Université Lille 1

Sciences et Technologies

Thèse

Présentée par

Sameh FAHIM FARAGALLAH SHEDED

Pour l'obtention du titre de

Docteur de l'Université Lille 1 Sciences et Technologies
Spécialité : Ingénierie des Fonctions Biologiques

PROCEDE DE PRODUCTION DE LIPOPEPTIDE PAR *BACILLUS SUBTILIS*

Préparée au Laboratoire ProBioGEM, UPRES-EA 1026

Soutenue le 21 Septembre 2011 devant le Jury composé de :

Philippe Jacques,	Professeur (Université Lille 1, France)	Co-directeur
Iordan Nikov,	Professeur (Université Lille 1, France)	Co-directeur
Henri Delmas,	Professeur (Université de Toulouse, France)	Rapporteur
Jacqueline Destain,	Professeur (Gembloux AgroBioTech-Université de Liège, Belgique)	Rapporteur
Frédérique Gancel,	Maitre de Conférences (Université Lille 1, France)	Examinateur
Ludovic Montastruc	Maitre de Conférences (Université de Toulouse, France)	Examinateur

Lille 1 University

Science and Technology

PhD Thesis

Engineering of Biological Functions

By

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Laboratory ProBioGEM, UPRES-EA 1026

Polytech'Lille

September, 21th 2011

Philippe Jacques,	Professor (University of Lille 1, France)	Co-supervisor
Iordan Nikov,	Professor (University of Lille 1, France)	Co-supervisor
Henri Delmas,	Professor (University of Toulouse, France)	Reviewer
Jacqueline Destain,	Professor (Gembloux AgroBioTech-University of Liege, Belgium)	Reviewer
Frédérique Gancel,	Assistant Professor (University of Lille1, France)	Examinator
Ludovic Montastruc	Assistant Professor (University of Toulouse, France)	Examinator

Résumé

Dans cette étude, plusieurs facteurs environnementaux influençant la production de lipopeptides ont été testés dans le but de développer un réacteur de type lit fluidisé inversé couplé à une alimentation par aspersion. La production de lipopeptides (appartenant aux familles des surfactines et des fengycines) a été étudiée en culture planctonique ou en culture immobilisée, chez la souche de *Bacillus subtilis* BBG 21, mutant spontané de la souche sauvage *Bacillus subtilis* ATCC 21332. Les résultats de production du mutant ont été comparés à ceux obtenus pour la souche sauvage. Les principaux paramètres pris en compte lors des fermentations sont le transfert d'oxygène, le pH et la température de culture ainsi que l'addition de particules solides. L'influence du coefficient de transfert d'oxygène (k_La) et de la dissipation de puissance (P) a été testée en faisant varier la géométrie des fioles de culture, les volumes de remplissage et la vitesse d'agitation de celles-ci. La production de surfactine est corrélée positivement à l'augmentation du transfert d'oxygène contrairement à la synthèse de fengycine qui est maximale pour de faibles taux de transfert. Pour chaque type de k_La (bas, modéré ou élevé), un rapport particulier surfactine/fengycine peut être déterminé. La meilleure productivité est observée pour un pH initial du milieu de culture fixé à 7. L'augmentation de la température de fermentation permet d'améliorer le taux de production de la surfactine mais réduit la synthèse de fengycine qui devient presque indétectable à 37°C. Dans la suite du travail, la production de lipopeptides, le transfert d'oxygène et l'apparition de mousse ont été étudiés - à l'échelle du laboratoire - dans un lit fluidisé inversé couplé à une alimentation par un ruissellement de liquide en tête de réacteur. Le k_La et le taux de moussage ont été déterminés pour un type de particules solides (non poreuses et de surface rugueuse), pour des vitesses superficielles de liquide (U_L) variant de 0,001 à 0,003 m s^{-1}, des vitesses de gaz (U_G) de 0,003 à 0,009 m s^{-1} et pour des tensions de surface (σ) de 72 à 42 mN. m^{-1}. Une forte diminution du k_La est observée quand la tension de surface chute de 72 à 42 mN. m^{-1}. L'importance du support a également été mise en évidence. Le réacteur, dans sa configuration initiale, générait de la mousse qui rendait difficile la maîtrise de la fermentation. Plusieurs modifications successives nous ont permis de proposer une configuration pour laquelle la mousse est absente. Plusieurs débits de gaz et plusieurs régimes d'écoulement ont été étudiés dans cette dernière configuration.

Abstract

The influence of several environmental factors on the lipopeptide production by *Bacillus subtilis* was first tested in view of its development in a new design of inverse fluidized bed bioreactor coupled with a liquid film top section. The production of lipopeptides - surfactin and fengycin - was performed with free and immobilized cells of *Bacillus subtilis* BBG21, which is a spontaneous mutant of the strain *Bacillus subtilis* ATCC 21332. The results were compared to those obtained with the wild-type strain. The main parameters tested were: oxygen transfer, pH, temperature and addition of solid carriers. The influence of volumetric oxygen transfer coefficient (k_La) and power dissipation (P) was tested in shake flasks by using different flasks geometries, relative filling volumes and shaking frequency. The surfactin production showed high correlation with the increasing of oxygen transfer rate, contrary to fengycin production which showed an optimum at relatively low oxygen transfer rate. Three different surfactin/fengycin ratios were observed with high, moderate, and limited k_La. The best productivity was obtained for the culture at initial pH 7.0. Interestingly the increasing of temperature improved the surfactin yield but reduced the fengycin production which was nearly undetectable at 37°C. Then, the optimization of production and the gas-liquid oxygen transfer and foaming were studied at laboratory scale in the inverse fluidized bed bioreactor with a liquid film top section. The k_La and foaming activity were determined at various conditions using model and real culture conditions and surfactant levels below, near and over the critical micelle concentration (CMC). The k_La values were determined for a selected type of modified surface roughness particles and for a range of superficial liquid velocities (U_L) (0.01 to 0.03 m s^{-}), gas velocities (U_g) (0.003 to 0.009 m s^{-1}) and surface tensions (σ) (32 to 72 mN m^{-1}). A strong reduction of this parameter has been observed in the real and model media when the surface tension decreased from 72 to 32 mN m^{-1}. The influence of support has also been demonstrated. In addition, in the first tested reactors the presence of surfactin and fengycin generated extensive foaming that imposed difficulties on the bioreactor performance. This parameter was controlled by the development of a new reactor design. Different ranges of gas space velocity, as well as no steady state regimes of fluid flows have been studied.

Acknowledgement

I would like to thank Professor Philippe Jacques for allowing me to work in ProBioGEM laboratory under his co-supervision and for his kind attention and continuous scientific guidance.

I would also like to thank Professor Iordan Nikov for his great support and for his follow-up throughout this work. I would like to thank him about all the knowledge I gained from him under his co-supervision.

I express my deepest gratitude to Dr. Frederique Gancel for supported me throughout this work, for giving me the benefit of her knowledge and for her valuable support during the writing of this thesis.

I do not forget Dr. Krasimir Dimitrov from ProBioGEM and Dr. Ludovic Montastruc for their cooperation which enabled me to be easily integrated.

Lastly, I thank my father, my mother, my wife, my brother and my colleagues in ProBioGEM laboratory for all the support, trust and words of encouragement they have given me.

And thank to any one helped me in all slices of life.

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Symbols and abbreviations

Nomenclatures

a		Gas-liquid superficial area	$m^2. m^{-3}$
CMC		Critical micellar concentration	$mg. L^{-1}$
d		Maximum inner shake flask diameter	m
d_0		Shaking diameter	m
D_t		Fluidization tube diameter	m
DO		Dissolved oxygen	$mg. L^{-1}$
g		Gravity acceleration	$m. s^{-2}$
k_L		Liquid-size Mass transfer coefficient	$m. s^{-1}$
k_La		Volumetric mass transfer coefficient	s^{-1}
Mo		Morton number	$Mo = \frac{g(\rho_l - \rho_s)\mu_l^4}{\rho_l^2 \sigma^3}$
N	..	Shaking frequency	r.p.m.
Ne		Newton number	$Ne' = \frac{P}{\rho N^3 d^4 V_L^{1/3}}$
OTR		Oxygen transfer rate	$mol. L^{-1} h^{-1}$
P		Power dissipation	W
P/V		Volumetric power dissipation	$W. m^{-3}$
PPch		Polypropylene with activated carbon coated particles	
PS		Polystyrene particles	
Re		Reynolds number	$Re = \frac{\rho N d^2}{\mu}$
T		Temperature	°C
TPIFB		Three phase inverse fluidized bed	
SDS		Sodium dodecyl sulfate	
SEM		Scanning electron microscope	
U_g		Superficial gas velocity	$m. s^{-1}$
U_l		Superficial liquid velocity	$m. s^{-1}$
V_G		Gas volume	m^3
V_L		Relative filling volume	m^3
V_{reac}		Reaction volume	m^3

Greek symbols

σ	Surface tensions	mN. m^{-1}
σ_w	Water surface tensions	mN. m^{-1}
ρ_l	Liquid density	kg. m^{-3}
ρ_s	Solid density	kg. m^{-3}
μ	Dynamic viscosity	Pa. s
τ	Time	s
ν	Cinematic viscosity	m^2. s^{-1}

CHAPTER I

General introduction

The actual level of knowledge on the biosynthesis of lipopeptides from *Bacillus subtilis* such as surfactin and fengycin and its regulation mechanism allows developing different techniques to overproduce the main active compounds and to reach yields that are compatible with industrial development of these bioactive surfactants (Jacques, 2011). Surfactin and fengycin are cyclic lipopeptides composed of, respectively, 7 or 10 amino acid residues linked to one β-hydroxy fatty acid. The length of the fatty acid chain varies from C13 to C16 for surfactin and from C14 to C18 in the case of fengycin (Ongena and Jacques, 2008). These long lipidic chains should be extended freely in solution below the critical micellar concentration (CMC, 10 $mg.L^{-1}$ for surfactin and 11 $mg.L^{-1}$ for fengycin (Deleu *et al*., 1999)) and strongly participate in intermolecular hydrophobic interactions in supramolecular structures such as micelles. These lipopeptides are effective biosurfactants: surfactin is known for its high foaming capacity while fengycin is less tensioactive and haemolytic than surfactin but shows a strong antifungal activity (Ongena and Jacques, 2008).

These compounds are mainly produced during the decelerating phase of growth (Jacques *et al*., 1999). Several studies reported that the production of lipopeptides is strongly influenced by oxygen transfer conditions (Hbid *et al*., 1996; Liu *et al*., 2007 and Guez *et al*., 2008). Recently, it has been communicated that the aerobic immobilized cultures of *Bacillus subtilis* ATCC 21332 produce 2-4 times more lipopeptides than planktonic cells (Chtioui *et al*., 2010 and Gancel *et al*., 2009).

These lipopeptidic compounds are strong biosurfactants responsible for foam formation in aerated bioreactor and the remaining challenges in the engineering of the aerobic bioreactors are thus related to oxygen mass transfer (the continuous gas flow regime in the reactor can be carried out applying a bubbling aeration and/or surface aeration).

When bubbling aeration is applied the main problem concerns the control of foaming. Two major factors particularly spoil the surfactant producing process: (1) stable foaming evolution causes liquid outflow and changes substrate concentration and mixing intensity; (2) presence of lipopeptide may decrease oxygen transfer rate and thus lowering bioreactor yields (Martinov *et al*., 2008).

It is well-known that surfactants affect bubble dynamics, gas hold -up and gas-liquid mass transfer in three ways. Firstly, they reduce the average of bubble size, increasing the bubble interfacial area and decreasing the rise velocity. Secondly, they immobilize the bubble surface: this effect tends to keep the average bubble size small and raises velocity low (Dimitrov, 2007).

Finally, they inhibit the bubble coalescence and provoke formation of fine foam; the complex heterogeneity of the flow due to foaming in these bioreactors is a key problem to associate oxygen transfer to cell response.

Development of a bubble-less process, by an oxygenation from continuous gas phase to a liquid surface in flasks, should lead to understand the relation between energy supplies, gas-liquid mass transfer and fermentation production and to facilitate the scale-up of lipopeptides production bioprocesses.

Indeed, chemical engineering parameters have been described in shake flasks to ensure defined culture conditions as mass transfer (Maier *et al*., 2004), power input (Maier and Büchs, 2001), hydro-mechanical stress and fluid movement (Peter *et al*., 2006) or influence of the power dissipation on cell response (Mehmood *et al*., 2010).

With such an approach, it should be possible to correlate the liquid-side mass transfer coefficient (k_L), the specific area (a) and the lipopeptide productivity and selectivity. These results should be also of interest to design bioreactor onto its selectivity as well as to develop adequate mathematical model.

Conforming to the above aspects, the first aim of this study was to investigate lipopeptides (surfactin and fengycin) production capacity with immobilised and planktonic cells of *B. subtilis* BBG21. Surface aerated cultures in shake flasks were used in order to establish the relationship between the power dissipation and k_La and to analyse the selectivity during the production process in function of several environmental parameters.

Lipopeptide production was thus studied in the following different conditions:

- different types of shake flasks (Pallone, cylinder, Erlenmeyer);
- different volumes of culture medium (50 to 1000 mL) and various relative filling volumes (0.05 to 0.4 mL media/ mL flask);
- different agitation rates (150 to 250 rpm);
- presence of solid particles;
- various temperatures (20 to 40 °C);
- various initial pH (6.8 to 7.2).

Kinetics of growth, pH evolution and lipopeptide production were established and compared for *B. subtilis* ATCC 21332 and its spontaneous derivative BBG21 known to produce higher concentrations of lipopeptides (especially fengycin) than the wild-type strain.

From all these data, relationships between $k_l a$ and power dissipation and $k_L a$ and lipopeptide production were established.

In the second part of this study, a three-phase inverse fluidized bed (TPIFB) biofilm reactor was designed for lipopeptide production. This process can operate in different configurations: (1) liquid batch regime (complete recycling) and continuous gas flow or (2) continuous regime of gas and liquid with partial liquid recycling.

Liquid recycling that induces bed expansion is relatively low and this is an advantage compared to the operating conditions in two-phase classical fluidized bed and two-phase inversed fluidized bed reactors (Nikov and Karamanev, 1991; Nikov and Delmas, 1992; Essadki *et al*., 2005 and Dimitrov, 2007).

Superficial gas velocity in IFB biofilm reactors is lower than in other equipments i.e. airlift and three-phase fluidized bed reactors, it decreases the energy cost for support expansion (Nikov and Delmas, 1987; Bang *et al*., 1998 and Dimitrov, 2007).

In comparison to classical fluidized beds and agitated reactors, higher oxygen transfer rates could be obtained in IFB reactors at lower gas flow and shear rates (Nikolov *et al*., 2000 and Essadki *et al*., 2005 and Dimitrov, 2007).

Conforming to the above aspects, the second aim of this study was to control gas-liquid mass transfer and foam formation through modifications of energy supply and structure of a three-phase inverse fluidised bed. The production capacity of a laboratory scale modified IFB biofilm device using different oxygen and energy transfer strategies during the production process was also analysed.

CHAPTER II

Review of literature

II.1. *Bacillus subtilis*

Historically, *Bacillus subtilis* was one of the first bacteria to be studied. Originally named *Vibrio subtilis* in 1835 by Christian Ehrenberg a contemporary of Robert Koch, this organism was renamed *Bacillus subtilis* in 1872 by Ferdinand Cohn (Fritze, 2004).

In *Firmicutes* phylum, which contains the most prominent types of endospore-forming bacteria (*Clostridia* and *Bacilli)*, the genus *Bacillus* belongs to the family of *Bacillaceae*. The genus consists in a group of diverse organisms as evidenced by the wide range of DNA base ratios from 32 to 69 mol % G + C (Garrity *et al*., 2007).

II. 1.1. Description and significance of *Bacillus subtilis*

As shown in Figure II-1, *B. subtilis* is a Gram-positive, rod-shaped (2 to 10 µm), endospore-forming bacteria. Under adverse environmental conditions the ability of *B. subtilis* to produce highly resistant spores and to grow under a wide range of conditions indicates that released strains likely survive outside of containment (Claus and Berkeley, 1986).The growth normally occurs under aerobic conditions but, in complex media in the presence of nitrate, anaerobic growth can occur (Perez, 2000).

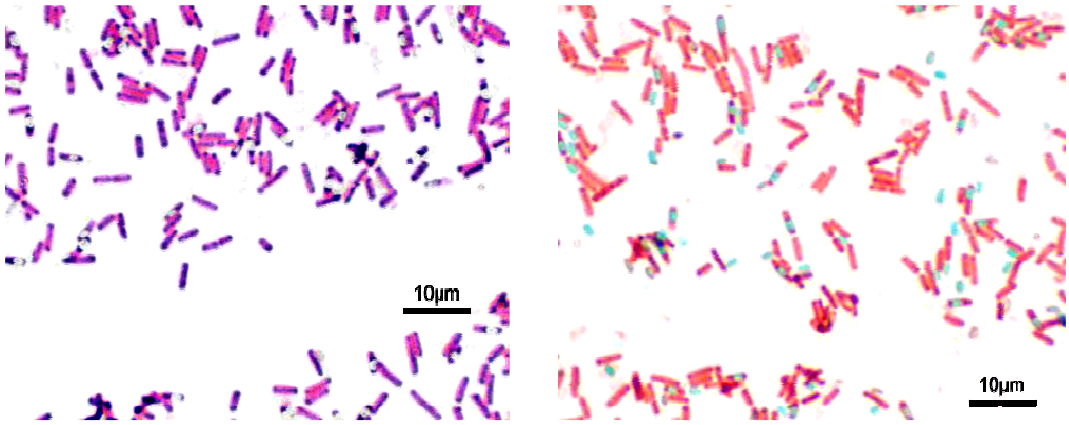

Fig (II-1): Optical microscope photo for Gram-stained and Spore-stained Bacillus subtilis. (http://en.wikipedia.org/wiki/Bacillus_subtilis)

B. subtilis grows in single cells or cells arranged in chains. Cells arranged next to each other can only swarm together, not individually and these arrangements of cells are called 'rafts' (Julkovska *et al*., 2005). *B. subtilis* strains use their flagella for a swarming motility that

occurs on surfaces, for example on agar plates, rather than in liquids. *Bacillus* strains also secrete slime layer which includes surfactin in order to swarm (Schaechter *et al*., 2006).

The optimal growing temperature is comprised between 25 and 35 °C whereas optimal production of antibiotics can occur at a higher temperature of 37 °C in specific strains. Strains of the genus are common saprophytic inhabitants of soil, including low-nutrient soil. Due to their association with soil particles, they reside predominantly in even marine and freshwater habitats, and are also inevitably transferred to plants, foods and animals and contribute to nutrient cycling due to synthesis of numerous enzymes. Strains of this species could also be isolated from fermented products (Jamil *et al*., 2007).

II. 1.2. Importance and applications of *Bacillus subtilis*

Currently, *B. subtilis* is a significant microorganism in the scientific research field, as well as in biotechnology and industry. It is considered as a laboratory model especially for studying endospore formation in bacteria. Endospores in *B. subtilis* are mostly formed in the tips of protuberances extending downward from liquid surface pellicles (Schaechter *et al*., 2006).

The stress and starvation are common in the environment and *Bacillus* strains have evolved a set of strategies that allow survival under these harsh conditions. A first strategy is the formation of stress-resistant endospores. Many strains produce spores with brown pigments. Depletion of carbon, nitrogen, or phosphorous stimulate the process of sporulation; however, the process needs to start before the entire exhaustion of nutrients (Perez, 2000).

When carbon- nitrogen- and phosphorus- nutrient levels fall below the bacterium's optimal threshold, spores are produced. Scientists have demonstrated that *B. subtilis* bacteria are also capable of secreting antibiotics in great numbers. Five signal peptidase genes were found to be important for this secretion function (Ara *et al*., 2007).

A lot of *B. subtilis* genes are responsible for antibiotic synthesis. The production of antibiotic increases the chance of *B. subtilis* to survive because they can kill surrounding microbes competing for the same nutrients.

B. subtilis forms rough biofilms which are dense organism communities at the air and water interface. These biofilms communities are beneficial as they allow the control of plant pathogen infections. The biofilms also form a mutualistic interaction with plant rhizome

systems and the preemptive colonization prevents other pathogens from infecting the plant (Morikawa, 2006).

Bacillus subtilis naturally supports plant growth. These bacteria often play a role in replenishing soil nutrients by supplying the terrestrial carbon and the nitrogen cycles. Also, the strains can act as biofungicides for benefiting agricultural crops (Kunst *et al*., 1997).

The scientists often use *B. subtilis* as a model organism in genetic area. *B. subtilis* 168 which is the reference strain, becomes naturally competent- able to bend and internalize DNA from a medium- during its transition between the exponential phase and the stationary phase of growth. Thus, this strain could be easily modified and acts as a good laboratory microorganism. Due to endospore-forming mechanisms, *B. subtilis* is also an excellent model for cell differentiation. Furthermore, *B. subtilis* produces a variety of enzymes and metabolites, including antibacterial and antifungal compounds, used in different industrial sectors. The use of *B. subtilis* in an industrial setting should not pose an unreasonable risk to human health or environment. First, human health and environmental hazards of *B. subtilis* are low. Second, the number of microorganisms released from the fermentation facility is low. In addition, *B. subtilis* is ubiquitous in the environment and the release expected from the fermentation facilities will not significantly increase populations of this bacterium in the environment (Katz and Demain, 1977; Parry *et al*., 1983).

Overall, *B. subtilis* has a low degree of virulence. Although the possibility of human infection is not non-existent, it remains low in the industrial setting. Its status of "generally recognize as safe" (GRAS) organism makes it an excellent industrial microorganism (Food and Drug Administration FDA, 2006). The FDA has reviewed three submissions for production of enzymes using genetically modified *B. subtilis* and found no unreasonable risks to human health or the environment from the use of this microorganism in fermentation facilities.

II. 1.3. Genome and metabolism of *Bacillus subtilis*

The first complete DNA sequence of *Bacillus subtilis* was achieved by Kunst *et al*. in 1997. The total size of all DNA is 4.2 Mbp representing 4.100 genes. Several families of genes have been expanded by gene duplication and represent 25 % of the chromosomic DNA. Most of the essential genes are involved in metabolism. The genome contains genes for the catabolism of many diverse carbon sources. Half of the essential genes are responsible for processing information. One-fifth of them are responsible for cell wall synthesis, cell division and shape,

and one-tenth of them are responsible for the energetic of the cell. 4% genes code for functions that are not known (Kobayashi *et al*., 2003 and Zeigler *et al*., 2008).

B. subtilis cells have been considered as strictly aerobic cells when glycolytic and Tricarboxylic Acid Cycle (TCA) pathways are complete and functional (figure II-2):

Cells seem to require oxygen to grow and cannot undergo fermentation. While, recent studies show that they can indeed grow in anaerobic conditions making them facultative aerobes.

The bacteria can make ATP in anaerobic conditions via butanediol fermentation as well as nitrate ammonification (Nakano *et al*., 1998).

Growth of *B. subtilis* in the presence of glucose results in a reduction in the pH of the culture medium. The pH generally reaches its lowest point at the end of the exponential phase and increased rapidly in stationary phase. Andrew *et al*. (2000) suggested that acetoin production is a mechanism to maintain internal pH of cells entering in the stationary phase and to accumulate pyruvic acid that is not required for biosynthesis.

Acetoin is a major extracellular product of *B. subtilis* grown on glucose or other fermentable carbon sources. The enzymes responsible for the formation of acetoin are acetolactate synthase (AlsS) and acetolactate decarboxylase (AlsD) are synthesized in detectable amounts only in cells that have reached stationary phase. AlsS and alsD genes appear to compose a single operon (Maria *et al*., 1993). It has been proposed that the formation of acetoin and its reduced product butanediol plays two important roles in the cell: the first one is the maintenance of intracellular pH, the second one is reduction of acetoin to generate butanediol may replenish pools of NAD (Gotfredsen *et al*., 1983 and Tsau *et al*., 1992).

Genes encoding enzymes that carrying out this reaction have not been found in *B. subtilis*. Although, it has been previously shown that a large percentage of glucose added to cultures of *B. subtilis* grown in complex medium is converted to acetate and pyruvate during vegetative growth. Excreted acetate is then used early during sporulation after glucose has been depleted. Acetoin production occurs 1 to 2 h following acetate production and utilization of acetoin occurs after acetate levels have dropped (Henkin *et al*., 1984).

B. subtilis has the ability to produce acid from a variety of sugars in response to nutrient limitation. It also produces several enzymes including proteases, amylases, cellulases, lipases and antibiotics. The enzymes produced degrade macromolecules to be used as energy sources while the antibiotics are used to limit bacterial competition for the newly discovered energy sources (Devine, 2000).

