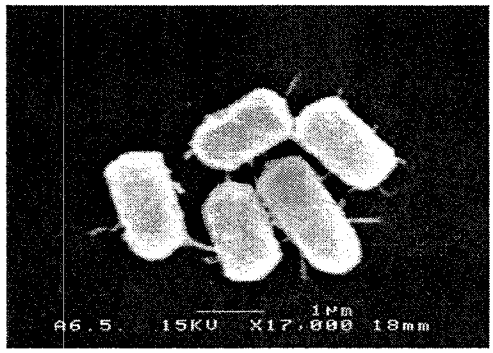
Figure 2. Standard curves

1. Sucrose, Beckman Model DU, 490 mμ, 100 mg. of phenol
2. Potato starch, Beckman Model DU, 490 mμ, 100 mg. of phenol
3. Dextran from *Leuconostoc mesenteroides* strain NRRL 512, Beckman Model DU, 490 mμ, 105 mg. of phenol
4. D-Glucose, Evelyn, filter No. 490, 80 mg. of phenol
5. L-Rhamnose, Coleman Jr., 480 mμ, 40 mg. of phenol
6. Raffinose, Beckman Model DU, 490 mμ, 100 mg. of phenol
7. D-Fructose, Beckman Model DU, 490 mμ, 200 mg. of phenol
8. 2-Deoxy-D-ribose, Coleman Jr., 480 mμ, 80 mg. of phenol

Annex 2:
Other interesting SEM pictures

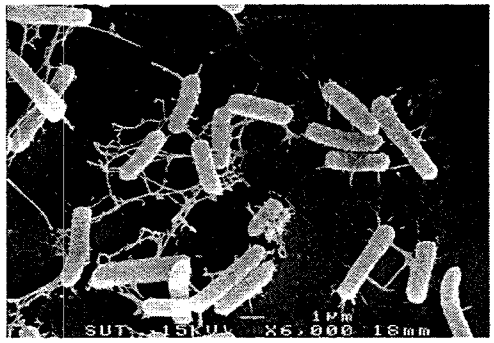


Bacillus A pH 6.5

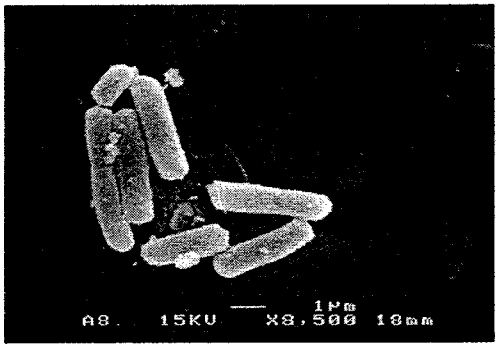


Bacillus A pH 6.5

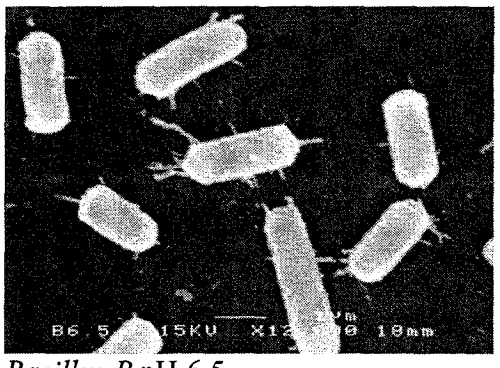
Bacillus A pH 7



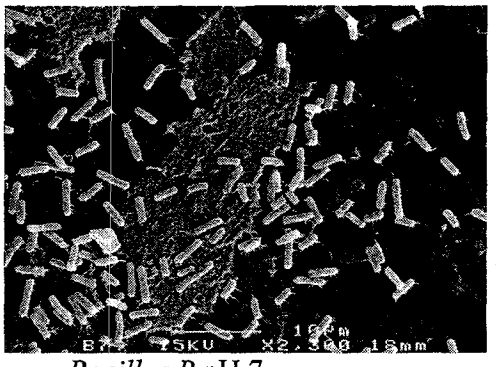
Bacillus A pH 7



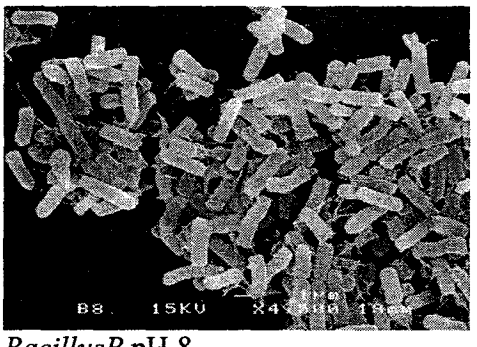
Bacillus A pH 8



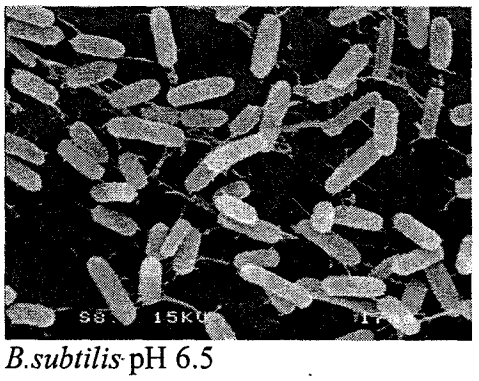
Bacillus B pH 6.5



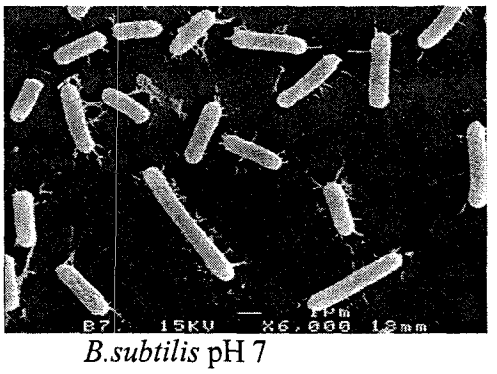
Bacillus B pH 7



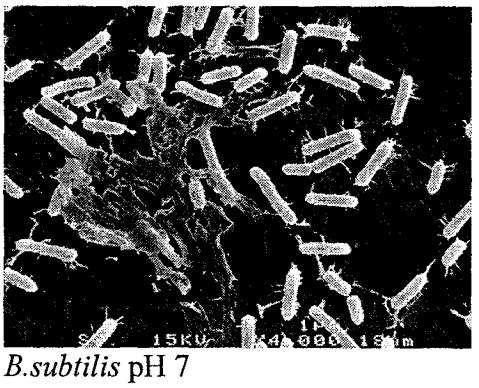
BacillusB pH 8



B.subtilis pH 6.5



B.subtilis pH 7



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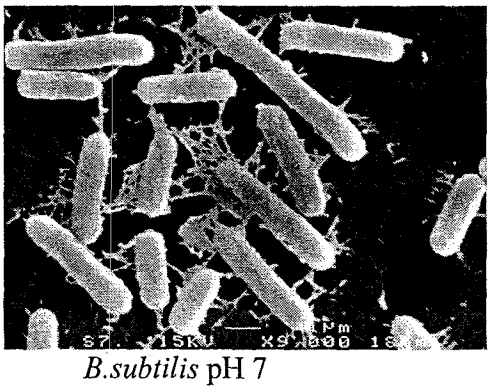

B.subtilis pH 7