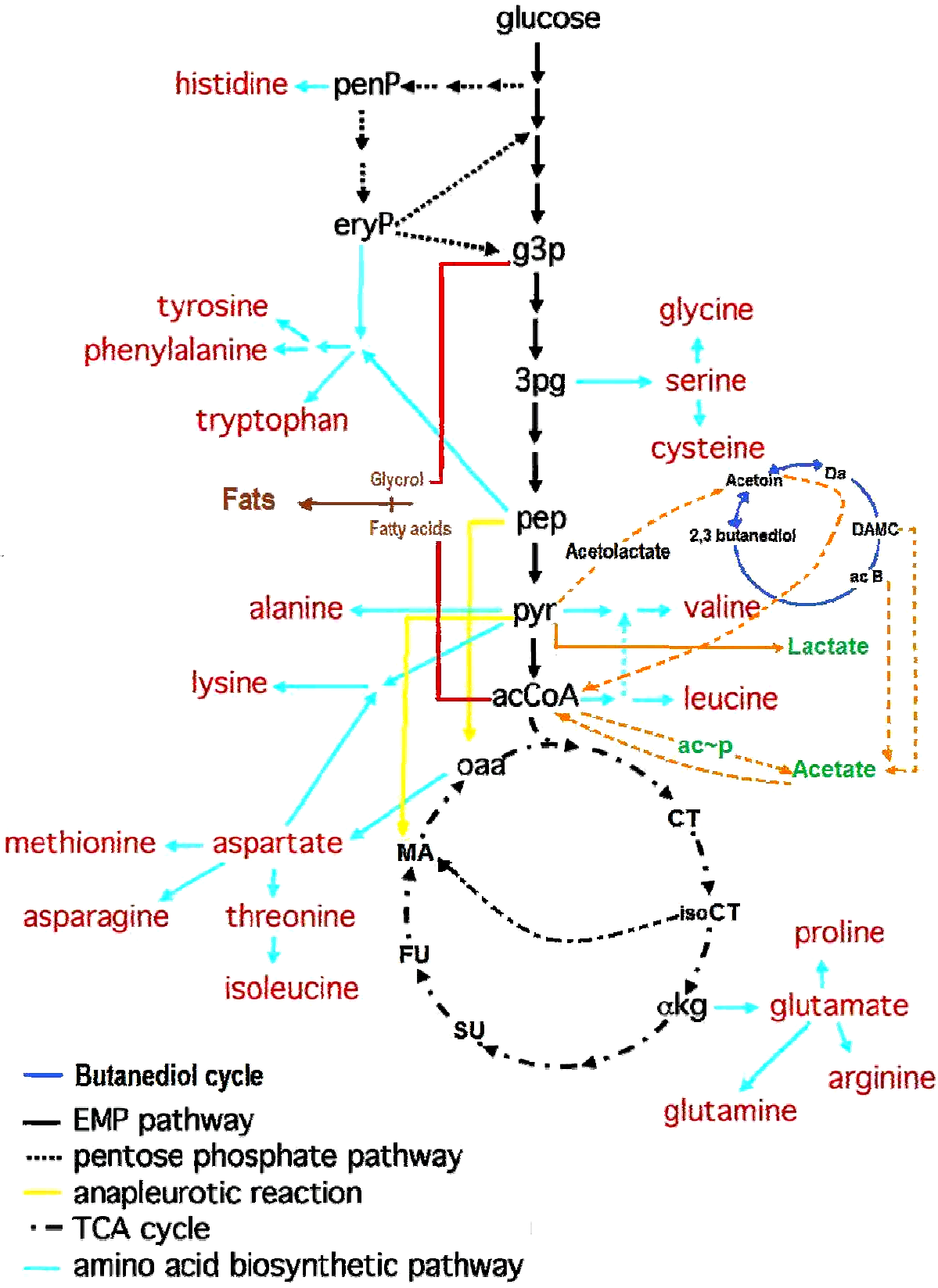

Fig (II-2): Proposal scheme of *Bacillus subtilis* central metabolism pathways.
(penP) pentose phosphate; (eryP) erythrose phosphate; (g3P) glyceraldehyde 3.phosphate; (3Pg) 3.phospho glycerate; (Pep) phospho enol pyruvate; (pyr) pyruvate; (acCoA) acytyl co-enzyme A; (Da) di-acytyl; (DAMC) di-acytyl methyl carbinol; (ac B) acetyl butanediol; (CT) citrate; (iso CT) iso-citrate; (αkg) α-ketoglutarate; (SU) succinate; (FU) fumarate; (MA) malate; (Oaa) oxalo-acetate.

Furthermore, *B. subtilis* contains 34 different two-component systems which can detect environmental conditions and initiate the appropriate responses to these conditions. This large number of two-component systems suggests that *B. subtilis* can respond to a variety of environmental conditions effectively with a wide range of responses (Devine, 2000).

B. subtilis is prototrophic for glutamate when grown with fumarate, the next intermediate of TCA cycle is transported into the cells by the same permease as succinate (Asai *et al*., 2000). Generally, glutamate is synthesized exclusively by the glutamate synthase and it can be degraded by the glutamate dehydrogenase. However, the major glutamate dehydrogenase (RocG) is expressed only in the presence of arginine and the bacteria are unable to utilize glutamate as the only carbon source. In addition to RocG a second cryptic gene (gudB) encodes an inactive glutamate dehydrogenase (Fabian *et al*., 2008).

Historically, in 1954, Alexander Keynan reported that during an investigation of the glutamine and glutamic acid metabolism of *B. subtilis,* it was observed that both of these compounds markedly stimulated the utilization of glucose and the analysis of the reaction products revealed an additional effect of the dicarboxylic acid and its amide i.e. stimulation of the non-oxidative conversion of pyruvic acid to acetoin with simultaneous decrease of the oxidative breakdown of the keto acid.

The percentage of stimulation was about the same in the pH region ranging from 6 to 8. Although, the rate of utilization of glucose was maximal at pH 7.4, it should be noted that the stimulation of glucose disappearance by glutamine could not be demonstrated under anaerobic conditions i.e. in a nitrogen atmosphere. In another investigation, Szulmajster and Hanson (1965) showed that the simultaneous presence of glucose and glutamic acid caused repression of aconitase synthesis in *B. subtilis* which in turn led to the inhibition of spore formation.

Under the latest conditions, glucose was metabolized very slowly at pH 7.4 with no effect of added glutamine at pH 7.0; the amount of glycerol produced was less than 1 per cent of the glucose utilized; acetoin, 2.3-butanediol, lactate and steam-volatile acids accounted practically quantitatively for all the glucose used.

In the case of *B. subtilis* grown in the presence of exogenous amino acids, the cells can produce glutamate not only by the glutamate synthase reaction but also by α-ketoglutarate-utilizing aminotransferase reactions and by degradation of certain amino acids, e.g.… glutamine, proline and arginine, the arginine catabolism to glutamate proceeds by the first

three steps of the Roc pathway through ornithine and α-glutamate semi-aldehyde (Fisher *et al*., 2002).

However, the transcription is high when cells are grown in glucose-ammonium medium and low when cells are grown in glucose-glutamate or glucose-proline medium. Also, an intermediate to high level of transcription is seen if glutamine or glutamate and ammonium or proline and ammonium are used as the nitrogen source (Bohannon *et al*., 1989 and Belitsky *et al*., 1995). While, various amino acids like glutamic acid have been reported to be allow the fermentation process (Nakano and Zuber, 1998).

Generally, the transcription of the *glt*A operon, encoding glutamate synthase, is controlled by two regulatory GltC and TnrA proteins; GltC is required for any significant expression of the *glt*AB operon, but TnrA can repress transcription even if GltC is fully active (Fisher, 1999).

The activities of these proteins depend on the nature of the nitrogen and carbon sources in the medium and respond to a variety of nutritional factors, primarily to availability of ammonium and amino acids of the glutamate family (Yoshida *et al*., 2003).

II. 2. Lipopeptides produced by *Bacillus subtilis*

Historically**,** the group of lipopeptides was discovered from *Bacillus* species during 1950s and 1960s. It gathers more than 30 different peptides linked to various fatty acid chains, more than 100 different compounds can so be described (Jacques, 2011).

Up to now, these lipopeptides were classified into three different families: surfactins, iturins and fengycins-plipastatins.

II. 2.1. Lipopeptide families: structure and relative operons

In 1968, Arima *et al*., first isolated an exocellular compound with an exceptional biosurfactant activity from the supernatant of a culture of *Bacillus subtilis*. This compound was named surfactin and its structure elucidated as that of a lipopeptide. Other lipopeptides were then found which differ in their fatty acid chain and their peptide moiety (Kakinuma *et al*., 1968). The resulting wide diversity of molecules can be used to study the relationships between the structure and function of the lipopeptides which are showed in figure (II-3).

The existence of several different compounds with the same molecular weight shows that it is essential to precisely characterize their structure by using, for example, LC-MS-MS techniques (Jacques, 2011).

II. 2.1.1. Surfactin family

The family of surfactins was composed of about 20 different lipopeptides (Bonmatin *et al*., 2003). With the exception of esperin (Thomas and Ito, 1969), they are heptapeptides with a chiral sequence LLDLLDL interlinked with a β-hydroxy fatty acid and with a D-Leu in position 3 and 6 and an L-Asp in position 4. Amino acid residues in position 2, 4 and 7 belong to the aliphatic group including Val, Leu and Ile (Peypoux *et al*., 1991; Itokawa *et al*., 1994 and Bonmatin *et al*., 1995).

Surfactin was characterized as a valuable inhibitor of fibrin clot formation, an antibacterial, antitumor and hypocholesterolemic agent. The *srf*A operon and the enzymes responsible for the biosynthesis of the surfactin were the first described for a lipopeptide from *B. subtilis* (Nakano *et al*., 1991 and Menkhaus *et al*., 1993).

This discovery established that this lipopeptide was synthesized by the non-ribosomal pathway (Konz *et al*., 1999).

II. 2.1.2. Iturin family

Mycosubtilin was the first discovered member of iturins family from *B. subtilis* mentioned in literature (Walton and Woodruff, 1949). A second similar compound named iturin was described by Delcambe in 1950. Its name is related to the Ituri, a region from Congo where the compound was isolated from a soil sample. Iturin A is the main studied lipopeptide of this family, it is a heptapeptide interlinked with a β-amino acid fatty acid with a length from C14 to C17 (Peypoux, 1978). Six other members of the iturin family were then described: iturin C, bacillomycin D, F, L and Lc and mycosubtilin (Bonmatin *et al*., 2003).

All iturins have the same LDDLLDL chiral sequence with a common part of the peptide cycle; β-amino acid L-Asx – D-Tyr – D-Asn, except for Iturin C in which first amino acid of the peptide chain is L-Asn according to the recent work from Volpon *et al*. (2007).

Iturin was first characterized as a strong antifungal agent with a restricted antibacterial activity against *Micrococcus* and *Sarcina* strains. The precise structure of similar compounds from the same species was described by Peypoux *et al*., in 1976 and 1986.

The genes involved in the biosynthesis of iturinic compounds have been first characterized for mycosubtilin in *B. subtilis* ATCC 6633 (Duitman *et al*., 1999), then for iturin A (Tsuge *et al*., 2001) and bacillomycin D (Moyne *et al*., 2004 and Koumoutsi *et al*., 2004).

II. 2.1.3. Fengycin family

In 1986, german and japanese teams (Vanittanakom *et al*., 1986 and Nishikiori *et al*., 1986) discovered simultaneously a third family of lipopeptides, fengycins, produced by *B. subtilis* and plipastatins produced by *B. cereus*.

Fengycins were determined as antifungal agents and plipastatins as phospholipase A2 inhibitors, the operon encoding fengycin - plipastatin synthetases was first described in *Bacillus subtilis* 168 in 1997 (Tosato *et al*., 1997).

Only small structural differences exist between these two compounds and a doubt still exists today about them, as well as the differences in their biological activities. Both molecules are lipodecapeptides which differ by their amino acid residue in position 6 that can be Ala (formA) or Val (formB). They display an internal lactone ring in the peptidic moiety between the carboxyl terminal amino acid (Ile) and the hydroxyl group in the side chain of the tyrosine residue in position 3.

Different β-hydroxy fatty acid chains (C14 to C18) are linked with an amide bond to the N-terminal amino acid residue (Glu) (Nishikiori *et al*., 1986 and Vanittanakom *et al*., 1986).

The main representative fatty acid chains are C15, C16 and C17. Two differences were initially identified between fengycin and plipastatin: Gln instead of a Glu in position 8 and the L and D forms of tyrosine, which are in position 3 and 9 respectively, for plipastatins and 9 and 3 for fengycins. In the different works describing fengycin structure since its discovery, the presence of a Glu in position 8 was never mentioned. However, Schneider *et al*. (1999) confirmed the existence of fengycin molecule with a D-Tyr in position 3 from the supernatant of *B. subtilis* S499.

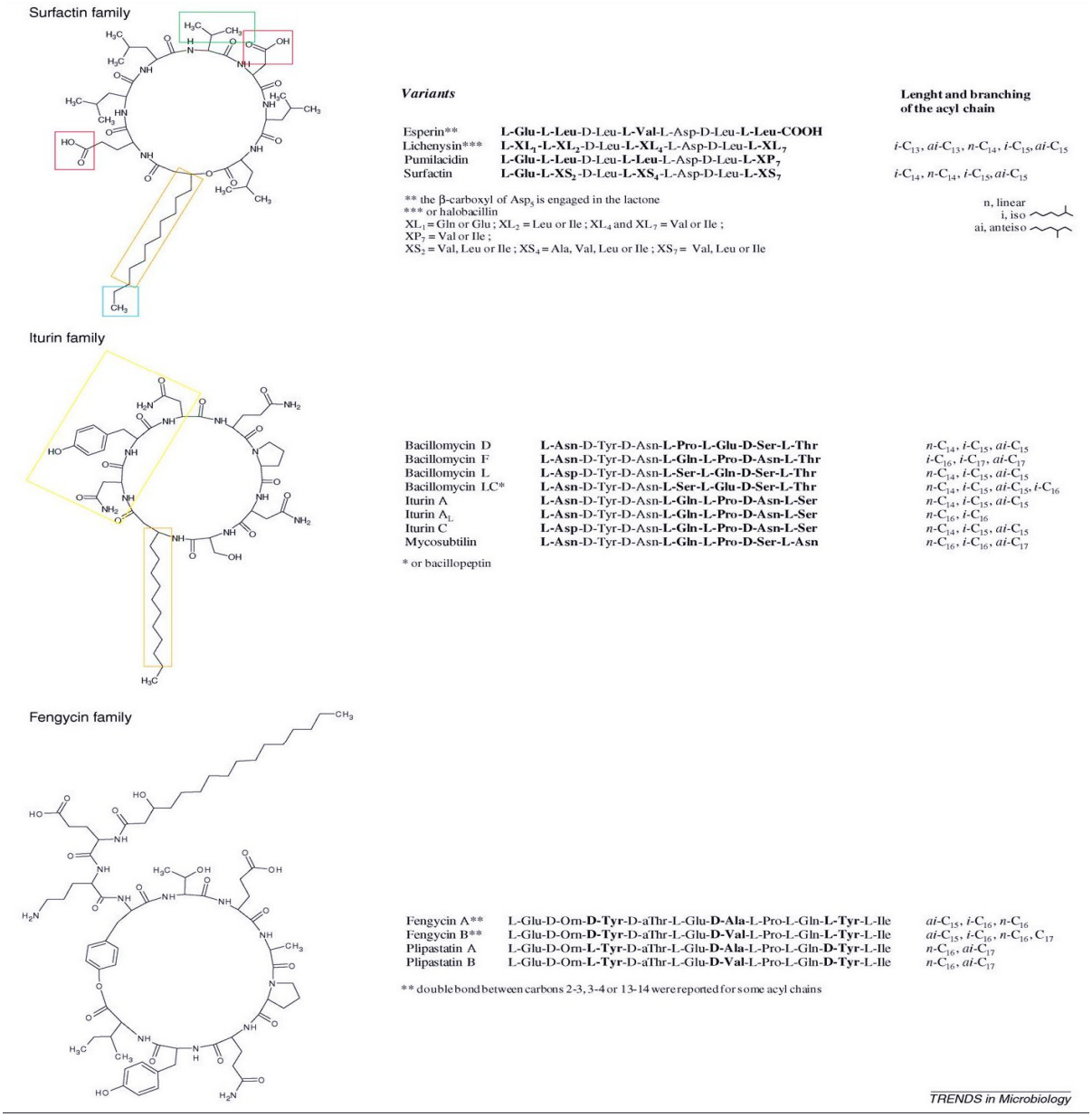

Fig (II-3): Structures of representative members and diversity within the three lipopeptide families synthesized by *Bacillus* species. Boxed structural groups are those that were shown to be particularly involved in interaction with membranes and are supposed to be important for biological activity in addition to the cyclic nature of the molecule. To the best of our knowledge, no clear data are available to date for fengycins in this context. Boxed blue type of branching (linear, iso, anteiso); boxed orange, acyl chain length; boxed red, ionisable or polar groups; boxed green, hydrophobicity of residue in position 4; boxed yellow, L-Asx(1)- D-Tyr(2)- D-Asn(3) sequence (Ongena and Jacques, 2008).

II. 2.1.4. New lipopeptides discovered in *Bacillus*

Since the discovery of iturin, more than 30 different lipopeptides produced by different strains of *Bacillus* have been characterized revealing the high potential of lipopeptide biosynthesis of this genus and the exponentially growing number of genome sequences should probably allow

discovering new families or new variants in the near future For example, kurstakin should be added as a new family of lipopeptides (Jacques, 2011).

The synthesis of kurstakin found in *B. thuringiensis* was described by Hathout *et al*., (2000) and Price *et al*. (2007). Authors show that the kurstakin family is composed of four partially cyclic heptalipopeptides with the same amino acid sequence: Thr-Gly-Ala-Ser-His-Gln-Gln. Lipopeptides differ only in their fatty acid chains and have pronounced antifungal activity against *Stachybotrys charatum*.

The L and D forms or the amino acid residues are not yet characterised. However, the recent identification of the genes involved in the biosynthesis of such or similar compounds indicates that amino acids in 1 and 6 positions could be in the D-form (Bumpus *et al*., 2009).

The kurstakin can locally modify the surface tension of the culture medium and facilitate by this way the invasive growth of the strain and Kurstakin could thus help the strain in colonizing different environment (Abderrahmani *et al*., 2011).

Fusaricidin (A, B, C and D) are new antibiotic lipodepsipeptides isolated from *Bacillus polymyxa* KT-8. The general structure of fusaricidin was determined to be L-Thr-X_1-X_2-D-*allo*-Thr-X_3-D-Ala. The fusaricidins are a family of antibiotics consisting of a β-hydroxy fatty acid linked to a cyclic hexapeptide. They contain 15-guanidino-3-hydroxypentadecanoic acid as a side chain attached to the N-terminal L-Thr via an amide linkage. The peptide is cyclised by an ester bond between the C-terminal D-Ala and the β-OH group of the N-terminal L-Thr (Kajimura *et al*., 1996).

The antimicrobial activity of the fusaricidins has been shown to vary depending on the amino acids present at three variable positions in the peptide moiety, and previous studies revealed that the antifungal activity against *L. maculans* was mainly attributable to production of a mixture of fusaricidins A and B; while, fusaricidins B, C and D are active against fungi and Gram-positive bacteria such as *Staphylococcus aureus* (Kajimura *et al*., 1997). The genes involved in the biosynthesis of fusaricidins have been identified by Li and Jensen (2007).

II. 2.2. Lipopeptide biosynthesis and non-ribosomal peptide synthesis system (NRPSs)

Historically, in 1968, Gevers *et al.* demonstrated for the first time using cell extracts of the producer strains that biosynthesis of gramicidin (a peptidic antibiotic) was possible in the

presence of RNases or inhibitors of the ribosomal machinery. It was an experimental key to show that another biosynthetic pathway exists for the biosynthesis of the peptides. Since this discovery many works were carried out to describe in details the non-ribosomal peptide biosynthesis (Finking and Marahiel, 2004).

The NRPS mechanism is responsible for the synthesis of more than 1.000 active bio-molecules that can be gathered in about 200 families. A recent database (NORINE) compiles most of the NRPS molecules (Caboche *et al*., 2008).

The peptide synthesis involves large multi-enzymatic proteins - called non-ribosomal peptide synthetases - which are organized in modules (Sieber and Marahiel, 2005).

Each module is responsible for the incorporation of one monomer into the growing peptidic chain. Each module can be subdivided in sections called domains and involved in a specific enzyme activity. These domains catalyze at least the steps of substrate activation, covalent binding and peptide bond formation of nonribosomal peptide synthesis (Stachelhaus and Marahiel, 1995).

The domains of equal function share a number of highly conserved sequence motifs. These "core-motifs" allow the identification of individual domains on the protein level which showed in figure (II-4) by Schwarzer *et al*. (2003).

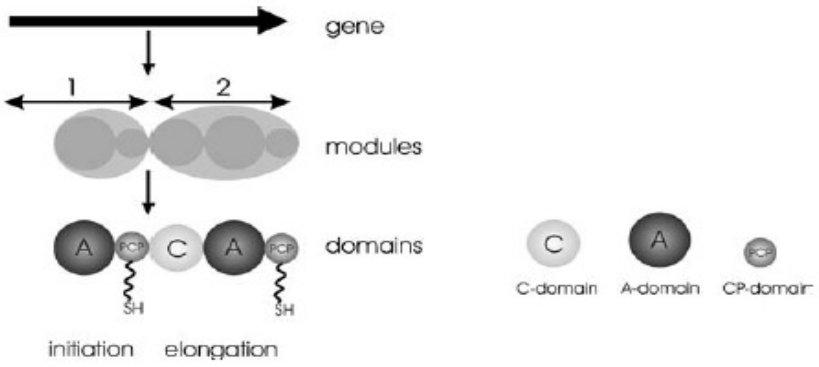

Fig (II-4): NRPS modules and domains coming from the gene (Schwarzer *et al*., 2003).

Four main domains are present in most of the NRPS: Adenylation (A) for substrate recognition (May *et al*., 2002). Peptidyl Carrier Protein (PCP) that holds the activated substrate (Ehmann et *al.,* 2000). Condensation (C) for peptide bond formation (Bergendahl *et al*., 2002) and Thioesterase (Te) domains (Sieber and Marahiel, 2003). These domains are summarized in figure (II-5) by Sieber and Marahiel (2005).

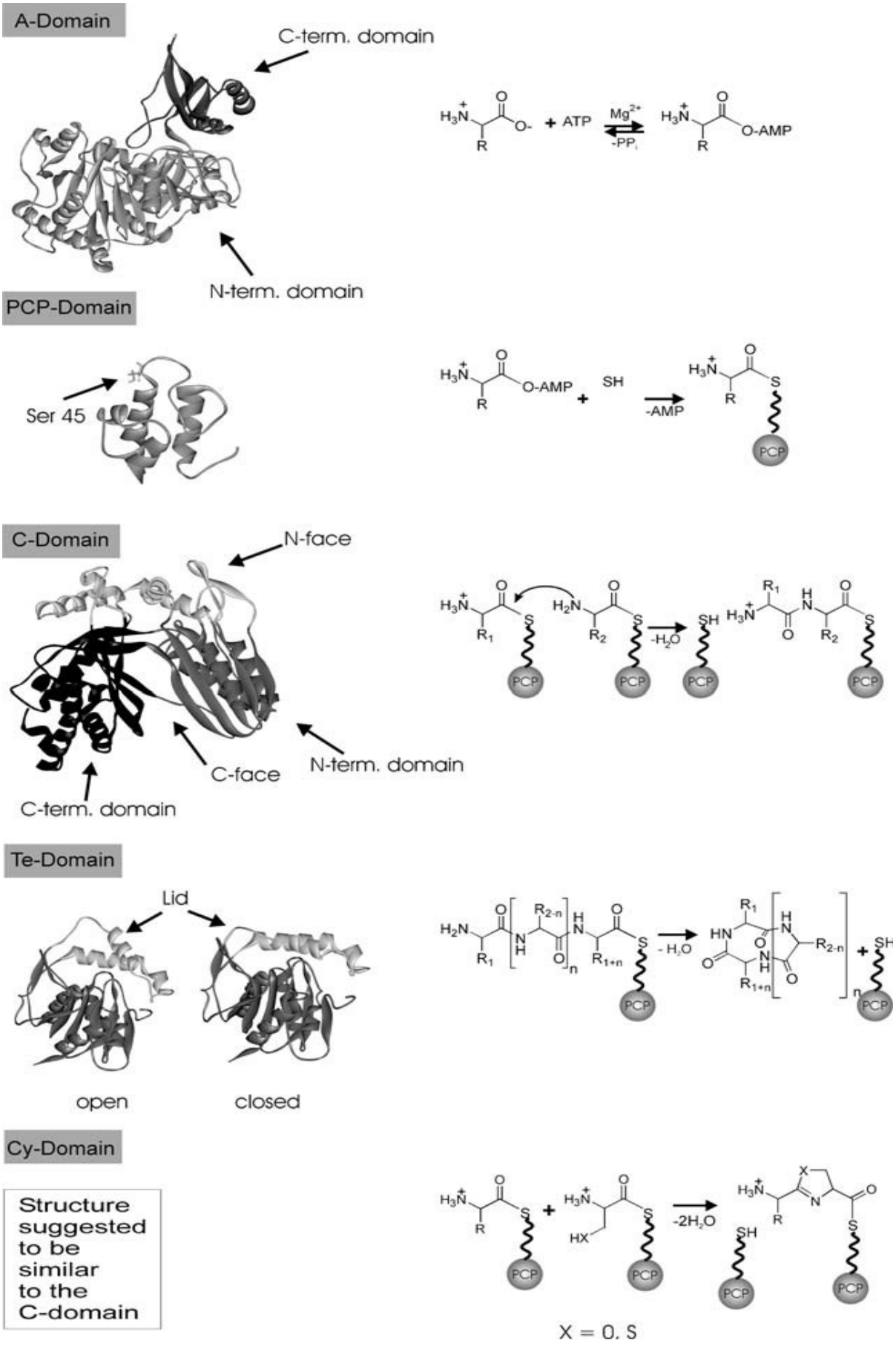

Fig (II-5): Non-ribosomal peptide synthesis system domains and modules (Sieber and Marahiel, 2005).

The (A) domain consists in 550 amino acids. It is responsible for the selection of the amino acids that make up the product and thus controls its primary sequence. The (A) domain activates amino acid substrate as amino acyl adenylate, while ATP is consumed (Mootz and Marahiel., 1997 and May *et al*., 2002).

The residues lie in 100 amino acid stretch between cores A4 and A5 of the A-domain analysis led to the introduction of the so-called nonribosomal code, which allows the prediction of A-domain selectivity on the basis of its primary sequence (Du *et al*., 2000).

The prediction of A-domain selectivity and the nonribosomal code was also recently exploited for the creation of two A-domains with altered selectivity. The site-directed mutagenesis within the core A4-A5 region can change the selectivity of surfactin from A to A1 type by changing the glutamic acid to glutamine and also from A to B2 type by changing the aspartic acid to aspargine (Eppelmann *et al*., 2002).

The (PCP) domain is a small domain containing between 80 to 100 amino acids; It represents the transport unit that accepts the activated amino acid that is covalently tethered to 4'-phosphopantetheinyl cofactor as thioester, this cofactor is post-translationally transferred to conserved serine residue of the carrier protein and acts as a flexible arm to allow the amino acyl bound and peptidyl substrate to travel between catalytic centers (Ehmann *et al*., 2000).

The (C) Domain. The 450 central entity of nonribosomal peptide synthetase is responsible for peptide bond formation between amino acyl substrates bound to (PCP) domain of adjacent modules (Bergendahl *et al*., 2002). The enzyme catalyzes the nucleophilic attack of the amino group of the activated amino acid bound to the downstream with respect to this domain onto the acyl group of the amino acid tethered to the upstream module according to the multiple-carrier thiotemplate model (Stein *et al*., 1996).

The (Te) Domain. In most cases the synthetase contains a (Te) domain which is important for the liberation of the product (Sieber and Marahiel, 2003).

The release of the product is achieved by a two steps process that involves an acyl-*O*-Te-enzyme intermediate that is subsequently attacked by either a peptide internal nucleophile (Kohli *et al*., 2001 and Trauger *et al*., 2001) or attacked by water (Miller *et al*., 2002).

While, the final peptide could be a macrocyclic product, as observed in the case of surfactin (Tseng *et al*., 2002)

Secondary catalytic domains are additional enzymes which can be involved in the biosynthesis of the peptide to modify the structure of monomers involved or to add some external compounds to the peptide. Some of these domains are integral parts of the NRPS and act in Cis-form whereas others are distinct enzymes acting in Trans-form before full maturation of the (NRPs) product. These tailoring domains include Cyclisation (Cy), Methylation (Me), Oxydation (Ox), Glycosylation (Gl) and Epimerisation (E) domains.

Addition of fatty acid chains are involved in lipopeptide biosynthesis in *Bacillus* spp. (Jacques, 2011). The addition of the fatty acid chain to the first amino acid of the peptide moiety is catalysed by a first specific condensation domain, also called starter condensation domain. The fatty acid chain can be partially synthesized by another main group of modular enzymes named the polyketide synthases (PKS). In this last case, a hybrid PKS/NRPs is required for the synthesis of the bio-molecules (Du *et al*., 2001).

Protein-protein interactions: In most multi-enzyme complexes, several proteins are involved in the complete assembly line. The biosynthesis of the right NRPs requires the proper protein-protein interactions between partner enzymes and concomitantly prevents undesired interactions between non-partner enzymes. The short terminal structures, referred to as NRPs communication-mediating (Com) domains are responsible, at least for the most part, for the correct channeling of reaction intermediates along the assembly line (Hahn and Stachelhaus, 2004).

A donor Com domain COMD X situated at the C terminus of an aminoacyl or peptidyl donating NRPS. “X”, an acceptor COM domain COMA Y located at the N terminus of the accepting partner enzyme “Y” form a compatible (cognate) pair that is crucial for establishing the productive interaction between both enzymes (Jacques, 2011).

II. 2.3. Biological activities and multifunctional applications of lipopeptides

Surfactins are powerful biosurfactants with exceptional emulsifying and foaming properties (Razafindralambo *et al*., 1996). They are able to reduce surface tension of water to 27 mN m^{-1} and show a low critical micellar concentration (CMC) of about 10 mg L^{-1}. They also exhibit

antifungal properties and moderate antibacterial properties (Bernheimer *et al.,* 1970 and Tsukagoshi *et al*., 1970).

Surfactins show hemolytic activity and inhibit the fibrin clot formation (Sheppard *et al*., 1991).

Surfactins also inhibit enzymes such as cyclic adenosine monophosphate phosphodiesterase (Hosono *et al*., 1983), exhibit antiviral and antitumor activities (Kameda *et al*., 1974) and inhibit starfish oocyte maturation (Toraya *et al*., 1995).

Surfactin can be also readily associated and tightly anchor into lipid layers; it can thus interfere with biological membrane integrity in a dose dependent manner.

Further addition of surfactin to reach the CMC leads to complete disruption and solubilisation of the lipid bilayer with formation of mixed micelles (Heerklotz and Seelig 2007 and Carrillo *et al*. 2003). The lipopeptide was found to be a selective inhibitor of lipopolysaccharide (LPS) signal transduction. The minimal cytotoxicity makes that surfactin may have a therapeutic potential for the LPS treatment of triggered syndromes such as Gram-negative bacterial sepsis as presented in figure (II-6) (Ohno *et al*., 2004).

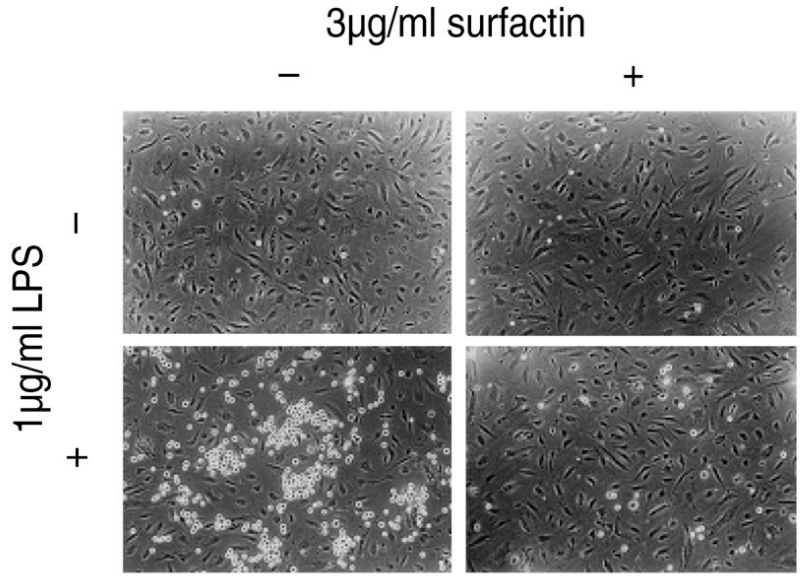

Fig (II-6): Inhibition of HL-60 cell adhesion to LPS-stimulated HUVEC by surfactin.
HUVEC were preincubated or not with 3 mg/ml surfactin for 2 hours, treated or not with LPS (1 mg/ml) for 4 hours, and after having been washed, incubated with HL-60 cells for 1 hour (Ohno *et al*., 2004).

Interestingly, the presence of cholesterol in the phospholipids layer attenuates the destabilising effect of surfactins, which suggests that the susceptibility of biological membranes may vary in a specific manner, depending on the sterol content of the target organisms. This could explain why surfactins display hemolytic, antiviral and antibacterial activities (Kracht *et al*. 1999). Antimycoplasma activity is shown in figure (II-7).

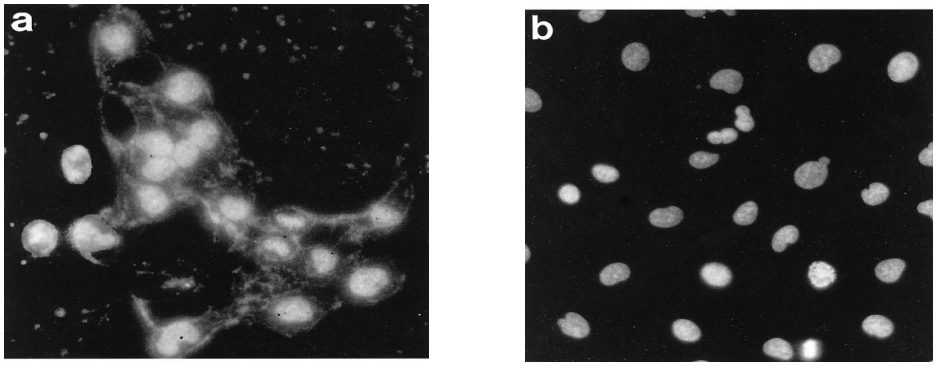

Fig (II-7): DNA fluorescence staining of surfactin-treated cells with DAPI: ML cells heavily contaminated with *Mycoplasma hyorhinis* (a) and *Mycoplasma*-free cultures (b) after treatment with surfactin (Vollenbroich *et al*., 1997).

Vollenbroich *et al*. (1997) and recent studies have shown that surfactin could induce plant systemic resistance (Ongena *et al*., 2007 and Jourdan *et al*., 2009).

Furthermore, surfactins also show anti-inflammatory (Kim *et al*., 1998) and anticancer activity (Kameda *et al*., 1974) and immunomodulatory effects (Park and Kim, 2009).

The linear surfactin is less hemolytic than nonlinear (Dufour *et al*., 2005). The emulsification properties of surfactin were also used to solubilise xenobiotic compounds (Lai *et al*., 2009) and enhance their biological degradation (Whang *et al*., 2009). This property also facilitates oil recovery from carbonate reservoirs (Zhang *et al*., 2000).

When linear surfactin was prepared by saponification of the lactone ring, its oil displacement activities decreased (Morikawa *et al*., 2000).

Several studies have also shown the role played by surfactin in pellicle formation of the producing *Bacillus* strains at the air-water interface (Hofemeister *et al*., 2004 and Chollet-Imbert *et al*., 2009); in swarming (Julkowska *et al*., 2005 and Debois *et al*., 2008) or in biofilm formed on roots (Bais *et al*., 2004). At concentrations between 5 and 50 $mg.L^{-1}$, it inhibits *Salmonella* biofilm formation in microtiter plates (Mireles *et al*., 2001) and

Pseudomonas syringae growth on roots (Bais *et al*., 2004). The adsorption of surfactin on stainless steel or Teflon substrata deeply modified the hydrophobicity of the surface and the *Bacillus cereus* spore attachment (Shakerifard *et al*., 2009), also surfactin modify the surface hydrophobicity of the producing strain (Ahimou *et al*., 2000).

Iturins are less potent surfactant than surfactin (Deleu *et al*., 1999). Iturin reduces the surface tension of water to 43 $mN.m^{-1}$ and form at CMC (about 20 mg L^{-1}) micelles with a Stokes radius of 1.3 nm and an aggregational number of 7. At concentrations slightly higher than CMC, iturin probably forms a fully interdigitated bilayer where each hydrocarbon tail spans the entire hydrocarbon width of the bilayer, resulting in multi lamellar vesicles with an average size of 150 nm (Grau *et al*., 2001).

Though, they are also strongly hemolytic, the biological activity of iturins differs from surfactin activity as they display a strong *in vitro* and *in vivo* antifungal action against a large variety of yeasts and fungi but have only limited antibacterial and no antiviral activities (Leclère *et al*., 2005; Mizumoto *et al*., 2007; Romero *et al*., 2007 and Fickers *et al*., 2009). The length of the fatty acid chain, the presence of an Asp in position 1 and tyrosine residue in position 2 are important structural traits for the antifungal activity of the lipopeptide (Bonmatin *et al*., 2003 and Fickers *et al*., 2009).

The fungitoxicity of iturins almost certainly relies on their membrane permeabilisation properties. The underlying mechanism is based on osmotic perturbation due to the formation of ion-conducting pores and not due to membrane disruption or solubilisation as caused by surfactins (Aranda *et al*., 2005).

In addition to the direct activity against fungi, iturin derivatives enhance the invasive growth of the producing strain and thus by these two mechanisms participate in plant protection against phytopathogens (Leclère *et al*., 2006).

Fengycins are less hemolytic than iturins and surfactins but retain a strong fungitoxic activity more specifically against filamentous fungi. Mechanistically, the action of fengycins is less known compared to other lipopeptides but they also readily interact with lipid layers and somewhat retain the potential to alter cell membrane structure (packing) and permeability in a dose-dependent way. Immunosuppressive activity of plipastatin was also described and patented (Umezawa *et al*., 1988).

These families of lipopeptides are known to act in a synergistic manner as suggested by several studies, on surfactin with iturin (Maget-Dana *et al*., 1992 and Razafindralambo *et al*.,

1997), surfactin with fengycin (Ongena *et al*., 2007) and iturin with fengycin (Romero *et al*., 2007). Also, Huang *et al*. (2008) using a response surface methodology observed the sensitivity of *Escherichia coli* to surfactin and fengycin.

In the context of biocontrol of plant diseases, the three families of *B. subtilis* lipopeptides (surfactins, iturins and fengycins) were mostly studied for their antagonistic activity against a wide range of potential phytopathogens including bacteria, fungi and oomycetes as presented in figure (II-8) by Ongena and Jacques (2008).

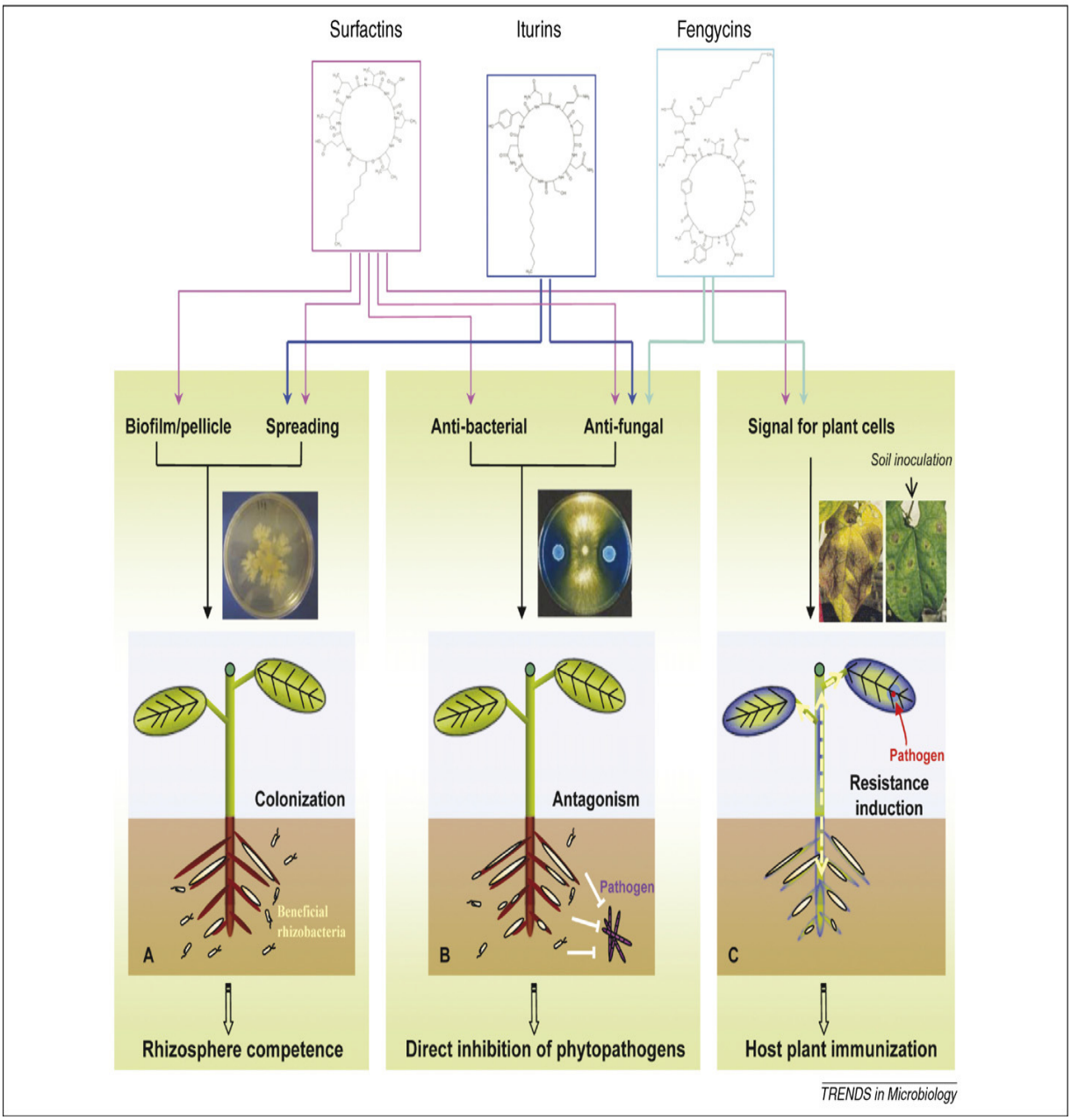

Fig (II-8): Overview of *Bacillus* lipopeptide interactions in the context of biological control of plant diseases.

From left to right, the three photographs show bacterial spreading, fungal growth inhibition through the production of fungitoxic compounds by blue bacterial cells and leaf disease reduction following inoculation of the beneficial bacterium on roots. They illustrate how to get experimental indications about the potential involvement of one particular strain in the three phenomena schematically represented in (A), (B) and (C). Establishment in biofilm and/or microcolonies of the rhizobacterium is represented in (A), (B) represents direct antibiosis that can be exerted by the established biocontrol strain toward pathogens sharing the same microenvironment. In (C), the arrows illustrate the emission of a signal following perception of the rhizobacterium at the root level. This signal moves over the entire plant leading to some systemic reinforcement allowing pathogen restriction at distal sites of infections (Ongena and Jacques, 2008).

II. 3. Parameters affecting lipopeptides production processes

In literature, numerous studies concerning free cells production of biosurfactants have been reported. Several parameters such as carbon sources, nitrogen sources, temperature, inorganic ions amount and addition of solid carriers may trigger both strain growth and lipopeptide synthesis (Gancel *et al*., 2009). Many of the physico-chemical parameters influence the biosynthesis of lipopeptides from *Bacillus* spp. due to the complex regulation of lipopeptide operon expression involving several pleiotropic regulators (Jacques, 2011).

II. 3.1. Cultivation and fermentation parameters

II. 3.1.1 Microbial strains

Biosynthesis of the lipopeptides is a property of most of the members of the genus *Bacillus*. From private or public collections, about 20 strains of *B. subtilis* have been listed as producers (Peypoux *et al.,* 1999). Interest in some of these strains derives from their origins e.g. injection brine (Jenneman *et al*., 1983) or deep-sea sediment (Trischman *et al*., 1994). Historically, *B. subtilis* IAM 1213 (Institute of Applied Microbiology) was the first strain discovered by Arima *et al*. (1968) which produces lipopeptide surfactant. This strain is now usually called *B. subtilis* ATCC 21332 and was known to produce a single surfactin lipopeptide (Davies *et al*., 1999 and Leclère *et al*., 2006). However, *B. subtilis* ATCC 21332 was recently proved in our laboratory to be a co-producer of surfactin and fengycin (Gancel *et al*., 2009; Tapi *et al*., 2010 and Coutte *et al*., 2010). Also, it has been communicated that the aerobic immobilized cultures of *B. subtilis* ATCC 21332 produce 2.0 to 4.3 times more lipopeptides than planktonic cells (Gancel *et al*., 2009 and Chtioui *et al*., 2010). By using UV mutagenesis, it was possible to isolate an over producing strain of *B. subtilis* ATCC 21332 (Roubin *et al*., 1989). This mutant lowers the isocitrate dehydrogenase activity to 30 times less than that of the parent and produces 4 times more surfactin. In our work, *B. subtilis* BBG21 is a spontaneous modified strain of *B. subtilis* ATCC 21332 which can overproduce lipopeptide biosurfactants.

II. 3.1.2. Culture media and its composition

A lot of studies have pointed out different environmental factors for their effect on surfactin production. For example, Duitman *et al*. (1999) reported that *B. subtilis* ATCC 6633 produced only surfactin when cultivated on Landy medium while mycosubtilin was detected with DSM medium. Mycosubtilin and surfactin were detected with ACS medium. Several experiments performed in our laboratory have shown that this effect can be strain dependent.

Regarding the carbon source, glucose was the most used substrate. In *Bacillus subtilis* C9, addition of glucose resulted in a higher specific production rate (biosurfactant/cell) than did the use of sucrose. The concentration of the biosurfactant increased almost linearly with increasing the initial concentration of the sugar (Hee-Sik *et al*., 1997).

The two main culture media mentioned in literature for surfactin production are Cooper's medium and Landy's medium (Cooper *et al*., 1981 and Landy *et al*., 1948).

Also, Jacques *et al*. (1999) showed that, fengycin was synthetised by *B. subtilis* S499 when grown in the Optimized medium (containing saccharose as carbon source).

For this synthesis, fructose has also been mentioned as efficient carbon source contrary to glycerol and hexadecane.

Studies of nitrogen requirement. Either mineral or organic nitrogen sources can promote lipopeptide synthesis in *B. subtilis*. Surfactin can be produced in Cooper's medium with mineral nitrogen (NH_4NO_3) or in Landy's medium which contains glutamic acid. In this medium, part replacement of glutamic acid by leucine or isoleucine promotes the synthesis of new isoforms of mycosubtiline (Guez *et al*., 2005).

Studies of the mineral requirement clearly established the need and the stimulatory effect of iron and manganese (Wei *et al*., 2004). $MnSO_4$, $FeSO_4$ and $Fe_2(SO_4)_3$ caused significant enhancement of surfactin (Yeh *et al*., 2005 and Davies *et al*., 1999). Small amount of $MnSO_4$ was necessary for the maximum growth and surfactin production (Wei *et al*., 2007). The salts such as $MgSO_4$, $CaCl_2$, Na_2HPO_4, KH_2PO4, $NaNO_3$, $ZrOCl_2$, $UO_2(C_2H_3O_2)_2$ or $VOSO_4$ have virtually no effect on either biomass or surfactin yield (Cooper *et al*., 1981). The presence of salt (NaCl 5%) was required for production of lichenysin by *Bacillus licheniformis* (Yakimov *et al*., 1995)

II. 3.1.3. Fermentation supply and environmental factors

Other environmental factors have been pointed out for their effect on lipopeptide production (Hbid *et al*., 1996 and Peypoux *et al*., 2000). The factors like oxygenation rate (Jenny *et al*., 1993; Hbid *et al*., 1996 ; Bandow *et al*., 2002 and Guez *et al*., 2008); pH (Volpon *et al*., 2000 and Andrew *et al*., 2000), temperature (Ohno *et al*., 1995a; Hbid *et al*., 1996 ; Kim *et al*., 1997; Jacques *et al*., 1999; Akpa *et al*., 2001 and Fickers *et al*., 2008) or immobilization of cells (Nikov *et al*., 1999 ; Donghee *et al*., 2005; Dimitrov *et al*., 2007b; Liu *et al*., 2007; Gancel *et al*., 2009; Coutte *et al*., 2010 and Chtioui *et al*., 2010) have been studied.

For continuous operation, a critical nitrogen/iron/manganese molar ratio of 920:7.7:1.0 was determined and was found to sustain surfactin production for at least 36 generations (Sheppard and Cooper, 1991).

Oxygen is considered as an important parameter. The replacement of Cooper's nitrogen source by NH_4HCO_3, and the introduction of oxygen limitation which redirects the energy flux into product synthesis have led to the highest productivity (7 $g.L^{-1}$) mentioned for surfactin production by *Bacillus subtilis* C9 (Kim *et al*., 1997).

Such a process appears not to be adapted to *Bacillus subtilis* S499 which produced more surfactin in better aeration conditions (Hbid *et al*., 1996 and Jacques *et al*., 1999).

In *Bacillus subtilis* NB22, a limited aeration reduced synthesis of iturin (Ohno *et al.,* 1993). On the contrary, oxygen limitation enhanced the mycosubtilin production in *B. subtilis* ATCC 6633 while no effect of oxygen was reported for *B. subtilis* BBG 100 (Guez *et al*., 2005) and Chollet *et al*. (2009) showed that the strain ATCC 9943 produced more fengycin when oxygen was limited.

II. 3.1.3.1. Effect of temperature and pH

Effect of temperature depends on the lipopeptides. Higher temperatures (37°C) favoured surfactin synthesis of *Bacillus subtilis* RB14 isolated from compost (Ohno *et al*., 1995a) but not this of *Bacillus subtilis* S499 (Jacques *et al*., 1999).

A 30-fold increase in mycosubtilin production was observed when the temperature was decreased from 37°C to 25°C. This phenomenon was observed for both strains ATCC6633 and its derivative BBG100. However, no significant difference in either the expression of the mycosubtilin synthetase encoding genes or in the intracellular synthetase concentration could

be found, suggesting that the observed phenotype originated from a higher mycosubtilin synthetase turnover at lower temperature (Fickers *et al*., 2008).

Production of lichenysin by *Bacillus licheniformis* was conducted in anaerobic conditions and mainly at higher temperatures (up to 45°C) than for surfactin (Yakimov *et al*., 1995).

pH of medium has been shown to affect surfactin synthesis in various strains of *Bacillus subtilis* (Cosby *et al*., 1998). According to these authors, when cells were grown in a rich DSM medium containing glucose and glutamine, pH was 5.0 or lower at the end of the stationary phase.

At these low pH the expression of the *srf* operon was reduced and the production of the surfactin synthetase subunits and of surfactin itself was also reduced. When pH rose to pH 7, surfactin production increases, this pH induction of *srf* expression required *spo0K* (encoding a permease which controls sporulation and competence), but not CSF, the competence-inducing pheromone that regulates *srf* expression.

The two-component regulatory pair ComP and ComA that stimulates cell-density-dependent *srf* transcription was required for optimal expression of surfactin operon at various pH. Synthesis of mycosubtilin in *B. subtilis* ATCC 6633 is also regulated by the pH of the medium and by kind of pH stabilizer used to buffer the culture medium. When grown in Landy medium buffered to pH 6.0 and 6.5 by MES, the strain produced only a very low quantity of lipopeptides and when grown in Landy MOPS (buffer solution) at pH 6.5 during 72h, the strain produced three times more mycosubtilin than in non-buffered Landy medium (Guez *et al*., 2005).

II. 3.1.3.2. Effect of biofilm

The attachment of bacteria to solid surfaces in aquatic environments in a complex community of microorganisms is referred as biofilm. The accumulation of microorganisms on the solid surface can be divided into three stages: immobilization, consolidation and colonization stages (Fletcher, 1996).

Biofilm reactors are used in the cases wherein the capacity obtained by freely suspended microorganisms is limited by the biomass concentration and hydraulic residence time. Another reason can be the use of slow growing organisms (whose growth in suspension requires long residence time or diluting feed streams) Dimitrov *et al*. (2007).

In addition, the use of immobilized bacteria leading to high cell concentrations within the reactor seemed a promising method to improve reactor performance and selectivity (Nikov *et al*., 1999; Donghee *et al*., 2005 and Liu *et al*., 2007).

However, the static biofilm reactors can be useful if the biomass retention is the main requirement and the enlargement of the biofilm specific area can lead to an essential reduction in the reactor volume and the area requirement of the process (Dimitrov *et al*., 2007a). The factors which come into play and the affect forces on microbial adhesion are summarized in figure (II-9) by Wiencek *et al*. (1995).

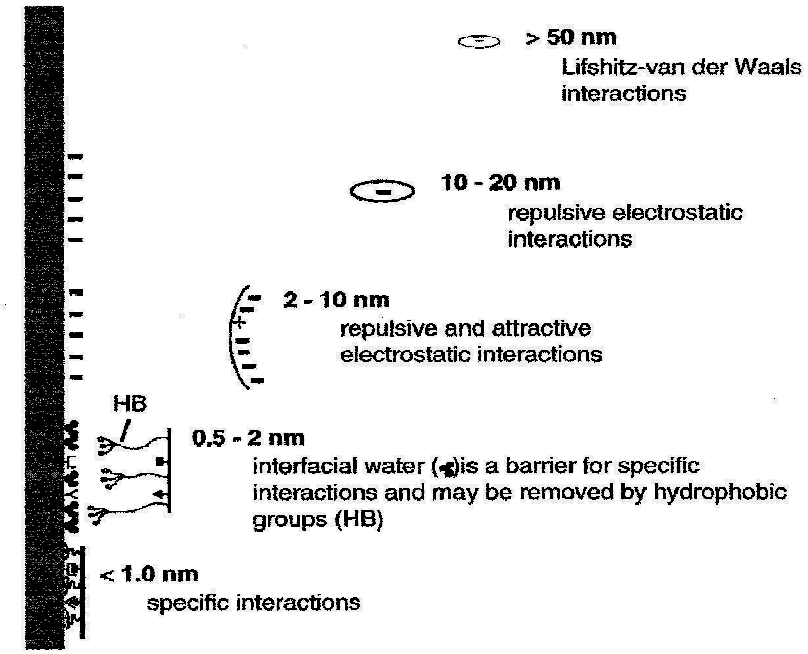

Fig (II-9): Forces involved in attractive and repulsive interactions between a bacterium and a substratum surface and approximate distances at which the forces are significant (Wiencek *et al*., 1995).

The transport pathway of the oxygen from the gas bubble to the cell - freely suspended or on the surface of the biofilm- is presented in figure (II-10) by Bailey and Ollis (1986).

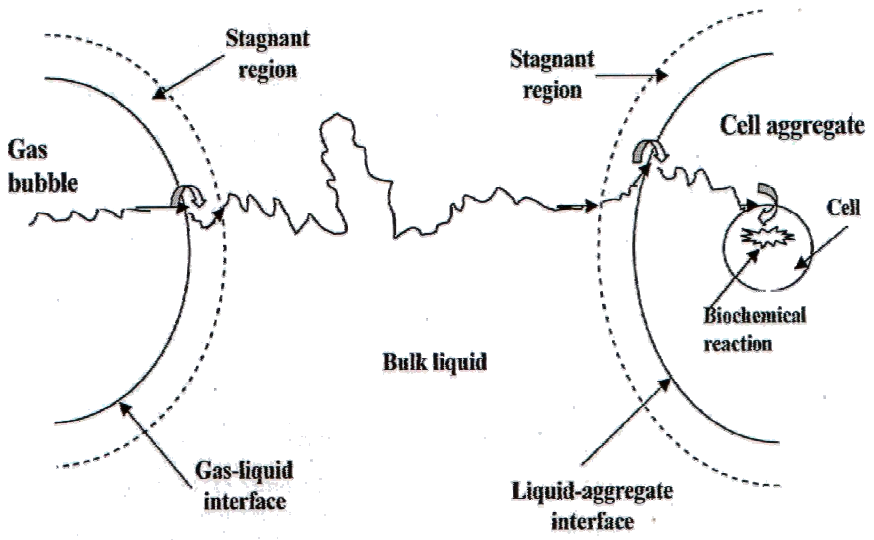

Fig (II-10): Schematic diagram of oxygen transport path from a gas bubble to inside a cell (Bailey and Ollis, 1986).

Recently, our laboratory demonstrated that, the environmental conditions can drastically modify the ratio between surfactin and fengycin produced by a derivative strain of *B. subtilis* ATCC 21332. Presence of coated polypropylene or polystyrene carriers increases the biosynthesis of fengycin (Gancel *et al*., 2009 and Chtioui *et al*., 2010).

II. 3.1.3.3. Culture supply and power dissipation effect

Many parameters which can directly influence biological cultures such as oxygen supply; carbon dioxide removal, degree of mixing and homogeneity and hydrodynamic stress are related to mechanical power dissipation (Shioya *et al*., 1999).

Up to now, a relationship between the volumetric power dissipation and the bio-molecules production like antibiotics was never demonstrated. The complex heterogeneity of the flow in gas-liquid stirred tank reactor is a key problem to precisely link power dissipation to cell response. However, typical engineering parameters have been described in shake flasks to ensure defined culture conditions (Belmar-Beiny *et al*., 1991). At last, nearly 90% of all laboratory microbial cultures and fermentation experiments are performed in such devices; as a consequence, the influence of the power dissipation on cell response can be advantageously studied in shake flasks (Buchs, 2001). For surface (film) aerated culture systems, the obtained volumetric gas-liquid mass transfer coefficient (k_La) values and production yields changed drastically with power dissipation increasing, especially in the case of microbial antibiotic

production (Seletzky *et al*., 2007). While, the increase in the power dissipation was not the sole parameter influencing the onset of the production, it had to be coupled with a transfer of oxygen sufficient to yield concentrations of biomass (Mehmood *et al*., 2010).

Oxygen transfer limitations occur commonly in bioreactors, leading to decreased performance; when oxygen is limited, the metabolic rate of the microorganisms decreases significantly and the culture may respond adversely to the resulting stress. An insufficient oxygen supply can lead to sub-optimal productivity rates, as well as products of low quality (Shu and Yang, 1990). Several methods have been used to try to enhance the gas-liquid oxygen transport such as modifying reactor designs, using oxygen vectors (Roels, 1990), increasing the oxygen composition or using pure oxygen in the inlet gas, increasing the agitation or aeration, and so on (Panda *et al*., 1989; Kawase and Tsujimura, 1994).

Numerous investigations have been performed to identify the effect of agitation and aeration on the rate of oxygen transfer, as evidenced by the large number of empirical formulations available in the literature (Lee, 1992).

II. 3.2. Fermentation tools and bioreactor type

The actual level of knowledge of the biosynthesis of lipopeptide from *B. subtilis* and their regulation mechanisms leads to the development of several strategies to overproduce the main active compounds and to reach yields that are compatible with industrial development of such compounds. Indeed, the microbial biosurfactants from *B. subtilis*, due to their biological and physicochemical properties, combined with their biodegradability are subject to significant commercial interest (Jacques, 2011). Most nutritional and production studies were done with batch cultures, usually in shake flasks, but occasionally in small-scale or large-scale fermentors (Mulligan and Gibbs, 1990 and Lin *et al*., 1994).

Three main types of original bioprocesses were developed for lipopeptide production: solid-state fermentation, foam fractionation and membrane bioreactors.

Solid state fermentation has been suggested for a long time by Japanese researchers. *B. subtilis* strains can easily grow on different food processing wastes and showed a high level of production by using this process (Ohno *et al*., 1995b).

3.2.1. Shake flasks fermentation

The production takes place in large bioreactors; therefore a scale-up strategy is required to reproduce the culture characteristics and the kinetic parameters from shake flasks (Gupta and Rao, 2003). Until lately, shake flasks have not been used to perform scale-up of fermentation processes due to the lack of knowledge of the conditions under which shake flask fermentations are performed. Sumino *et al*. (1993) pointed out, that a better understanding and a thorough utilization of shake flasks should make a direct method for scaling-up a fermentation process possible. In recent years, the knowledge about the conditions in shake flasks has increased; Maier and Büchs (2001); Maier (2002) and Maier *et al*. (2004) characterized the mass-transfer; Büchs *et al*., (2000a; 2000b) and Peter *et al*. (2004) characterized the power input and the hydro-mechanical stress; the power dissipation and the fluid movement were studied by Peter *et al*. (2006) and Mehmood *et al*. (2010).

Whoever, Clark *et al*. (1995) stated that, oxygen limitation acts is an analogous manner to substrate limitation. Katzer *et al*. (2001) pointed out that, the production of secondary metabolites is influenced not only by factors like nitrogen or phosphate limitation but may also be dependent on oxygen limitation.

Also, if the gas-liquid mass transfer is the limiting step the efforts aiming at the improvement of. e.g., the enzymatic activity of the metabolic pathway leading to the desired product will not be of much benefit (Büchs *et al*., 2001).

II. 3.2.2. Bioreactor fermentation

The development of strategies for high throughput and low cost production of surfactin is an urgent demand. Two major factors spoil the surfactant producing process: (1) stable foaming evolution causes liquid outflow and changes substrate concentration and mixing intensity, (2) lipopeptides presence may decrease oxygen transfer rate, thus, lowering bioreactor yields. It has been suggested previously (Sheppard and Cooper, 1990) that lipopeptide concentration, while lower than critical micelle concentration (CMC) enhances the substance spontaneous migration towards the air-water interface and surfactin concentration at the interface increases.

Recent finding shows that addition of solid carriers (such as activated carbon) into fermentation culture could enhance surfactin production significantly (Yeh *et al*., 2005); Mao-

Sung *et al*. (2006) found that the supply of sufficient dissolved oxygen and mechanical agitation played an important role in the efficiency of surfactin production in a carrier-assisted surfactin fermentation system as shown in figure (II-11).

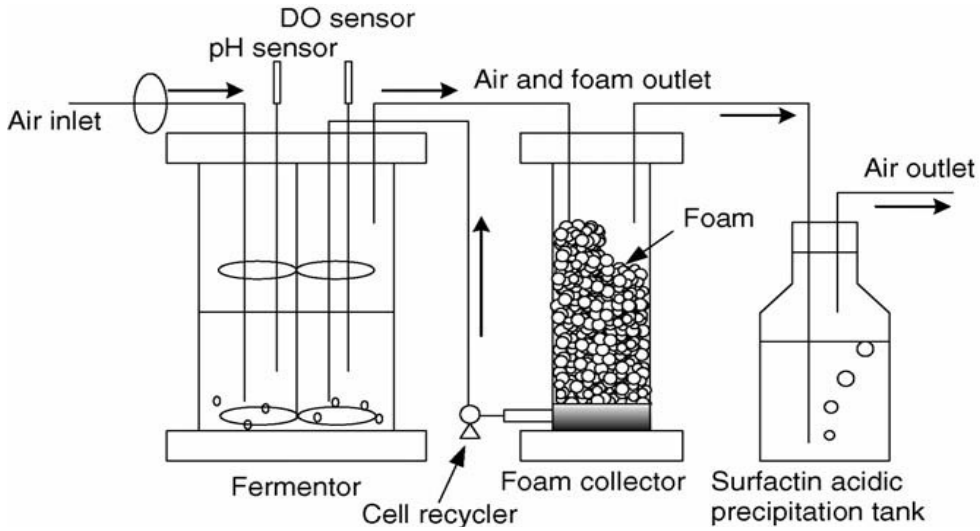

Fig (II-11): Carrier-assisted bioreactor design for enhanced surfactin production by *B. subtilis* (Mao-Sung *et al*., 2006).

Another strategy for the production of biosurfactants with aerobic immobilized cultures of *B. subtilis* in fed batch process (Melo *et al*., 2002; Chtioui *et al*., 2010) is shown in figure (II-12).

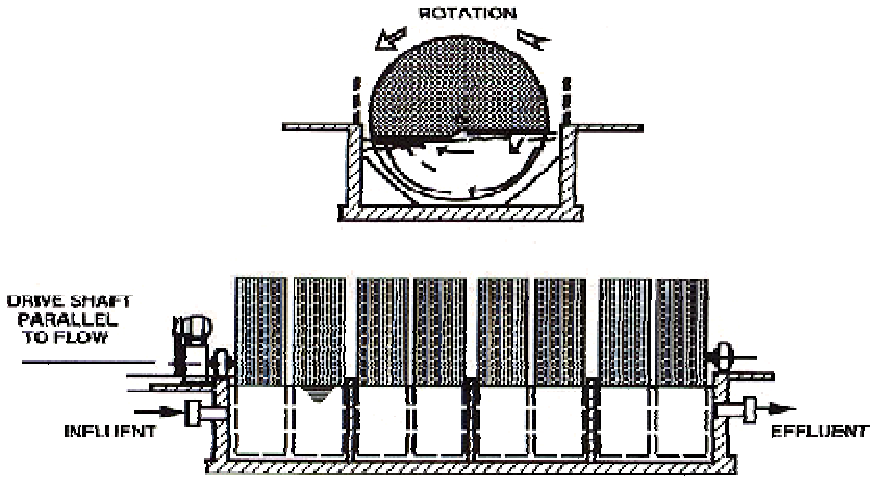

Fig (II-12): Rotating biological contactor (John and Cookson, 1995).

The technique of foam fractionation was first suggested by Cooper *et al*. (1981); it offers the double advantage of continuous in situ removal of produced surfactin from the fermentation broth and the prevention of any possible feedback inhibition (Davis *et al*., 2001).

The same strategy was also applied for the production of mycosubtilin with an overflowing exponentially fed batch process (OEFBC) which allowed a continuous extraction of the mycosubtilin with high efficiency (Guez *et al*., 2007).

In order to develop a continuous process which combines production and extraction of surfactin and fengycin, an integrated bioprocess was developed by Coutte *et al*. (2010). In this bioprocess, the use of a membrane ensure the oxygen transfer in bubble less conditions avoiding foam formation and leading to the development of a membrane bioreactor with cell recycling by microfiltration and lipopeptide concentration by ultra-filtration.

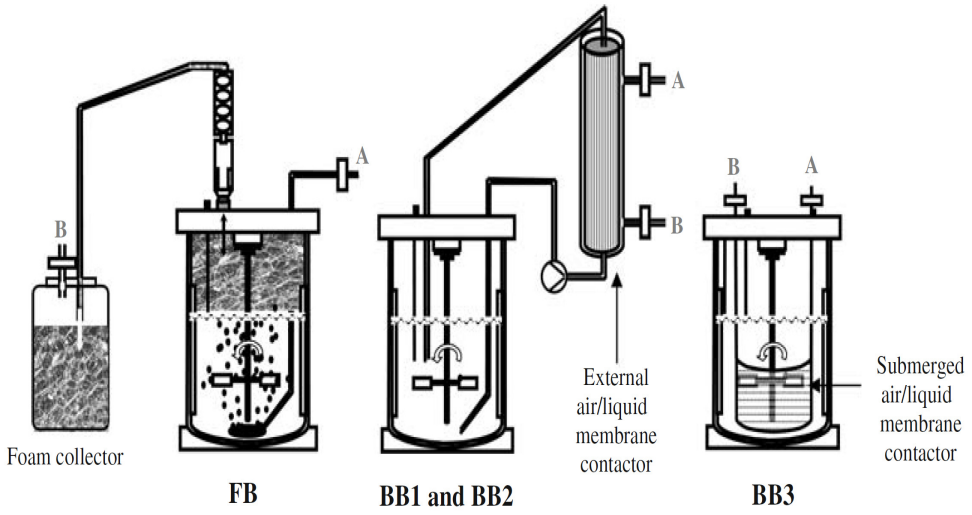

Fig (II-13): Schematic representation of bioreactors used for the production of lipopeptides by *B. subtilis*; (FB) foaming bioreactor. (BB1) and (BB2) bubble-less bioreactor 1 and 2 using an external hollow fiber membrane module; BB3 bubble-less bioreactor 3 using a submerged hollow fiber membrane: A air inlet, B air outlet (Coutte *et al*., 2010).

Three phases inverse fluidized bed reactors (TPIFB) have been studied in the past two decades mainly in relation to anaerobic or aerobic wastewater treatment (Nikov *et al*., 1999; Buffiere *et al*., 2000; Loch and Liu. 2001; Donghee *et al*., 2005; Sowmeyan and Swaminathan, 2008).

When micro-organisms with low growth rates and yields are present, the use of these reactors offers several advantages. Leading to their advantages, high cell concentrations per unit volume and low shear stress, the use of biofilm reactors is a promising technique for

productive systems (Nikov *et al*., 1999). One other advantage of inverse fluidized beds is the high solid-liquid mass transfer coefficient (Nikov *et al*., 2000)

The (TPIFB) reactor designed for lipopeptide producing process can be used in different configurations and in particular for liquid batch regime (complete recycling) and continuous gas flow or for continuous regime of gas and liquid with partial liquid recycling. However, the physical properties of such systems differ considerably from those found in most industrial units which usually contain multifarious liquid components subject to foaming. The hydrodynamic mass and energy transfer in the three-phase contactors is a complex function of physical properties of the phases operating conditions and reactor geometry (Nikov *et al*., 1999). There is a significant amount of research on the hydrodynamic, heat and mass transfer properties in three phase inverse fluidized beds under ambient operating conditions which devolved by Dimitrov *et al*. (2007) and the design is presented in figure (II-14).

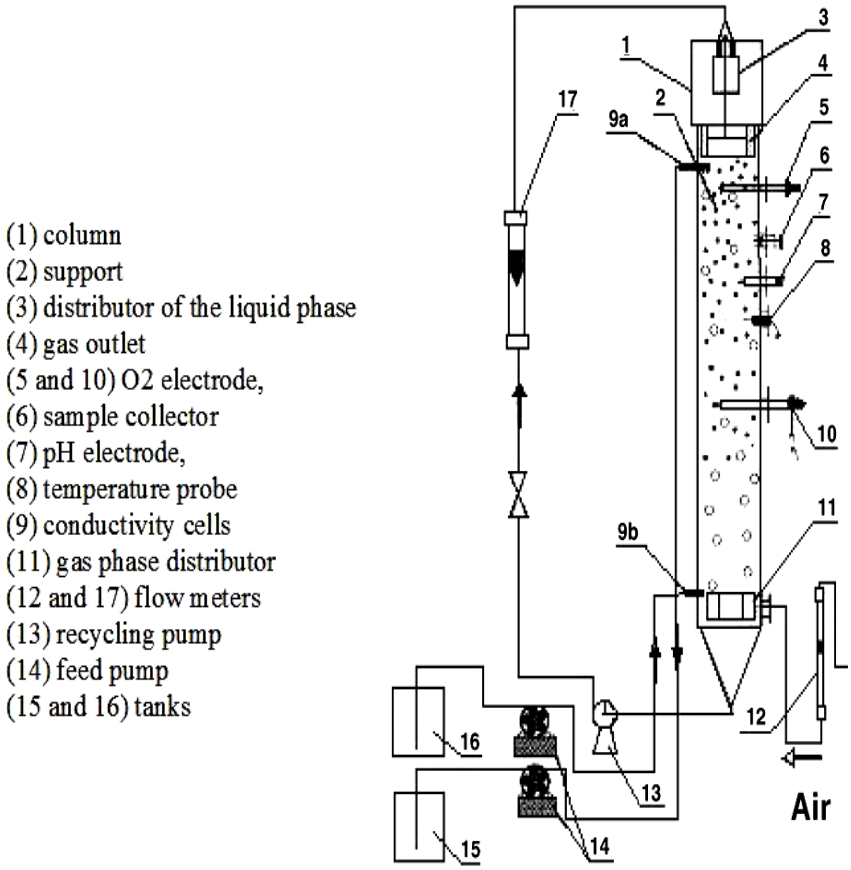

Fig (II-14): Three-phase inverse fluidized bed (TPIFB) reactor (Dimitrov *et al*., 2007).

The inverse fluidized bed biofilm reactors have attached great attention for potential applications in the bioprocesses because they provide better solid and liquid mixing, higher oxygen transfer, easier CO_2 removal, easier cell regeneration and more uniform cell population (Dimitrov *et al*., 2007).

The remaining challenges in the engineering of this aerobic reactor are related to oxygen mass transfer and control, biofilm characteristics and their optimisation, and on the other hand: the major problem concerning the foaming control.

II. 3.3. Fermentation supplying and problem appropriation

Providing sufficient oxygen to biological culture is vital for efficient bioprocess development (Hilton *et al*., 1999). However, in small liquid batch processes, that critical parameter is gas exchange or the balance between "respiration of the culture" oxygen uptake rate (OUR) and oxygen transfer rate (OTR). The OUR may be limited if it exceeds the maximum oxygen transfer capacity (OTR_{max}) in the gas-liquid interface (Maier *et al*., 2004).

II. 3.3.1. Hydrodynamic and mass transfer supplying

II. 3.3.1.1. Hydrodynamic and mass transfer in shake flasks

In recent years, many research studies have been published about traditionally shaken flasks sealed with different types of closures. The use of a sterile closure leads to a reduction of O_2 and an accumulation of CO_2 in the gas phase of the headspace as presented in Figure (II-15) by (Royce and Thornill; 1992 and Amoabediny and Büchs, 2010).

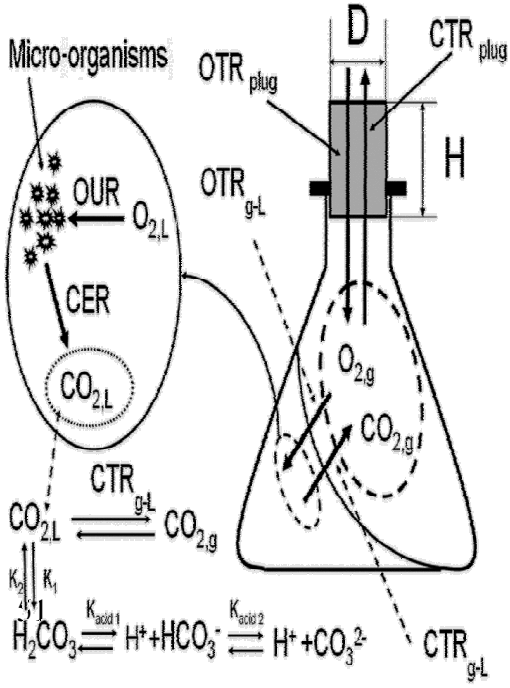

Fig (15): Oxygen and carbon dioxide transfer in ventilation flask (Royce and Thornhill, 1992).

To harmonies screening experiments, suitable operating conditions (flask size, shaking frequency, filling volume and shaking diameter) ensuring sufficient oxygen supply to cultures must be determined (Büchs, 2001).

The increase of the maximum oxygen transfer capacity of a given shake flask can be achieved by increasing the shaking frequency (N), reducing the filling volume (V_L), increasing the shaking diameter (d_0) and reducing the maximum flask diameter (d) which is summarized in figure (II-16) by Maier *et al.* (2004).

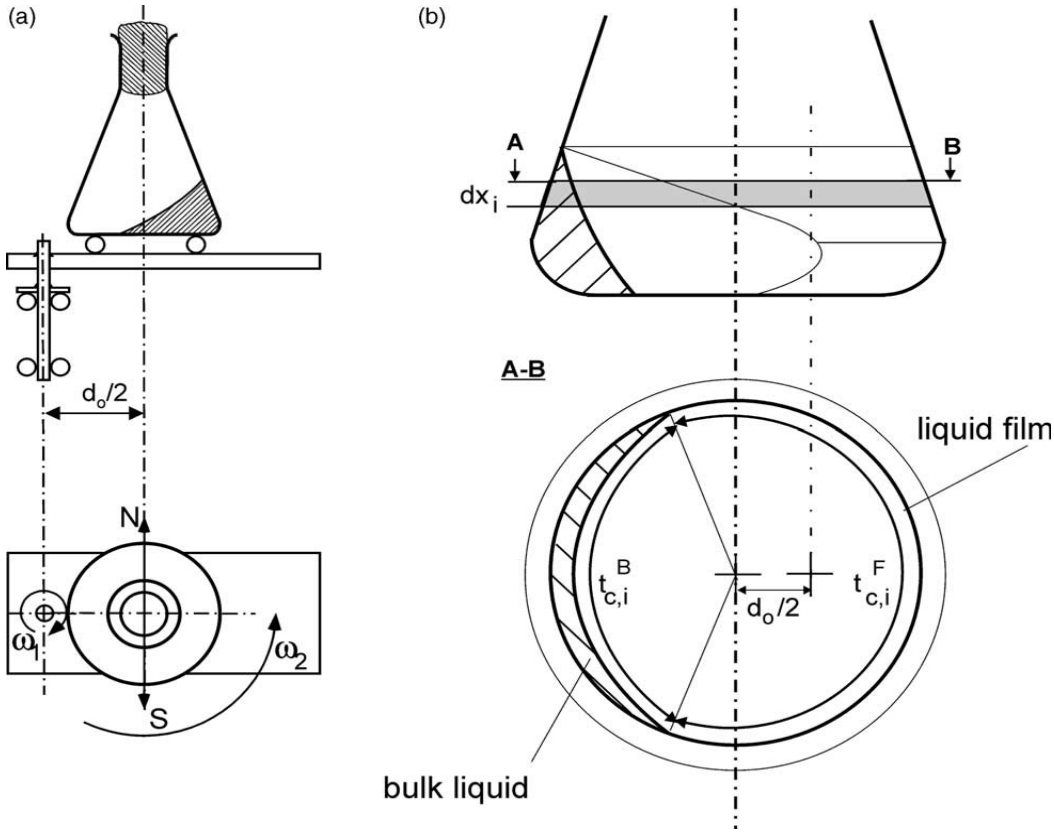

Fig (II-16): Model of the liquid distribution and mass transfer in shake flask:
(a) division of the shaking movement into two partial movements, ω_1 rotation of the shaking table and ω_2 counter orientation of the shake flask; the combination of both movements results in a unchanged orientation of the shake flask relative t the ground; (b) application of Higbie's penetration theory to differential slice in the shake flask. d_0 shaking diameter, $t^F_{c.i}$ film contact time, $t^B_{c.i}$ bulk contact time according to (Maier *et al*., 2004).

Flask geometry understanding and modeling the (k_La) in shake flask has for a consequence that the influence of the volume mean power dissipation on cell response could be advantageously studied in shake flasks (Mehmood *et al*., 2010).

II. 3.3.1.2. Hydrodynamic and mass transfer in (TPIFB) reactor

Description of the behaviour of TPIFB reactor involves design parameters such as gas hold-up, liquid and gas velocity, mixing time, mass transfer rate and power dissipation and there is a large degree of interaction among these parameters. However, very little has been known about the gas-liquid mass transfer rate in inverse fluidized bed reactors. The most utilized criteria for scale-up of aerobic fermentations are based on empirical or semi-empirical equations which correlate the volumetric oxygen transfer coefficient (k_La) and total or gassed power per unit liquid volume (P/V). The reasons to expect some differences between classical and inverse fluidization in mass transfer coefficient are the opposed directions of the gas-liquid flows and their different inertial effects on the fluidized particles (Nikov *et al*., 1992).

Liquid recycling that induces bed expansion is a relatively low advantage compared to the operating conditions in two-phase classical fluidized bed and two-phase inversed fluidized bed reactors (Nikov and Karamanev, 1991; Nikov and Delmas, 1992; Essadki *et al*., 2005). Superficial gas velocity (U_g) in IFB reactor is lower than in other equipments, i.e. airlift and three-phase fluidized bed reactors (Nikov and Delmas, 1987; Bang *et al*., 1999; Essadki *et al*., 2005 and Dimitrov, 2007).

However, the inverse fluidization decreases the energy cost for support expansion. Further, higher oxygen transfer rates can be realized in IFB reactors at lower gas flow and shear rates in comparison to classical fluidized beds and agitated reactors (Nikolov *et al*., 2000).

The mass transfer characteristics of the IFB reactors have received less attention than its hydrodynamic behavior; the air-liquid bubbling mass transfer rate without liquid recycling has been measured in order to determine qualitatively the influence of the solid hold-up, gas velocity and liquid flow rate (Nikolov *et al*., 2000 and Sànchez *et al.,* 2005).

The TPIFB reactors with low density bio-particles have different hydrodynamic characteristics than do those with dense particles traditionally used in three phase fluidization. Furthermore, the fluid flow rates in bio-fluidization are relatively low compared to those found in traditional fluidization, corresponding to relatively slow biological reactions rates and the need to prevent excess erosion of the biofilm or bio-particle (Tang *et al*., 1990).

The hydrodynamic characteristics of TPIFB bioreactors depend on a numerous factors including particle properties (size, density, wet ability and roughness); fluid properties (flow rates, surface tension, viscosity, presence of surfactants, electrolyte concentrations) and reactor design (column geometry, gas distributor design) as described by Tang *et al*. (1990).

It has generally been found that, liquid mixing in TPIFB depends on the regime in which the fluidized bed is operating. The diameter of reactor has an influence on mixing; the influence of the gas phase on liquid mixing increases as the diameter increased. The bio-particle size and density have been demonstrated to strongly influence solid mixing in TPIFB bioreactors (Gommers *et al*., 1986; Fan, 1989 and Bly *et al*., 1990).

Small and light particles were more readily drawn into bubble wakes than were large and heavily particles subject to greater mixing (Tang *et al*., 1990). Also, in the case of inverse fluidization it has been shown that, the hydrodynamics is similar to those in classical fluidization (Chern *et al*., 1983).

The estimates of gas holdup and expected bubble size can also assist in predicting oxygen transfer, a critical factor in aerobic bioprocesses, by providing an estimate of interfacial area for gas-liquid mass transport (Charles and Wilson, 1994).

Davison (1989) observed that, the gas holdup decreases at the top of a three phase low density particles system in the region where it becomes a two-phase system. However, the presence of surfactant tends to accumulate at the gas-liquid interface and lower interfacial tension. Thus the effect of the surfactants to bubbles dynamic results in reducing the average bubble size and decreasing of rise velocity (Fan, 1989).

Also, Dargar and Macchi (2006) found that, the presence of surface active agents increases the gas holdups while available correlations predict a greater impact.

Similarly to classical fluidization, the parameters affecting the liquid-solid mass transfer in inverse fluidization are the velocity and physicochemical properties of the liquid and solid phase, the density of the solid phase, the diameter of the solid particles, the superficial velocity of the gas phase and the size of the gas bubbles The volumetric oxygen transfer coefficient (k_La) in classic fluidization has been generally measured by fitting the axial dissolved oxygen concentration to the axial dispersion model. At it was always found, the bubbling in the inverse fluidisation can be achieved at gas flow rates much lower than those in co-current fluidisation, this lower superficial gas loading to small bubbles and essential gas holdup results in considerable mixing in the gas phase (Nikov *et al*., 2000 and Dimitrov, 2007).

Thus, it is more reasonable to assume ideal mixing of the gas phase. Therefore, the methods used for determination of k_La in classical fluidization can not be applicable for an inverse fluidized bed (Kim *et al*., 1990).

Regarding oxygen transfer, the methods developed to evaluate the performance of stirred and aerated vessels are based on either the employment of dynamic (Dang *et al*., 1977 and Linek *et al*., 1987).

The steady-state methods, as the classical sodium sulphite oxidation method, are based on steady-state absorption of oxygen from the gas phase with simultaneous oxygen removal from the liquid phase through a chemical reaction. In the dynamic measurement of k_La in aerobic fermentation usually a dissolved oxygen electrode was applied. A well-mixed liquid and gas phase in IFBR was approached closely, as confirmed Dimitrov (2007). This approach gives the following expression for gas-liquid transfer:

$$dC/dt = k_La\ (C^* - C) \qquad (II.1)$$

The k_La was calculated as the slope of linear equation; in (E) = k_La (t - t_0) where (E) was the fractional approach to equilibrium is given as:

$$E = (C^* - C)/(C^* - C_0) \qquad (II.2)$$

In this equation C* is the saturation concentration of dissolved oxygen, C_0 the initial concentration of dissolved oxygen at time t_0 when a hydrodynamic steady-state has been re-established (< or = 1 min) upon commencement of aeration and C the dissolved oxygen concentration at any time. The oxygen transfer rate, the power consumption and the mixing time were shown to provide an engineering basis for scale-up (Chisti and Ulises, 2002).

The volumetric oxygen transfer coefficient and the power consumption data could be correlated on different hydrodynamic and aeration conditions. The gassed and a gassed (no aerated) power consumption values (P_M and P_G) for Newtonian (*Ne*) and non-Newtonian fluids were related through a well-known equation based of Kolmogoroff's theory of isotropic turbulence and Bernoulli's equation.

There are two inputs of the power delivered to a reactor: the specific power input (P/V) due to aeration (P_G/V) and the mechanical power input due to the pump (P_M).

On the other hand, there are two sources of gassed power delivered to a liquid reactor volume: (1) isothermal expansion of gas as it moves up the vessel, and (2) the kinetic energy (ε) of gas imported to the gas-liquid dispersion at the point of gas injection.

The two sources of gassed power are considered separately; the contribution of kinetic energy to the total power input is shown obviously to be negligible.

Thus, the specific power input due to aeration could be calculated using the equation

$$P_G/V_L = U_g \times g \times \rho_l \quad \text{in W. m}^{-3} \qquad \text{(II.3)}$$

where; (P_G), in Watt is the power input due to aeration, (V_L) in m^3 is the culture volume, (g) the gravitational acceleration (gravity) and (U_g) is the gas velocity. While, the energy provided by the pump forms the mechanical power (P_M). In the hydraulic field, the load of a pump is expressed in theory in height of water.

The absorptive energy by the pump breaks up: Mechanical energy provided to the fluid (closed loop). It is hydraulic power communicated to the liquid of its passage through the pump. The mechanical power is given by the following formula:

$$P_M = Q \times H_f \times 9810 \qquad \text{(II.4)}$$

with, P (in Watt) power transmitted to the fluid by the pump, (Q) flow in $m^3.s^{-1}$ and (H_f) energy or pressure loss of the hydraulic network expressed in m.

$$P_{Mec} = P_{fl} \,/\, (R_V \times R_T) \qquad \text{(II.5)}$$

where, (P_{Mec}) is mechanical power necessary to the pump, (P_{fl}) is power transmitted to the fluid; (R_V) is output of ventilator and R_T is output of the transmission.

The total specific power input in the fluid could be obtained as:

$$P/V_L = (P_G + P_M) / V_L \quad \text{(II.6)}$$

where, (P) in Watt is total power input and (V_L) is the volume of fluid.

II. 3.3.2. Foam controlling problem

During the microbial production of lipopeptides, excessive foam is produced in the bioreactor, when the solution is aerated and agitated because the lipopeptide lowers surface tension; the process of recovering the lipopeptide using a foam separation was attempted as solution for foam adapting (Davis *et al*., 2001).

Very often this is the case of the microbial producing systems which are foaming because of the presence of cell proteins and/or surface active products of a specific nature, e.g. biological surfactants such as glycolipids, lipopeptides, polysaccharides-protein complexes, phospholipids, fatty acids, and neutral lipids (Peypoux *et al*., 1999).

The excessive foaming phenomenon in a bioreactor causes some problems in most microbial cultivation; on a small scale, it presents a problem in operation and process safety, and in large process plants, foaming jeopardizes economic viability. Additionally, reactants and products accumulate in the foam and may be lost from the reactor with the escape of foam; as well, the aeration-agitation rate may be limited unless foaming is controlled by some means (Noble *et al*., 1994).

Effect of fermentation broth composition on foam coalescence behavior is difficult to evaluate, since several parameters can influence this process.

While, the variable composition due to metabolites produced by the cells or dissolved proteins from dead organisms which have become liberated by cell lysis can have significant effects on the coalescence (Prins and van't Riet, 1987).

This is especially true when excessive foam was generated by excretion of surface-active agents into the broth such as microbial surfactants, proteins, peptides and fatty acids that were produced by microorganisms (Al-Masry, 2001).

Both chemical and mechanical methods are available for foam control. Mechanical foam-control is preferable to chemical foam-control because it avoids problems occurred by the addition of antifoam agents such as lowering of the mass transfer rate, blockage of the spin-

filter mesh, reaction inhibition, cell toxicity and adverse effects on separation and purification of products (Andrew, 1982).

However, mechanical methods of foam control include high operating costs, complicated designs, possible shear damage to the product or microorganisms and risk of disturbances to the unit operations (Vardar-Sukan, 1998; Deshpande and Barigou, 1999).

The physical properties of such systems differ considerably from those found in most industrial culture media which usually contain numerous liquid components subject to severe foaming; vigorous agitation and aeration may lead to severe foaming and damages on solid carriers, causing unstable and inefficient fermentor operation as well as the requirement of antifoam agent addition (Davis *et al*., 1999, 2001; Kim *et al*., 1997 and Lee and Kim, 2004).

For advanced models, there are two major factors which particularly spoil the surfactant producing process. First, the stable foaming evolution causes liquid outflow and changes substrate concentration and mixing intensity. Secondly, the presence of lipopeptides may decrease oxygen transfer rate; thus, lowering bioreactor yields (Martinov *et al*., 2008).

The agitation and aeration strategies need to be optimized not only to meet the requirement of sufficient oxygen and mass transfer, but also to minimize the side effects of intensive foaming and breaking of solid carriers. The bioreactor should also be tailored to cope with the foaming problems, avoiding massive addition of costly and probably cell-growth-inhibiting antifoam agents. In particular, the valid reactor volume decreases and the process become hard to control (Grangemard *et al*., 2001).

II. 3.4. Summary of the main processes for lipopeptide production by *Bacillus subtilis* ATCC 21332

The lipopeptide production obtained with *Bacillus subtilis* ATCC21332 in different culture conditions are summarized in table (II-1).

Table (II-1): Lipopeptide production of *Bacillus subtilis* ATCC21332 and foam controlling methods under different culture conditions.

Equipments &bioreactor type	**Culture media**	**Sur mg.L^{-1}**	**Feng mg.L^{-1}**	**Foam controlling**	**Authors**
Shaking flasks (Plank-tonic)	Nutrient broth	100	ND	--	**Arima *et al*., 1968**
Bench-top reactor	Landy	760	ND	Foam removal	**Cooper *et al*., 1981**
	Cooper minimum salts	800	ND	Foam removal	
Bench-top reactor with foam collector unit	MSI	1010: 6450	ND	Foam collection	**Yeh *et al*., 2005**
Bench-top reactor with adsorption unit	Landy	800	ND	Foam separation	**Liu *et al*., 2007**
Shaking flasks (Plank-tonic)	Landy	50	166	--	**Gancel *et al*., 2009**
	Modified Landy	143	326		
	Landy and pp_{fe} carrier* with fe (0.35 and 0.74 %) respectively	215, 183	609, 336		
Shaking flasks (Biofilm)	Modified Landy and pp_{fe} carrier* with fe (0.0, 0.35 and 0.74 %) respectively	300, 390, 310	310, 680, 510		
Bench-top reactor with foam collector unit	Landy	744	43	Foam collection	**Coutte *et al*., 2010**
Bench-top reactor with External Ps fiber unit*		722	142	External separation	
Bench-top reactor with External PP fiber unit*		690	622	External separation	
Bench-top reactor with submerged PP fiber unit*		469	982	Internal separation	
Shaking flasks (Plank-tonic)	Modified Landy	160	30	--	**Chtioui *et al*., 2010**
Shaking flasks (Biofilm with PP_{ch} carriers)		350	340		
Rotating discs contactor	Landy	162	452		

PP polypropylene; PP_{fe} polypropylene with ferric; PP_{ch} polypropylene with activated carbon. Ps Polyethersulfone.

CHAPTER III

Definition of culture conditions of lipopeptide production by *B. subtilis*

III.1. Introduction

In this first part of the thesis, different physico-chemical parameters such as pH, temperature, presence of solid supports, nitrogen source as well as typical engineering parameters, which potentially influence lipopeptide production by *Bacillus subtilis* were studied in flasks. Indeed, the complex heterogeneity of the flow in gas-liquid mixed reactor is a key problem to precisely link oxygen and energy supply to cell response. Typical engineering parameters, like oxygen transfer, power consumption, mixing time, shear rate, have thus been studied in shake flasks to ensure defined culture conditions. In addition, the behaviour of *Bacillus subtilis* ATCC21332 and its spontaneous mutant strain BBG21 was compared in these different cultivation conditions

III.2. Materials and methods

III. 2.1. Microbial strains

Two strains of *Bacillus subtilis* were used in this work, ATCC 21332 and BBG 21. *Bacillus subtilis* BBG21 is a strain from the collection of ProBioGEM laboratory (University Lille 1, France). It was described as a spontaneous modified strain of wild type ATCC 21332 which was recently proved to be a co-producer of surfactin and fengycin (Gancel *et al*., 2009; Tapi *et al*., 2010 and Coutte *et al*., 2010); This strain was used as a model in order to estimate the conditions of lipopeptide production, the influence of power dissipation and oxygen transfer on lipopeptide production.

III. 2.2. Culture media

III. 2.2.1. Landy medium (Landy *et al*., 1948)

Landy medium was used for the production of lipopeptides and it contains:

Glucose 20 g l^{-1}; glutamic acid 5 g l^{-1}; yeast extract 1 g l^{-1}; K_2HPO_4 1 g l^{-1}; $MgSO_4.7H_2O$ 0.5 g l^{-1}; KCl 0.5 g l^{-1}; $CuSO_4$ 1.6 mg l^{-1}; $Fe_2(SO_4)_3$ 1.2 mg l^{-1}; $MnSO_4$ 0.4 mg l^{-1}. It is buffered by the addition of 3-[N-morpholino]-propane sulfonic acid (MOPS) 1M. The initial pH was adjusted at 6.8, 7 or 7.2 by adding solution of KOH 5 M and medium was autoclaved at 110°C for 30 min.

Stock solutions: the concentrated stock solutions were stored at 4°C:

Glutamic acid: The solution glutamic acid 4× (20 g l^{-1}) was adjusted to pH 7 by KOH 5M and sterilized by filtration through 0.2 µm filter.

Mineral solutions:

- The mineral solution (1): was made 40× (K_2HPO_4 40 g l^{-1}; $MgSO_4$ 20 g l^{-1}; KCl 20 g l^{-1}). To dissolve the salts, solutions were acidified by addition of H_2SO_4 and sterilized at 110 °C for 30 min or sterilized by filtration through 0.2 µm filters.

- The mineral solution (2): was made 40× ($CuSO_4$ 64 mg l^{-1}; $Fe_2(SO_4)_3$ 48 mg l^{-1}; $MnSO_4$ 16 mg l^{-1}) and acidified by addition of H_2SO_4 up to the dissolution of the salts and sterilized either by autoclave at 110 °C for 30 min or by filtration through 0.2 µm filter.

The solution MOPS 1M was prepared by adding 20.9 g to 100 mL distilled water.

However, the pH was adjusted to 6.8, 7.0 or 7.2 by KOH 5M and sterilized by autoclave at 110 °C for 30 min or filter-sterilized (on 0.2 µm filter).

III. 2.2.2. Landy modified medium (Chollet-Gancel *et al.*, 2009)

Landy modified medium was also used for the production of lipopeptides; 2.2 g $(NH_4)_2SO_4$ was added to 1 liter Landy medium.

To prepare 1L of Landy modified medium, the following solutions were added:

- Landy Base: glucose 20 g; yeast extract 1 g; $(NH_4)_2SO_4$ 2.2 g; 25 mL (40×) mineral solution (1); 25 mL (40×) mineral solution (2) in 650 mL distilled water.

- (MOPS): 100 mL 1M solution.

- Glutamic acid: 250 mL (4×) solution.

III. 2.2.3. Luria-Bertani medium (Sambrook and Russel, 2001)

Luria-Bertani (LB) medium contains tryptone 10 g L^{-1}; yeast extract 5 g L^{-1}; NaCl 10 g L^{-1}.

For solid medium it should be added 17.0 g of agar-agar to 1 liter of LB medium. The pH is adjusted to 7.2 by KOH 3M and it were sterilized by autoclave at 121°C for 20 min.

III. 2.3. Solutions and standards

- **Sodium dodecyl sulfate (SDS)** has been studied as a model to understand foam formation. The sodium dodecyl sulphate was supplied by Biomedical (Ohio, USA) with purity 98% and was used without further purification.

- **The standard of surfactin** was purchased from Sigma (USA).

- **The standard of fengycins** was kindly provided by Dr. Magali Deleu from the Agricultural University of Liege, Belgium (Deleu *et al*., 1999).

III.2.4. Influence of flask geometry: flask shape and size

In this study, three shapes of standard shaking flasks were used for batch culture: Erlenmeyer, Cylinder and Pallone shape (Pyrex- France). Each size 50, 100, 250, 500 and 1000 mL has a specific diameter (*d*) as summarized in Table (1).

Table (1): The flasks shapes, sizes and maximum diameters were used in this study.

Maximum flasks diameters (cm)	50 mL	100 mL	250 mL	500 mL	1000 mL
Erlenmeyer flask	2.8 cm	4.8 cm	8 cm	10 cm	12 cm
Cylinder flask	2.4 cm	3.6 cm	5 cm	7 cm	9 cm
Pallone flask	2.6 cm	4.2 cm	7 cm	9 cm	11 cm

In addition, different relatives filling volumes (V_L) of (0.05, 0.1, 0.2, 0.3 and 0.4 mL/mL flask) and three different shaking frequencies (N) were used (150, 200 and 250 revolution per minute (rpm) in horizontally shaking incubator; the fixed shaking diameter (d_0) was 5 cm.

All the samples were taken at least in duplicate, at regular time intervals to serve the different kinetic analysis and lipopeptide determinations during four days under incubation temperatures from 20 to 40 °C.

The strain *Bacillus subtilis* BBG 21 was cultivated in three different types of flasks of five different sizes (50, 100, 250, 500 and 1000 mL), agitated at 150, 200 or 250 rpm and filled with five different relative filling volumes (0.05, 0.1, 0.3 and 0.4 mL medium/mL flask).

The lipopeptide production obtained in different flasks shapes were measured by HPLC (Gancel *et al*., 2009) with standard deviation percentage between 3.34 and 9.96 % for surfactin values and between 2.03 and 59.55 % for fengycin values.

III.3. Results

III.3.1. Influence of flask geometry: flask shape and size

Bacillus subtilis BBG21 was cultivated in the different conditions of flask sizes and shapes described in the paragraph III.1.1.4. The lipopeptide productions obtained are shown in figure (III-1a) and (III-1b).

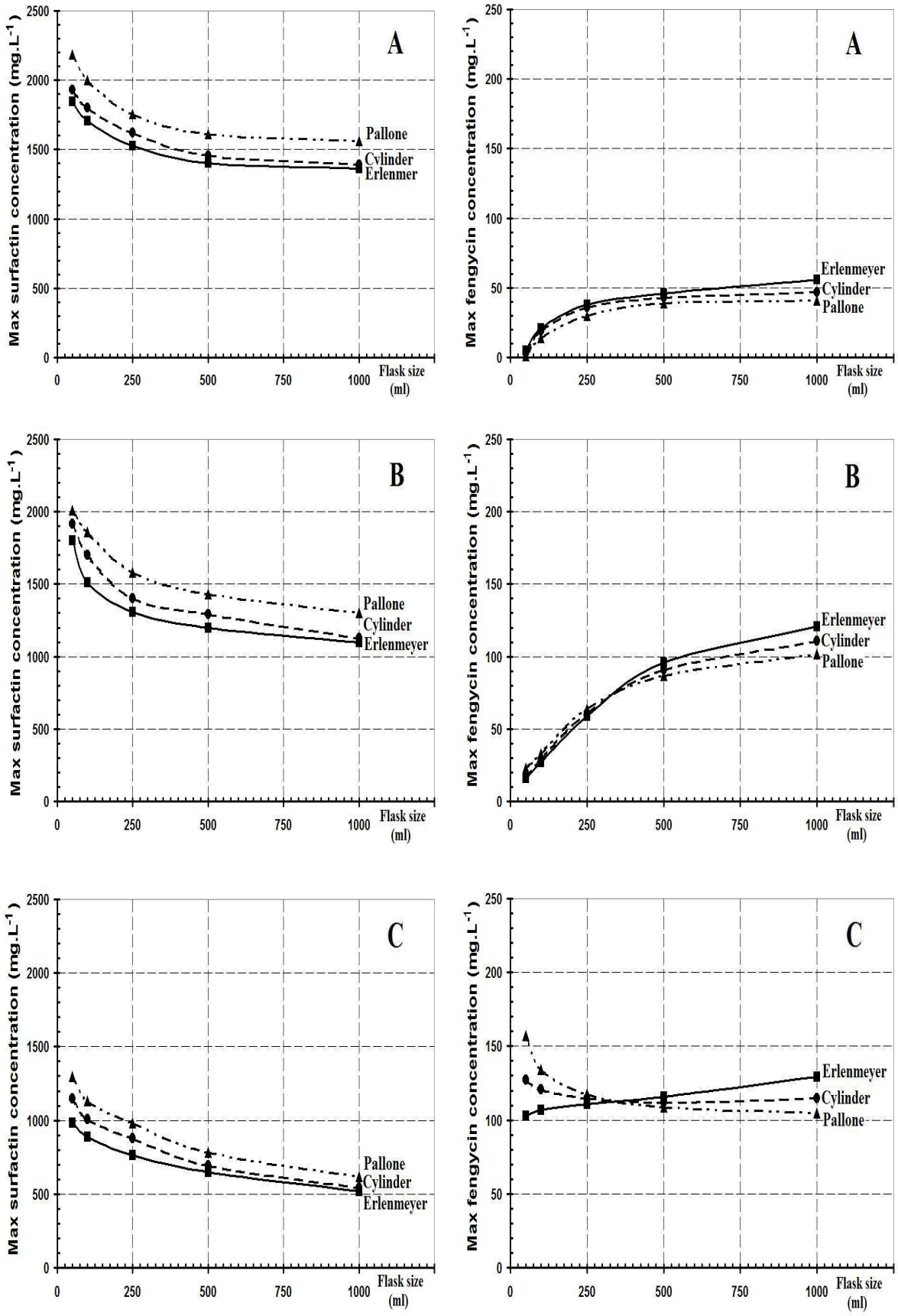

Fig (III-1a): Flask shape effect on lipopeptide production. *B. subtilis* BBG 21 was grown in Landy medium at pH 7.0 at 30 C° and 200 rpm with three flasks shapes (Erlenmeyer, Cylinder and Pallone) in three relative filling volumes (**A**: 0.05, **B**: 0.1 and **C**: 0.2 mL/mL flask); SD (surf ± 3.34 to 9.96 % and feng ± 2.03 to 59.55 %)

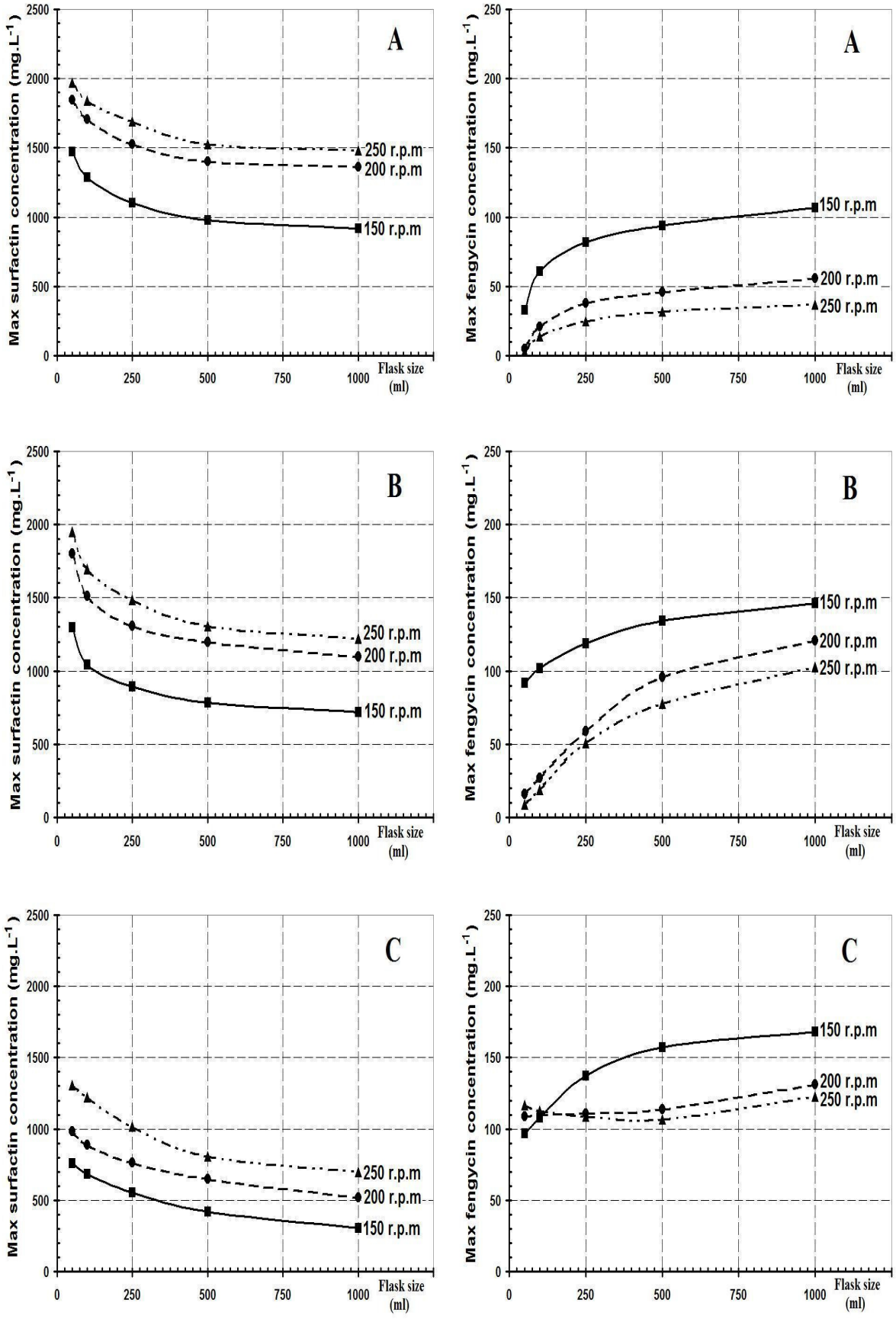

Fig (III-1b): Flask size effect on lipopeptide production. *B. subtilis* BBG 21 was grown in Landy medium, at pH 7.0 at 30 °C and three different agitation rates 150, 200 and 250 rpm. Three relative filling volume were studied (**A**: 0.05, **B**: 0.1 and **C**: 0.2 mL/mL flask); SD (surf ± 3.34 to 9.96% and feng ± 2.03 to 59.55%)

The first main conclusion deduced from these results was the quite different influence of the different tested parameters on surfactin or fengycin production. Results were thus analyzed independently for both lipopeptide families.

Results on surfactin:

Whatever the tested conditions, surfactin production was higher in Pallone flask than in Cylinder and Erlenmeyer ones.

The increase of the oxygen transfer conditions led, in every case, to the increase of surfactin production. The highest surfactin production (2.2 $g.L^{-1}$) was observed in the following conditions: Pallone flasks of 50 mL with a relative filling volume of 0.05 mL/mL flask.

Increasing shaking frequency and decreasing flask size and relative filling volume were favorable for surfactin production. A maximal production (2 $g.L^{-1}$) was observed in flask of 50 mL filled with 2.5 mL of culture medium and shaked at 250 rpm. On the contrary, surfactin was nearly undetectable in flask of 1L filled with 400 mL and shaken at 150 rpm.

Results on fengycin:

The flask shape differently influenced the fengycin production in function of the relative filling volume. There are no significant differences between the production observed in the three different flask types at relative filling volume of 0.05 and 0.1 mL/mL flask except in 1L flask. In this case, Erlenmeyer flasks were the best recipients.

At a relative filling volume of 0.2 mL/mL flask, the production is the highest in Pallone flask in 50 mL and in Erlenmeyer flasks in 1L.

The highest production of fengycin was observed in flasks of 50 ml filled with 20 ml of culture medium and shaked at 200 or 250 rpm. Extreme conditions of the tested experimental field were clearly unfavorable for fengycin biosynthesis. Fengycin was nearly undetectable on one side, in flasks of 500 or 1000 mL with relative filling volume of 0.4 mL/mL flask and shaked at 150, 200 or 250 rpm, on the other side, in flasks of 50 mL filled with 2.5 mL of culture medium and shaked at 200 to 250 rpm. Biosynthesis seemed thus optimal in a narrow range of micro-aerobic conditions.

The flask geometry or the changes of relatives filling volumes and shaking frequencies can modify the liquid distribution within the shaking flask and thus the overall absorption rate of oxygen (Maier *et al*., 2004 and Seletzky *et al*., 2007).

This new set of experiments confirmed that the production of the two families of lipopeptides was differently influenced by the tested conditions.

In all flask sizes, a significant correlation between the surfactin production and the increasing of oxygenation rate was observed contrary to the production of fengycin which can be clearly correlated with the decrease of oxygenation rate.

Generally, this first set of experiments showed the strong influence of chemical engineering parameters, especially oxygen transfer, on lipopeptide production. Indeed a factor of 4.4 and 150 was observed between the lowest and highest productions of surfactin and fengycin, respectively. The differences observed between both families are indications of a main difference in regulation of lipopeptide production into the cells.

III.3.2. Influence of initial pH and temperature

The temperature is considered as an important parameter for lipopeptide production. For example, a 30-fold increase in mycosubtilin production was observed when the temperature decreased from 37 to 25 °C; this was observed both for strain *B. subtilis* ATCC 6633 and its derivative BBG 100, which is a constitutive mycosubtilin over-producer.In addition, Cosby *et al*. (1998) showed that an alkaline pH is favorable for surfactin synthetase expression and thus surfactin production.

The effect of three initial pH (6.8, 7 and 7.2) and five different temperatures (20, 25, 30, 35 and 40 °C) on the surfactin and fengycin concentrations were tested in the different production regimes previously described. Results are shown in figure (III-2).

In the first regime with high oxygen transfer rate; the enhancement of temperature from 20 to 35°C enhances surfactin production with the three tested pH.

The optimal temperature was 35 °C. At higher temperature, the yield of surfactin decreased especially when pH values were 7.2. As expected the best production of surfactin was observed at this regime and also the lowest fengycin production; this last one increase with temperature to reach a maximum value at 40°C for the three pH. An initial pH of 7.0 or 6.8, is optimal for surfactin or fengycin production respectively.

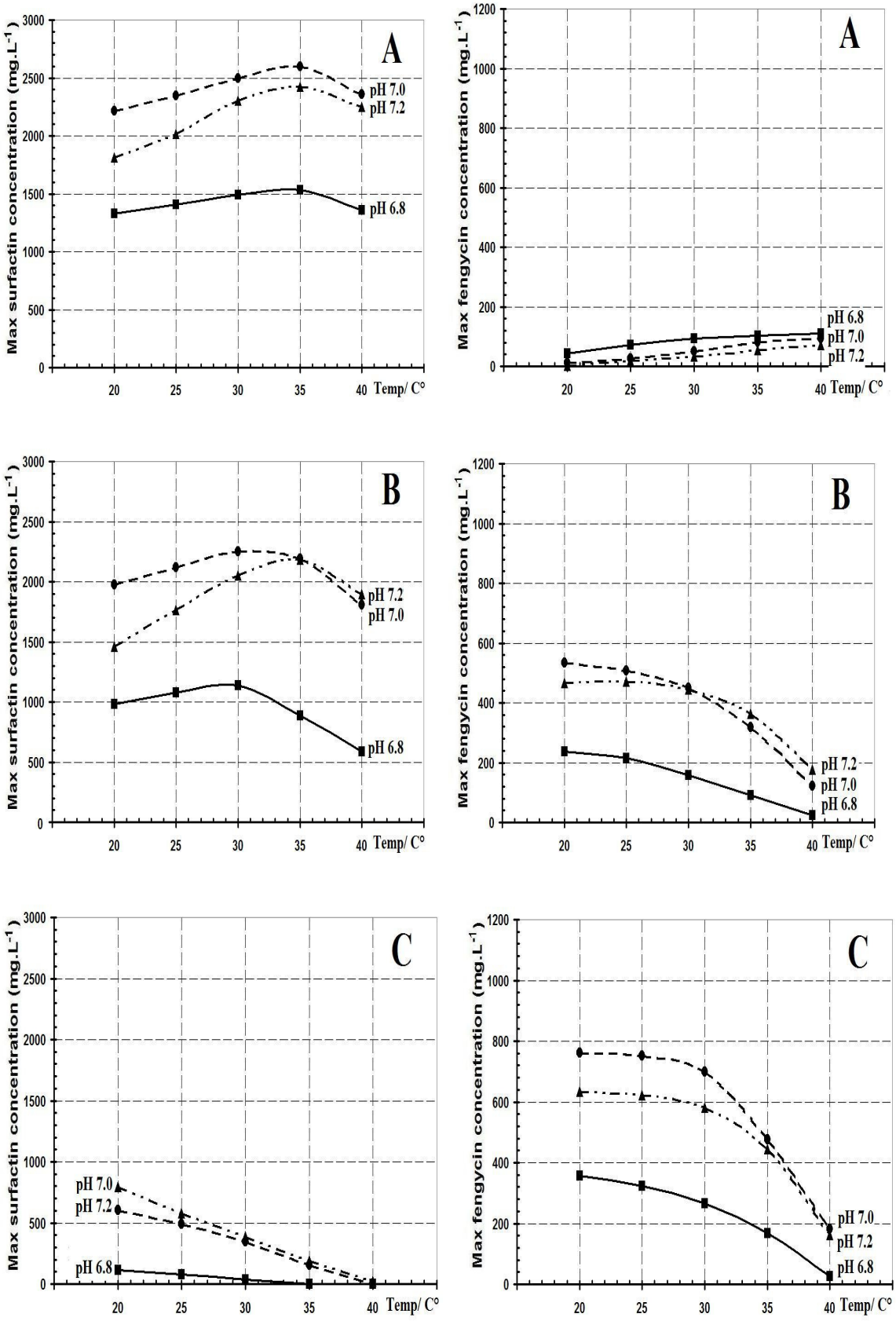

Fig (III-2): Effect of initial pH and temperature on lipopeptide production. *B. subtilis* BBG 21 was cultivated in Landy modified medium at five different temperatures (20, 25, 30, 35 and 40°C), three different initial pH 6.8, 7.0 and 7.2 with three different regimes of oxygen transfer (**A**: high, **B**: moderate and **C**: limited). SD (surf ± 1.31 to 23.57% and feng ± 4.31 to 23.14%)

In the second regime with moderate oxygen transfer rate, an optimal biosynthesis of surfactin was observed at 30°C for initial pH of 6.8 and 7.0 and at 37°C for pH 7.2. For fengycin, the increasing of temperature led to a decrease of the lipopeptide production at the three pH. For both lipopeptides pH 6.8 is less favorable than pH 7.0 and 7.2.

In the third regime with limited oxygen transfer rate; a positive correlation between the lipopeptide production and the temperature decrease was observed for both molecules. The highest lipopeptide production was observed at 20°C and pH 7.0.

III.3.3. Influence of medium additions to lipopeptide production

III.3.3.1. Supports particle additions

For biosurfactant production in inverse fluidized bed reactor, cells need to be immobilized on beads. In a previous work, *Bacillus subtilis* cells immobilization was tested on different support, PPch was shown as one of the most efficient one.

The effect of addition, in the culture medium and of PPch solid carriers on lipopeptide production was thus tested in 50 mL and 500 mL flasks at the different relative filling volume and shaking frequencies. Results are shown in Figure (III-3).

Support addition did not change the pattern of the curves but a clear positive effect on the production of both lipopeptides was registered for all experimental tested conditions.

The surfactin concentration increases up to 20 % with the addition of the carriers, reaching in 50 mL flask and for a relative filling volume of 0.05 mL/mL flask, the concentration of 2.1 $g.L^{-1}$. The effect of solid carriers addition was more important on fengycin concentrations, the fengycin production increases from 1.3 up to 5 times in function of the relative filling volume except in 500 mL flask at a relative filling volume of 0.4 mL/mL flask.

In these last conditions the fengycin production is undetectable without carrier and reached 150 $mg.L^{-1}$ in the presence of the carrier.

These results are in agreement with those previously obtained in our laboratory (Gancel *et al*., 2009 and Chtioui *et al*., 2010) showing that immobilized cells of *B. subtilis* BBG21 produce more lipopeptides than planktonic cells.

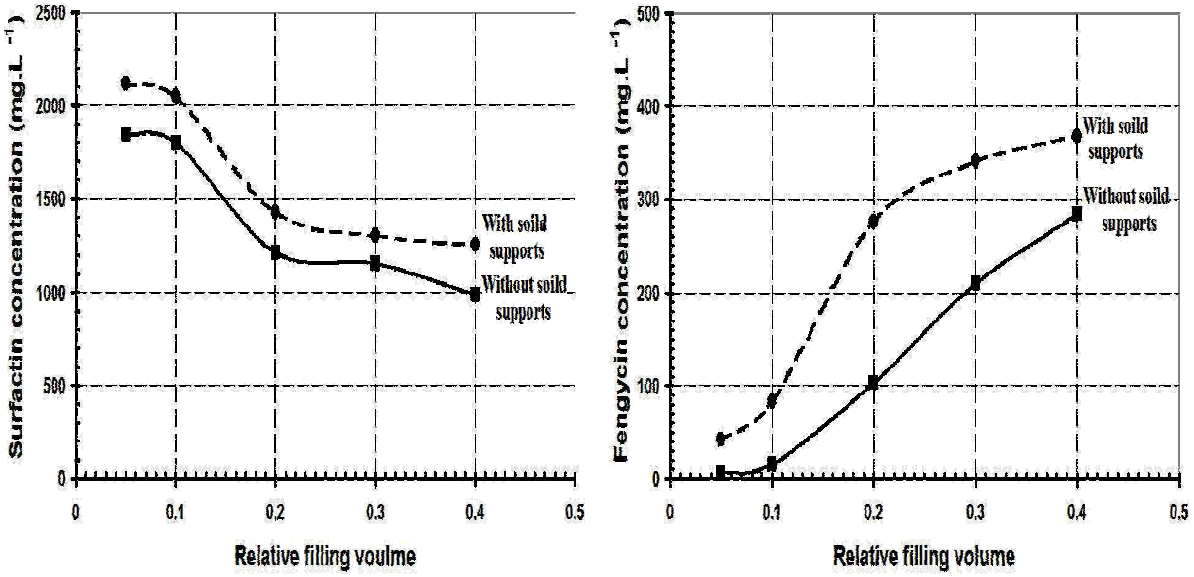

Fig (III-3): Effect of supports carrier addition in lipopeptide production, *B. subtilis* BBG 21 growth in Landy medium, pH 7.0, 30 °C, 200 rpm, flasks of 50 Ml; SD (surf ± 1.54 to 4.18% and feng ± 7.03 to 13.49%)

III.3.3.2. Influence of nitrogen source addition on lipopeptide production

Nitrogen source is important for lipopeptide production. The Landy medium contained glutamic acid as nitrogen source. However, the optimal growth and mycosubtilin production occurred in a Landy modified medium in which glutamic acid was replaced with a combination of glutamic acid and $(NH_4)_2SO_4$ (Guez *et al*., 2008).

In the same medium, an increase in the biosynthesis of fengycin was observed (Gancel *et al*., 2009; Chtioui *et al*., 2010 and Coutte *et al*., 2010).

The lipopeptide productions were thus measured for *Bacillus subtilis* BBG21 cultivated in this Landy modified medium in three different flask sizes (50, 500 and 1000 mL) and with five different relative filling volumes (0.05, 0.1, 0.2, 0.3 and 0.4 mL/mL flask).

The results are shown in Figure (III-4) and compared to results obtained in Landy medium. The addition of ammonium sulfate increased the surfactin production for all the experiments conducted with a high oxygen transfer rate (relative filling volumes lower than 0.2 mL/mL flask).

At a relative filling volume of 0.4 mL/mL flask, the Landy medium was more favorable than the modified Landy medium.

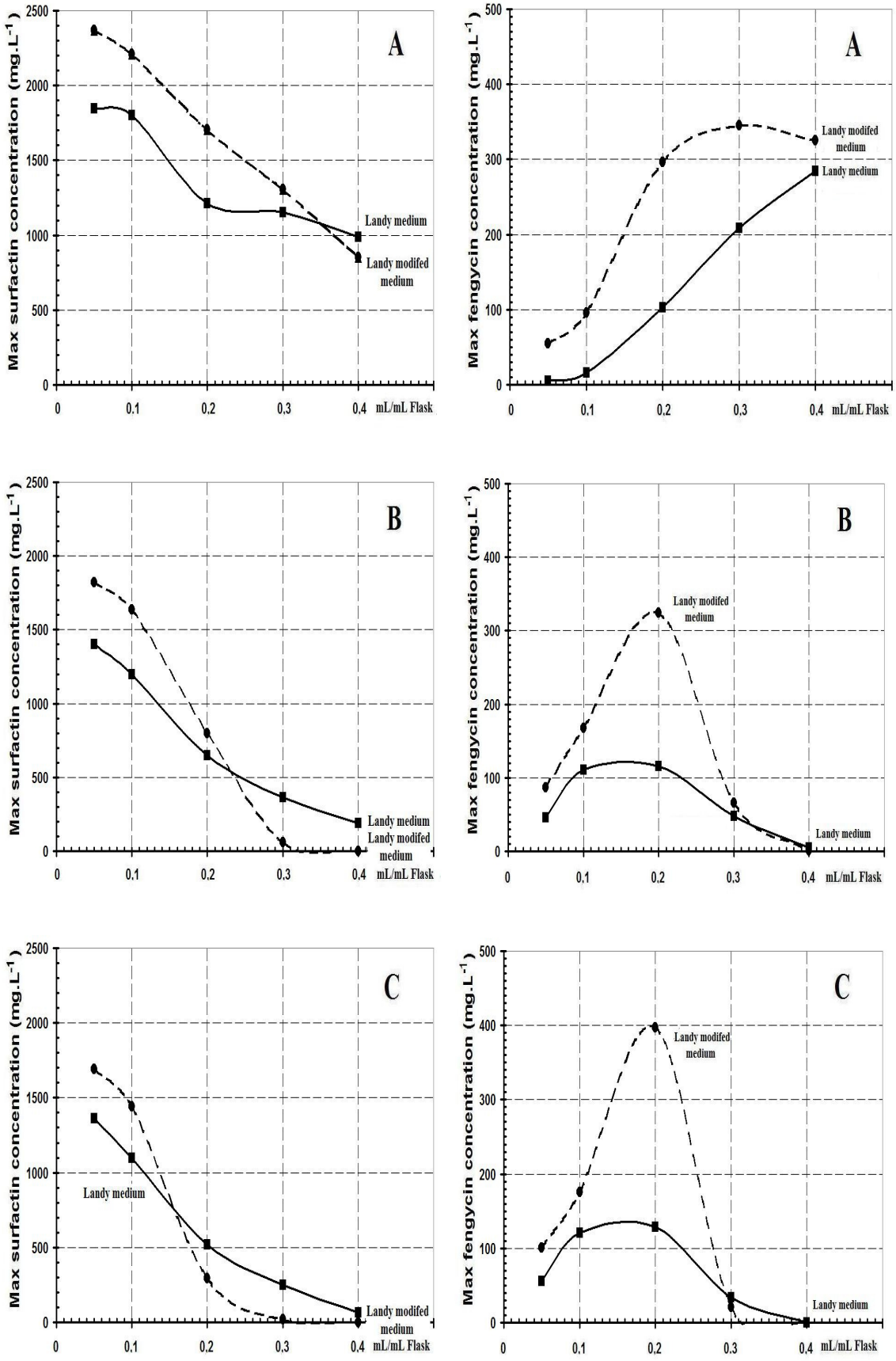

Fig (III-4): Effect of modified medium on lipopeptide production. *B. subtilis* BBG 21 was grown in Landy and Landy modified medium. pH 7.0 and 30 C° with three different relative filling volumes; three flask sizes were studied (**A**: 50, **B**: 500 and **C**: 1000 mL); SD% (surf ± 1.31: 23.57% and feng± 4.31: 23.14%)

An optimal production of 2.35 $g.L^{-1}$ was obtained for a culture in a flask of 50 mL filled with 2.5 mL of modified Landy culture medium.

The effect of the addition of ammonium sulfate to the culture medium is more remarkable for fengycin production.

Interestingly in this case an optimal production of about 400 $mg.L^{-1}$ was observed in a flask of 1L filled with 200 mL of modified Landy culture medium.

Nevertheless, the increasing of the relative filling volume up to 0.3 mL/mL flask dramatically fengycin yield reduced; this phenomenon is more important with increasing the flask sizes.

On the other hand, the positive effect of ammonium sulphate addition in surfactin producing can be only achieved when a good oxygenated transfer rates conditions were observed.

III.3.4. Influence of oxygen supply and power dissipation

The influence of the oxygen supply and power dissipation will be presented in form of a publication (see here after). The attached work was accepted in SFGP Congress-2011 to be submitted for publication in the international journal Process Biochemistry. On the basis of the obtained results, three different regimes covering the range of tested oxygen transfer conditions and leading to three different patterns of lipopeptides produced were defined:

Regime 1:

Flask size of 50 mL with relative filling volume of 0.05 mL/mL flask), agitation rate of 250 rpm. In these conditions, k_La is higher than 0.06 s^{-1} and the ratio between the surfactin and fengycin concentrations ($R_{surf/feng}$) reached 50.

Regime 2:

Flask size of 50 mL with a relative filling volume of 0.2 mL/mL flask and an agitation rate of 150 rpm. In these conditions k_La is less than 0.003 s^{-1} and ($R_{surf/feng}$) is about 5.

Regime 3:

Flask size of 1000 mL with a relative filling volume of 0.4 mL/mL flask and an agitation rate of 250 rpm. In these conditions k_La is about 0.015 s^{-1} and ($R_{surf/feng}$) is about 0.5.

Récents Progrès en Génie des Procédés, Numéro 101 - 2011
ISSN 1775-335X - ISBN 2-910239-75-6, Ed. SFGP, Paris, France

Influence de l'apport d'énergie et du transfert d'oxygène sur la production sélective de lipopeptides par *Bacillus subtilis* ATCC 21332

Impact of energy supply and oxygen transfer on selective lipopeptide production of *Bacillus subtilis* ATCC 21332

FAHIM Sameh, DIMITROV Krasimir, GANCEL Frédérique, JACQUES Philippe, NIKOV Iordan*
Laboratoire ProBioGEM EA 1026
Polytech'Lille, Université Lille 1, Villeneuve d'Ascq, France

Résumé

L'influence de la puissance dissipée sur la productivité de surfactants lipopeptidiques (surfactine et fengycine) par *Bacillus subtilis* a été étudiée en fioles agitées dans l'optique d'une montée en échelle du procédé de production. Les expériences réalisées en fioles de différents volumes ont confirmé clairement que la puissance dissipée influence, via la surface d'échange gaz-liquide, la production de biosurfactants. A la base des différentes concentrations en surfactine et fengycine obtenues, trois catégories de conditions expérimentales pour la production de ces lipopeptides ont été définies. Lorsque la puissance dissipée moyenne et le coefficient de transfert de masse gaz-liquide, k_La, sont faibles ($k_La < 0{,}003$ s^{-1}), les deux lipopeptides ne sont pas synthétisés. Lorsque ces deux paramètres sont élevés ($k_La > 0{,}07$ s^{-1}), seule la surfactine est produite. Une synthèse des deux biosurfactants est observée lorsque de faibles valeurs de k_La sont compensées par une puissance dissipée élevée et vice-versa. La production de fengycine est optimale pour des valeurs de k_La relativement faibles (autours de 0,01 s^{-1} ou inférieures). A agitation constante, la réduction du rapport entre les volumes de culture et de la fiole favorise la production de surfactine dont la concentration pouvant atteindre 2 $g.L^{-1}$. Dans toutes les conditions testées, l'addition d'un support solide pour l'immobilisation cellulaire augmente la production des deux lipopeptides.

Abstract

The influence of power dissipation on *Bacillus subtilis* lipopeptidic surfactant (surfactin and fengycin) productivity was studied in shake flasks in view of scaling-up of this fermentation process. The experiments performed in different working volumes of flask, confirm clearly that power dissipation changes, via interfacial gas-liquid contact surface, the biosurfactant production. From the collected data on the surfactin and fengycin concentrations, three zones of conditions for lipopeptides' production were established. At low k_La (less to 0.003 s^{-1}) and volume mean power dissipation values, lipopeptides were practically not produced. When both parameters were very high ($k_La > 0.07$ s^{-1}) only surfactin was produced. A mixed production was observed when low values of k_La were compensated by a relatively high value of power dissipation and vice versa. Fengycin production was favored at relatively low k_La (about and less to 0.01 s^{-1}). At constant shaking frequency, the reducing of the relative filling volumes from 0.4 to 0.05 increased the surfactin concentration up to 2 $g.L^{-1}$. In all tested conditions, the addition of a solid carrier for cell immobilization improved both surfactin and fengycin productions.

Mots-clés : Surfactine ; Fengycine ; k_La ; Puissance dissipée ; *Bacillus subtilis*

Key-words : Surfactin; Fengycin; k_La; Power dissipation; *Bacillus subtilis*

1. Introduction

The actual level of knowledge of the biosynthesis of lipopeptides from *Bacillus subtilis* such as surfactin and fengycin, and its regulation mechanism allows developing different techniques to overproduce the main active compounds and to reach yields that are compatible with industrial development of such

* Corresponding author: Tel.: + 33 3 28 76 74 10 ; Fax: + 33 3 28 76 74 01 ; E-mail: Iordan.Nikov@polytech-lille.fr

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2-910239-75-6, Ed. SFGP, Paris, France

compounds (Jacques 2011). A lot of studies have pointed out different environmental factors for their effect on surfactin production using planktonic and immobilized cells (Jacques 2011). Several studies demonstrate that their production is strongly influenced by oxygen transfer conditions (Hbid et al. 1996; Guez et al. 2008; Gancel et al. 2009). Recently, it was also demonstrated that cell immobilisation can drastically modify the ratio between surfactin and fengycin produced by the same derivative strain of *B. subtilis* ATCC 21332 (Gancel et al. 2009; Chtioui et al. 2010; Coutte et al. 2010). The complex heterogeneity of the flow and foaming in real gas-liquid reactor is a key problem to associate oxygen transfer to cell response. Typical engineering parameters have been described in shake flasks to ensure defined culture conditions: mass transfer (Maier et al. 2004), power input (Büchs et al. 2000), hydro-mechanical stress and fluid movement (Peter et al. 2006), and the influence of the power dissipation on cell response (Mehmood et al. 2010). Development of a bubble-less process by an oxygenation from continuous gas phase to a formed liquid surface in flasks will allow to understand the relation between energy supplies, gas-liquid mass transfer and fermentation production and to facilitate the scale-up of lipopeptides fermenters. The study of this relation focuses the comprehension of the mechanism of the gas-liquid reacting production, respectively the liquid-side mass transfer coefficient k_L, specific area a, and lipopeptide productivity and selectivity. These data could be also of interest to design bioreactor onto its selectivity, as well as for the developing of adequate mathematical model. Conforming to the above aspects, the aim of this study was to investigate the production capacity of surface aerated immobilised and planktonic cells of *B. subtilis* ATCC 21332, relation between the power dissipation and k_La and to analyse the selectivity during the production process.

2. Materials and methods

2.1. Culture and production conditions

In this study, *B. subtilis* BBG21, a spontaneous modified strain of ATCC 21332, was used. Cultures were performed in Erlenmeyer flasks of different sizes (50, 500 and 1000 mL) with various shaking frequency conditions (150, 200 and 250 min^{-1}). Different relative filling volumes (Rv, volume of medium / volume of flask) from 0.05 to 0.4 were tested in each kind of flask. The cultures were performed 96 h at 30°C in Landy medium buffered at pH 7 with MOPS 100 mM. When supports were used, *B. subtilis* BBG21 was previously immobilized on solid carriers, selected on the basis of previous studies (Dimitrov et al. 2007; Chtioui et al. 2010). The supports, polypropylene foamed beads (PPch) were produced by using supercritical carbon dioxide as a foaming agent and coated with powdered activated carbon. These coated supports had enhanced roughness. The particle size of the supports was 3.0-3.5 cm in diameter and their average density (ρ_s) was 350 $kg.m^{-3}$. All experiments were performed in triplicate.

2.2. Analytical measurement methods

2.2.1. Quantitative analysis of lipopeptides

The surfactin and fengycin concentrations were determined by reverse phase C18 HPLC (600 s, Waters, USA) equipped with a Merck C18 column (5 mm, Merck, Germany) as previously described (Gancel et al. 2009; Chtioui et al. 2010; Coutte et al. 2010). The standard of surfactin was purchased from Sigma (USA) with purity of 98%. The standard of fengycin was kindly provided by Dr Magali Deleu from the Agricultural University of Gembloux (Belgium).

2.2.2. Oxygen transfer and mean power dissipation quantifications

To determine volumetric gas-liquid mass transfer coefficient k_La in shake flasks, the empirical correlation of Maier and Büchs (2001) was used:

$$k_La = 6.67\text{x}10^{-6}\, N^{1.16}\, V_L^{-0.83}\, d_0^{0.38}\, d^{1.92} \quad (1)$$

where N is the shaking frequency [min^{-1}], V_L the working volume [mL], $d_{0\ the}$ shaking diameter [cm], and d the maximum inner shake flask diameter [cm]. This correlation is valid for standard glass Erlenmeyer, Cylinder and Pallone flasks with hydrophilic walls, shaking frequencies of 150-250 min^{-1}, relative filling volumes of 0.05-0.4, shaking diameters of 0.25-10 cm and nominal flask volume of 50-1000 mL with maximum flask diameter of 2.5-12 cm. The power dissipation P was calculated according to the equation proposed by Büchs et al. (2000) using the modified Newton number (Ne_{mod}):

$$P = Ne_{mod}\, \rho\, N^3\, d^4\, V_L^{1/3} \quad (2)$$

$$Ne_{mod} = 70\, Re^{-1} + 25\, Re^{-0.6} + 1.5\, Re^{-0.2} \quad (3)$$

with the Reynolds number:

$$Re = \rho\, N\, d^2 / \mu \quad (4),$$

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ISSN 1775-335X - ISBN 2-910239-75-6, Ed. SFGP, Paris, France

where ρ (kg.m^{-3}) is the liquid density , and μ (Pa.s) the dynamic viscosity (water values at 30°C).
The power dissipation for different relative filling volumes (*Rv* of 0.05, 0.1, 0.2, 0.3 or 0.4) was estimated in each kind of flask.

3. Results and discussions

3.1 Influence of flasks size, relative filling volume and shaking frequency on lipopeptide production

Bacillus subtilis BBG21 was cultivated in flasks of different sizes (50, 500 and 1000 mL) with various relative filling volumes (*Rv* 0.05 to 0.4) and in different shaking frequency conditions (150, 200 and 250 min^{-1}). The results shown in Fig. 1 confirm clearly that the fengycin and surfactin productions are influenced by these parameters.

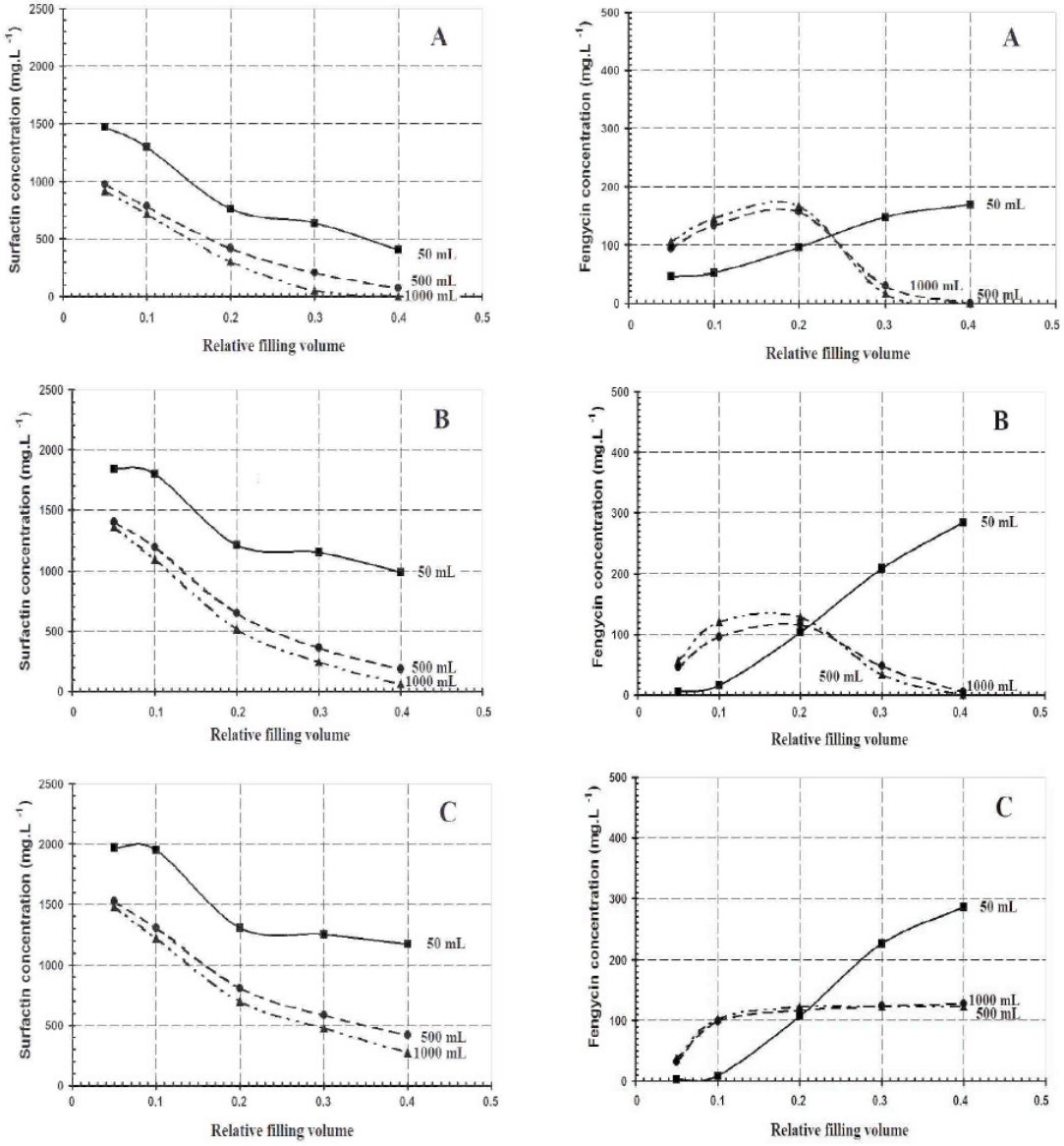

Fig. 1. Influence of filling volume on lipopeptide production by B. subtilis BBG 21 growth in Landy medium, pH 7.0, 30 °C with three different flask sizes 50, 500 and 1000 mL at constant mechanical power (A: 150 min^{-1}, B: 200 min^{-1}, C: 250 min^{-1})

From the collected data, a positive correlation between the surfactin produced and the reduction of the flask sizes from 1000 to 50 mL, the reduction of *Rv* from 0.4 to 0.05, and the increase of the shaking

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frequencies from 150 to 250 min^{-1} was observed. A different behaviour was obtained for the fengycin production which was optimal (286 $mg.L^{-1}$) in a 50 mL flask with a *Rv* of 0.4 and shaked at 250 min^{-1}. In all oxygen transfer studies in shake flask, there are two types of liquid hydrodynamic behaviours: bulk liquid and film liquid. The bulk liquid generally rotates with the direction of the centrifugal acceleration. The film liquid is distributed on the flask wall as sickle rotating within the flask. In film liquid regime, the oxygen is primarily absorbed in the thin film of liquid thrown up on the flask walls and the gas-liquid mass transfer coefficient k_La is higher. Consequently, the oxygen transfer is directly coupled with reducing the relative filling volume which strongly affects lipopeptide production. At low mechanical power (150 min^{-1}) in 1 L flask, the surfactin production increases several hundred times between a *Rv* of 0.4 and this of 0.05. Similar behavior was observed for fengycin production in the same conditions except a decrease of fengycin production between a *Rv* of 0.1 and a *Rv* of 0.05. At high mechanical power (250 min^{-1}) in 50 mL flask, completely different behavior was observed for the two lipopeptides: fengycin production increases from nearly undetectable concentrations at a *Rv* of 0.05 to 286 $mg.L^{-1}$ at *Rv* of 0.4 while surfactin production decreases from 1.97 $g.L^{-1}$ to 1.17 $g.L^{-1}$ in the same range of *Rv*.

3.2. Biofilm formation and productivity

The effect of addition, in the culture medium, of PPch solid carriers, on lipopeptide production was tested in the same different conditions. The obtained results are shown in Fig. 2.

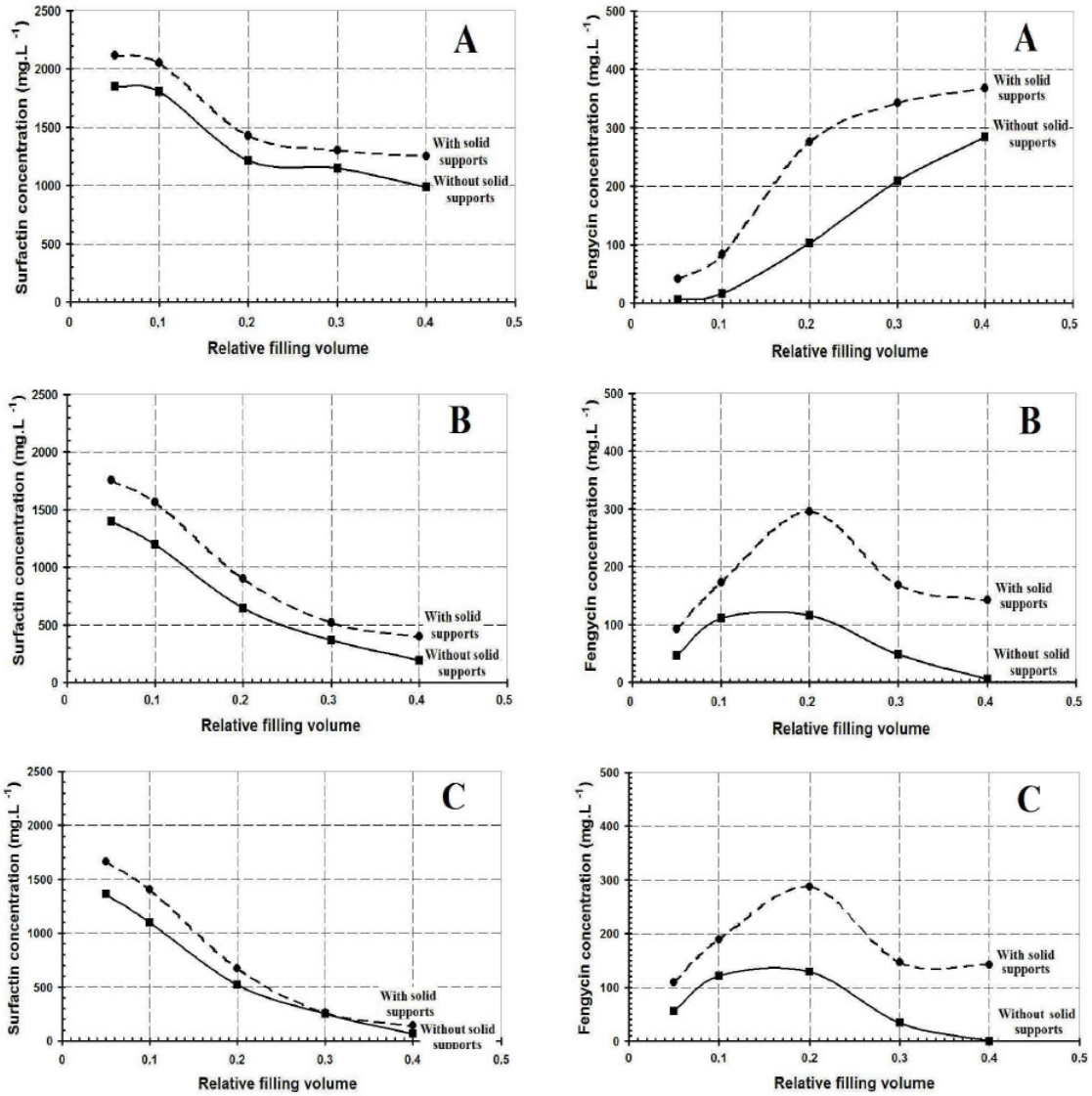

Fig. 2. Influence of carrier-biofilm formation on lipopeptide production by B. subtilis BBG 21 growing in Landy medium, pH 7.0, 30 °C, 200 min^{-1} (A: flasks of 50 mL, B: flasks of 500 mL, C: flasks of 1 L)

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ISSN 1775-335X - ISBN 2-910239-75-6, Ed. SFGP, Paris, France

Support addition did not change the pattern of the curves but a clear positive effect on the production of both lipopeptides was registered for all experimental tested conditions. The surfactin concentration increased up to 2.2 times with the addition of the carriers, reaching in 50 mL flask and for a *Rv* of 0.05, a concentration of 2.1 $g.L^{-1}$. The effect of solid carriers' addition on fengycin production was even more important. The fengycin concentration increased from 1.3 up to 8.4 times in function of the *Rv* except in 500 mL and 1 L flasks at a *Rv* of 0.4. In these last conditions the fengycin production was undetectable without carrier and reached about 0.14 $g.L^{-1}$ in the presence of the carrier. These results are in agreement with those previously obtained (Gancel et al. 2009; Chtioui et al. 2010) showing that the immobilized cells of *B. subtilis* ATCC 21332 produce more lipopeptides than the planktonic cells.

3.3. Influence of oxygen transfer and power dissipation on surfactins and fengycins concentrations

Generally, for gas-liquid bioreactors, k_La results are usually reported in terms of gas power input and mechanical power per unit liquid working volume (Bang et al. 1998). In the flasks only mechanical power could be estimated (no gas flow rate). From the data collected on the surfactant production, a net correlation between k_La and mechanical power dissipation was established (Fig. 3).

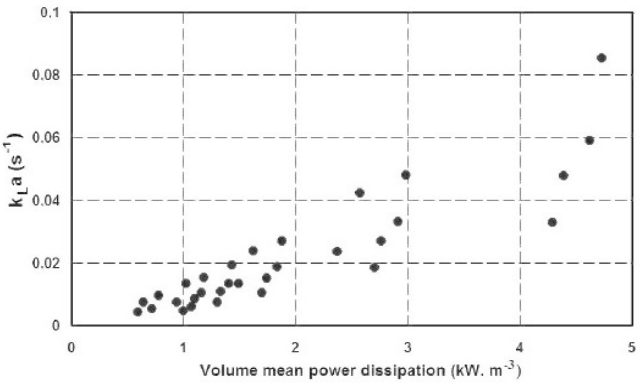

Fig. 3. Relationship between k_La and volume power dissipation (various flasks volumes, filling volumes and agitation conditions)

The movement of the shaking table is divided into two partial movements, the movement of the flask around the eccentric axis and the opposite movement around the flask axis. The liquid distribution results as the intersection between the rotational paraboloid and the flask wall. The overall absorption rate in shaking flasks is dependent on the frequency of replacement of thin layer or the rate of generation of fresh liquid surface. By this description we found that, the oxygen transfer is also directly coupled with increasing the shaking frequency *N* which affecting on lipopeptide production and power dissipation in shake flask.

When k_La ranged from 0.003 to 0.08 s^{-1}, a net positive correlation between the surfactin concentration and the k_La was established (Fig. 4).

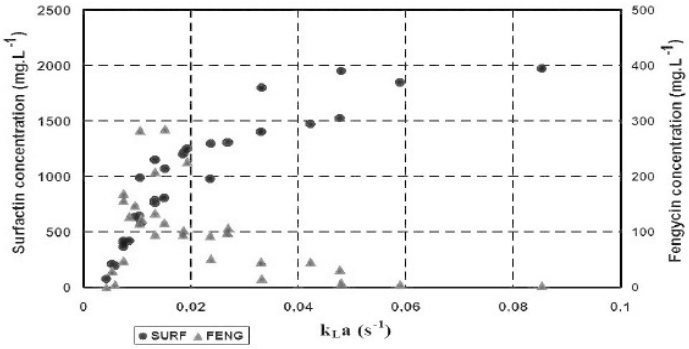

Fig. 4. Relationship between k_La and lipopeptides production (various flasks volumes, filling volumes and agitation conditions)

The concentration of surfactin increased strongly with k_La increase, especially for k_La values lower than 0.015 s^{-1}. Fig. 4 shows that the production of fengycin was enhanced at relatively low k_La (about and less to 0.01 s^{-1}). Obviously, the optimal conditions for the production of the two lipopeptides are not the same. In fact, the increasing of power dissipation increases indirectly the lipopeptide production, trough a modification of the mechanism of gas-liquid mass transfer and, therefore, the oxygen supply to *B. subtilis* cells.

4. Conclusion

From the collected data on the surfactin and fengycin concentrations three zones of conditions for lipopeptides' production could be established. When both mechanical power per unit working volume and gas-liquid mass transfer coefficient k_La values remained very high ($k_La > 0.07$ s^{-1}), only surfactin could be produced. When both k_La (less than 0.003 s^{-1}) and volume mean power dissipation values reminded very low, the conditions are unfavourable for both surfactin and fengycin production. A mixed production could be obtained when low values of k_La were compensated by a relatively high value of power dissipation and *vice versa*.
Surfactin production is clearly favoured at good oxygenation of the cells (high k_La and power dissipation values), while optimal fengycin production could be obtained at moderate oxygen supply.
The immobilization of *B. subtilis* on supports of low density allows a higher production of both lipopeptides, and could improve strongly fengycin production even in the conditions of limited oxygen supply.

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Acknowledgments

Financial support for this work by ARCIR program of the regional council of Nord-Pas de Calais, France, is gratefully appreciated. We are particularly grateful to Dr M. Deleu from the Agricultural University of Gembloux (Belgium) for the kindly provided standards of fengycin.

III.3.5. Comparison of *B. subtilis* ATTC 21332 and BBG21

B. subtilis BBG21 is a spontaneous mutant obtained in our laboratory from *B. subtilis* ATCC 213332. Previous experimentations showed that both strains differently produce lipopeptide in different conditions. The kinetic study of the evolution of biomass, pH and lipopeptide production of both strains was carried out in Landy modified medium at pH 7.0 and 30°C. Detailed results are presented in figures group (III-7 and III-8).

While, figure (III-6) summarizes the maximum lipopeptide concentrations observed for both strains in the different conditions.

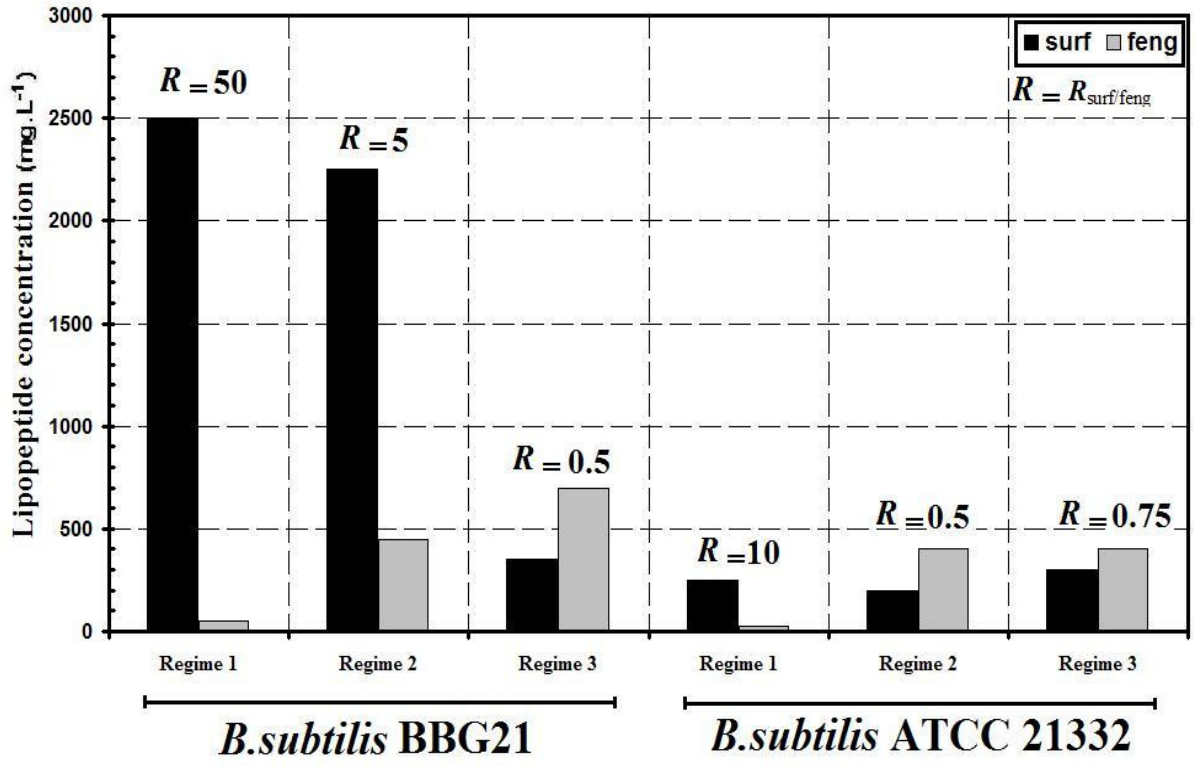


Fig (III-6): Comparison between the lipopeptide productions obtained in the three oxygen transfer regimes for *B. subtilis* BBG 21 and *B. subtilis* ATCC 21332.

At regime 2, small differences were observed between the growth curves of both strains and the pH evolution. At regime 3, pH patterns in function of time seemed more different but following the same oscillation. The highest differences were observed with regime 1 after 24h of culture. Growth of strain ATCC 21332 reached a maximum after 72h and then decreased while growth of strain BBG21 reached a maximum at 36h of culture decreased up to 72h and then re-increased. The strongest pH oscillation was observed for ATCC21332.

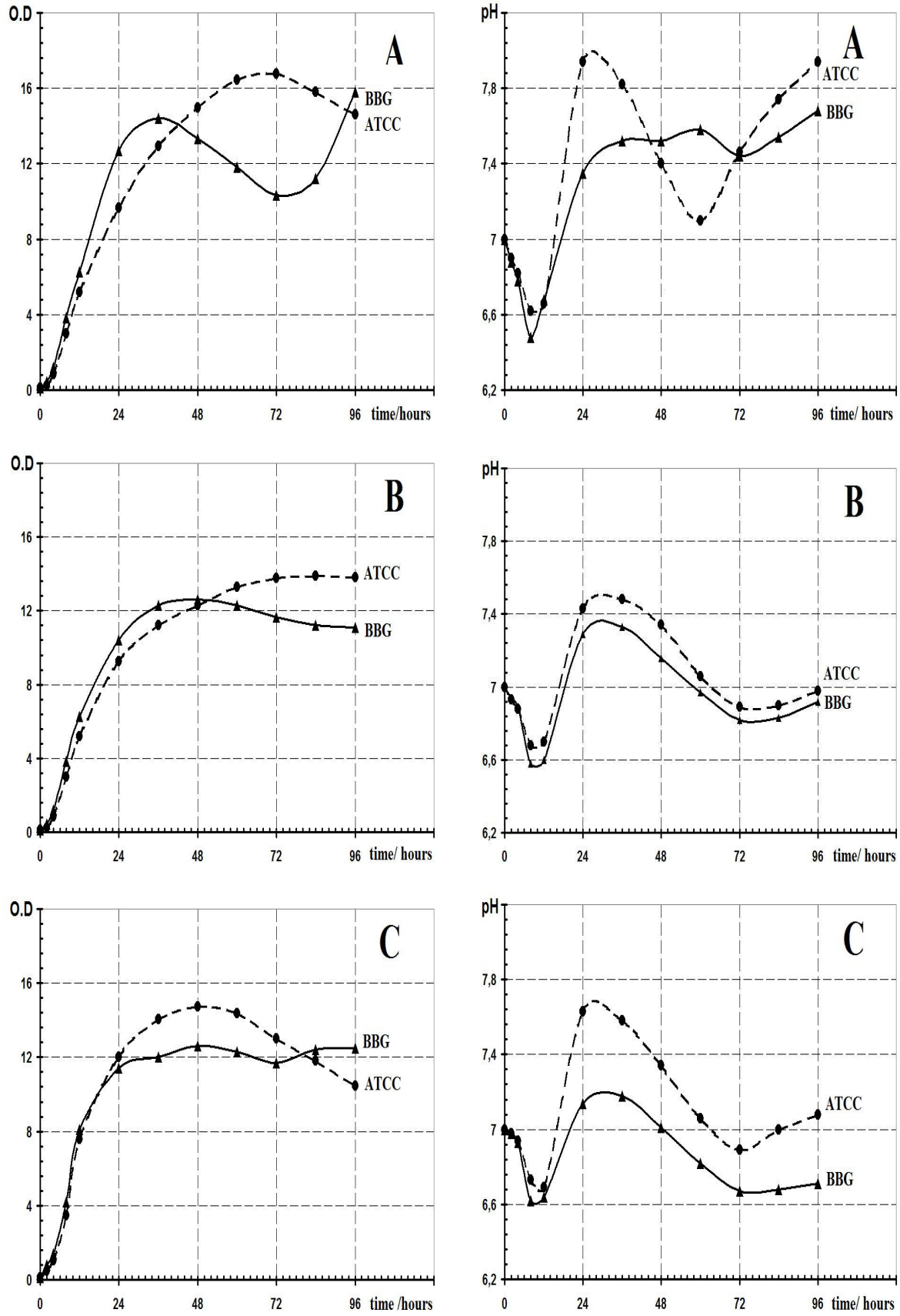


Fig (III-7): Growth curves and pH evolution in function of time for *B. subtilis* ATCC21332 and its spontaneous mutant BBG21 cultivated in Landy modified medium at 30 °C with initial pH 7.0 in three different regimes of oxygen transfer (**A**: regime 1, **B**: regime 2 and **C**: regime 3);
SD (O.D ± 2.42 to 3.69% and pH ± 0.41 to 1.24%)

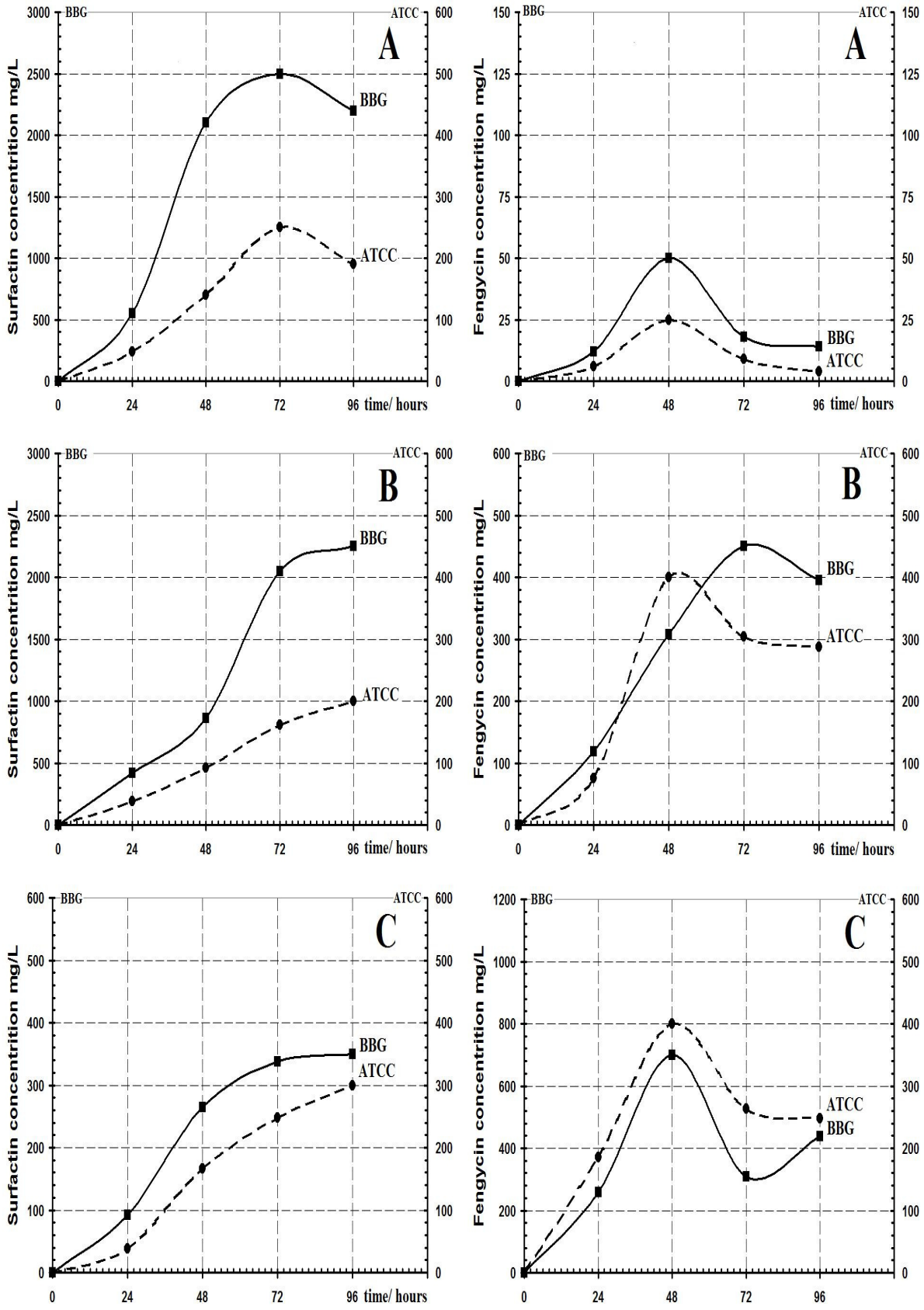


Fig (III-8): The kinetic lipopeptide production of *B. subtilis* ATCC21332 and its spontaneous mutant BBG21 cultivated in Landy modified medium at 30 °C with initial pH 7.0 in three different regimes of oxygen transfer (**A**: regime 1, **B**: regime 2 and **C**: regime 3); SD (surf ± 1.31 to 23.57% and feng ± 4.31 to 23.14%)

Regarding lipopeptide production kinetics, surfactin was in every case more produced by BBG21 than ATCC21332 and for both strains, regime 1 was the best for this biosurfactant. The situation was more complex for fengycin production which is slightly higher in regime 3 for ATCC21332 compared to BBG21.

While at regime 1, BBG21 produced two times more fengycin than ATCC21332. The different behavior of the strains was well illustrated by the figure (III-6). Surfactin/fengycin ratios were not identical for these strains in the different regimes. These results confirmed that the spontaneous mutation obtained in BBG21 probably involves a pleiotropic regulator which modifies the primary and secondary metabolism of the strain.

CHAPTER IV

From flask to bioreactor:

Lipopeptide production in TPIFB bioreactor

IV.1. Introduction

This second part of the work is dedicated to the transfer of the production of lipopeptides from shake flasks to three phase inverse fluidised bed (TPIFB) bioreactor.

Firstly, the oxygen transfer in TPIFB, in real fermentation conditions, will be studied and then, the impact of k_La on the lipopeptide production in different power dissipation conditions and in the presence and in the absence of solid carriers will be analyzed.

Finally, the original TPIFB process with control of oxygen supply and foam formation will be proposed.

IV.2. Materials and methods

IV.2.1. Pilot installation

The Three-Phases Inverse Fluidized Bed Reactor (TPIFB) designed for lipopeptide producing process can be utilized in different configurations.

In particular: for liquid batch regime (complete liquid recycling) and continuous gas flow or for continuous regime of gas and liquid with partial liquid recycling.

The final optimized experimental set-up of the three phases inverse fluidized bed (TPIFB) bioreactor equipped with a top gas-liquid section used in this work is described in figure (IV-1)

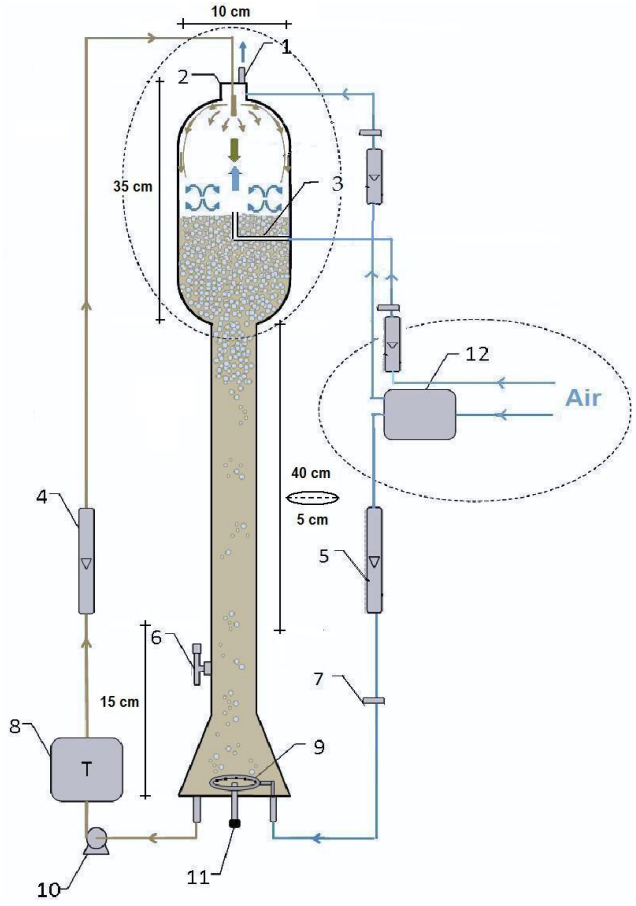


Fig (IV-1): Schematic of the final optimized experimental set-up of the three phases inverse fluidized bed (TPIFB) bioreactor equipped with a top gas-liquid section.

The lab scale fluidized bed device presented in figure (18) consists of a glass column with an internal diameter of 5 cm and a length of 40 cm equipped with a top gas-liquid section with an internal diameter of 10 cm and a length of 35 cm and a bottom conic calm section with a length of 15 cm. The total volume of the reactor is 5 liter.

The gas (3,9) and liquid (2) distributor supply lines are equipped by flow meters (4,5). There are other measurement units: i.e. a dissolved oxygen concentration probe (11), thermo-regulator (8) and pH probe. Also, there are sampling point (6), micro air filters (7), electronic valve for air follow regulation and air outlet point (1).

The height of the fluidized bed section between the air injection point and the liquid level was up to 45 cm.

The reactor can be sterilized at 110 °C for 30 minute; different gas superficial velocity (U_g) was used from 0.003 to 0.015 m s^{-1} coupled with different liquid superficial velocity (U_l) from 0.025 to 0.045 m s^{-1}; the temperature was regulated at 30°C.

For bioreactor maintenance, two types of configuration were designed by modifying the top section according to the filling volume case (20% for essential design or 50% for extreme design); these designs are summarized in figure (IV-2); the top section controls the safety level of relative filling volume and foam.

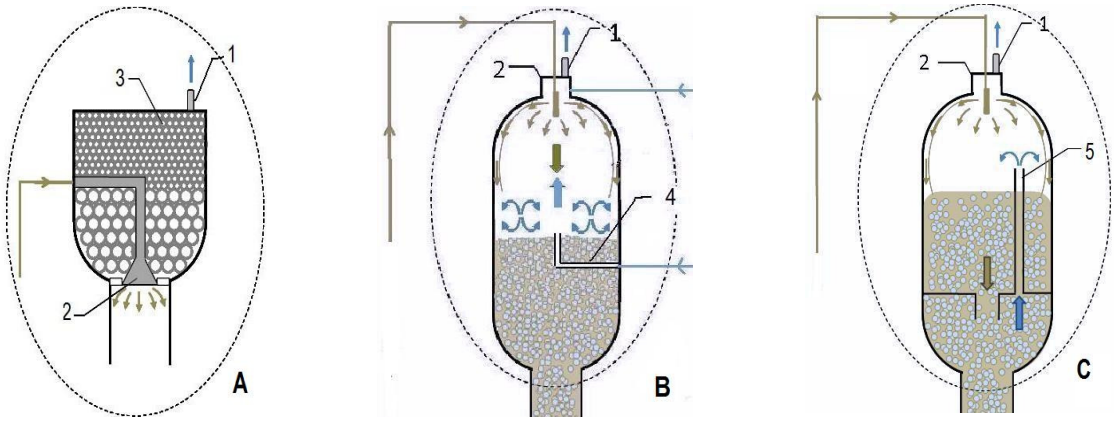


Fig (IV-2): The developed steps of top section for the three phase inverse fluidized bed bioreactor (TPIFB) (1). Gas out let. (2). Liquid distributor. (3). Different particle size. (4). Gas distributor. (5). Foam out let **A** Original design; **B** Design: V_L 20%; **C** Design: V_L 50%

IV.2.2. Solid carriers, culture media and model fluids

The solid carriers used in this study were selected based on our preliminary experience (Dimitrov *et al*., 2007). The three supports were non porous and of various diameters between 0.3 to 0.35 cm. Two supports types have been coated to enhance the surface roughness: the first was polystyrene coated with a layer containing powdered activated carbon (PSch) and the second was polypropylene foamed with powdered activated carbon (PPch). The third support was non-coated expanded polystyrene (PS).

The (PPch) microcellular supports were produced by using supercritical carbon dioxide as a foaming agent (Gancel *et al*., 2009 and Chtioui *et al*., 2010) and the (PSch) was obtained as described in our previous study (Dimitrov *et al*., 2007a). The average density of the carriers (ρ_s) used for mass transfer measurements was 350 kg.m^{-3}.

The experiments were related to *Bacillus subtilis* fermentation, the typical biofluid involved in lipopeptide production contained surfactin. Consequently, real fermentation solutions of surfactin with active cells were used in the analysis (see chapter III).

In parallel, runs were also carried out using water solution with surface tension σ = 71.2 mN.m^{-1} and density ρ_L = 998 kg.m^{-3} as a reference. The surface tension of all solutions used for substratum conditioning was measured by Du Nöuy ring method according to Bonmatin *et al*. (1995). A tensiometer (TD1-E LAUDA. Germany) was used. In this method, the interaction of a platinum ring with the surface is used. The ring is submerged below the liquid interface and subsequently raised upwards; as the ring moves upwards it raises a meniscus of the liquid. Prior to this event. the volume and the force exerted of the meniscus passes through a maximum value which directly thus corresponds to the surface tension of the measured liquid in mN.m^{-1}.

IV.2.3. Validation of the dynamic method

The k_La was determined using the dynamic response of dissolved oxygen concentration following nitrogen injection switched on to air injection. Initially this method has been applied for studying oxygen transfer in continuous stirred tank reactors known to exhibit considerable gas phase mixing; as it is more realistic to assume complete mixing for the gas phase in the reactor, the method can be successfully used in the case of this study. The point of zero oxygen concentration was always ensured before air injection began and identical hydrodynamic conditions were maintained. While, the increase of the dissolved oxygen concentration in the real and model liquid was measured using an oxygen probe (Mettler Toledo), the dissolved oxygen (DO) probe response could be represented according to equation (IV. 1) proposed by Nikolov *et al*. (2000) as follows:

$$\alpha = \frac{1}{K_L a} + \frac{RT}{He}\frac{V_L}{V_G}\tau_G + \tau_G + \tau_E + \tau_F \qquad \text{(IV. 1)}$$

In this equation, (α) is the first moment of the impulse response curve; (R) the gas constant; (T) the absolute temperature; (He) the Henry constant and the values of (τ) indicate of the time constants for electrode and film lag. (τ_G) is the time constant for the response signal lag of the gas layer, (τ_E) is the time constant of the oxygen transfer lag through the electrode membrane and (τ_F) is the time constant of the response lag through the liquid film surrounding the probe.

The combined ($\tau_F + \tau_E$) were measured at various agitation speeds. Physically, (τ_E) mean the time in seconds necessary for the electrode response to reach 63.2 % of its steady state value. In order to do that, the (DO) probe was instantly moved from one highly turbulent medium to another with different (DO) concentrations. Under vigorous mixing the impedance and the capacity of the liquid film surrounding the electrode membrane could be neglected. While, the impedance certainly increased with the softening in the fluid dynamic conditions. The resistance was represented by the value of the liquid film constant (τ_F). Since both film and membrane resistances were represented in series, the cumulative time constant could be represented by the sum of the two time constants involved. (V_L) and (V_G) in the equation represent the liquid and the gas volumes in the fluidized bed. These variables were determined by the displacement method. The solid weight of 0.14 kg being measured previously by weight analysis and the formed fixed solid bed in the reactor was 0.15 m highest. While, the total gas volume (V_G) was determined by the difference of global bed height in aerated and non-aerated conditions.

IV.3. Results

IV.3.1. Development of a correlation for the estimation of oxygen transfer rate in real culture conditions

IV.3.1.1. Analysis of the parameters involved in model and culture conditions

The effectiveness of oxygen transfer is a critical factor determining successful application of any aerated bioreactor. On the basis of the first set of results, we have seen that the oxygen transfer rate is very important in flasks for lipopeptide biosynthesis.

An understanding of the various factors affecting oxygen transfer in TPIFB reactor is thus important for reactor scale-up; this oxygen transfer was already characterized in TPIFB by Dimitrov (2007) but in model solutions. In this chapter, we characterize it in real fermentation conditions. In parallel, runs were also carried out using water solution with surface tension σ = 71.2 mN.m^{-1} as a reference.

It can be assumed that the oxygen transfer is governed by the system physico-chemical properties (liquid and solid densities, liquid viscosity, surface tension, gravity…), the bead diameter, the reactional volume and hold-up and the liquid and gas phase flow rates i.e. the throughputs, etc... Thus,

$$k_l a = f\left(\rho_l, \rho_s, \mu_l, \sigma, g, d_p, V_{reac}, U_g, U_l\right) \qquad \text{(IV-2)}$$

In our experiments, six basic parameters were varied, because the gravity, the particle diameter and the viscosity were determined as constants.

IV.3.1.2. Influence of liquid and gas velocity on k_La

The oxygen transfer rate coefficient k_La was first evaluated in inverse fluidized bed reactor at three different liquid superficial velocities (U_l = 0.02, 0.025 and 0.03 m.s^{-1}) and one gas velocity (Ug = 0.006 m.s^{-1}). Results were compared to those obtained by (Nikolov *et al*., 2000 and Dimitrov, 2007) and are presented in figure (IV-3).

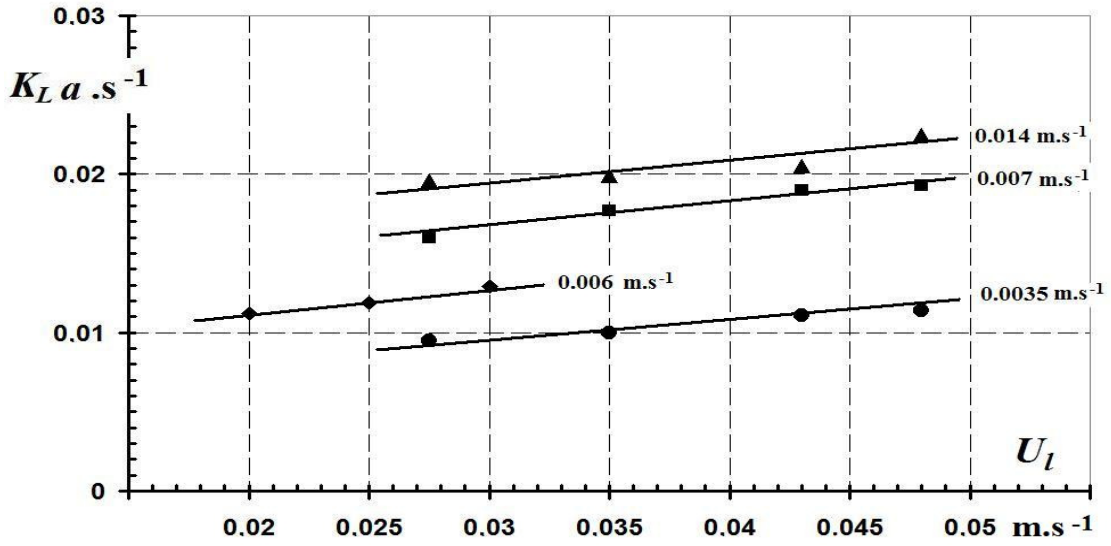


Fig (IV-3): Effect of gas and liquid superficial velocities on (k_La) at different (U_g)
Data concern (Ug = 0.006 m.s^{-1}); SD% (k_La ± 4.28: 7.37%)

These results showed that the k_La is not strongly affected by the liquid velocity (U_l) compared to the gas velocity (U_g) which showed positive influence on (k_La) values.

The same effect in classical fluidization was observed by Tang *et al*. (1990) and Kang *et al*. (1991) and was attributed to an increase in mass transfer coefficient (k_L). It was also assumed that the gas bubbles holdup and break-up in the bed increases and amplifies the gas-liquid superficial area (a).

IV.3.1.3. Effect of the presence of carriers and carriers' surface coating

Similarly, the oxygen transfer rate coefficient k_La was evaluated in inverse fluidized bed reactor at three different liquid superficial velocities (U_l = 0.02, 0.025 and 0.03 m s^{-1}) and one gas velocity (Ug = 0.006 m s^{-1}) in the presence of expanded polypropylene carriers with surface covered with an activated carbon powder layer.

These results were compared to those obtained by (Nikolov *et al*., 2000 and Dimitrov, 2007) (figure (IV-4)) with similar carrier modified or not at different liquid superficial velocities (U_l = 0.028, 0.035, 0.042 and 0.048 m s^{-1}) and one gas superficial velocity (U_g = 0.014 m s^{-1}).

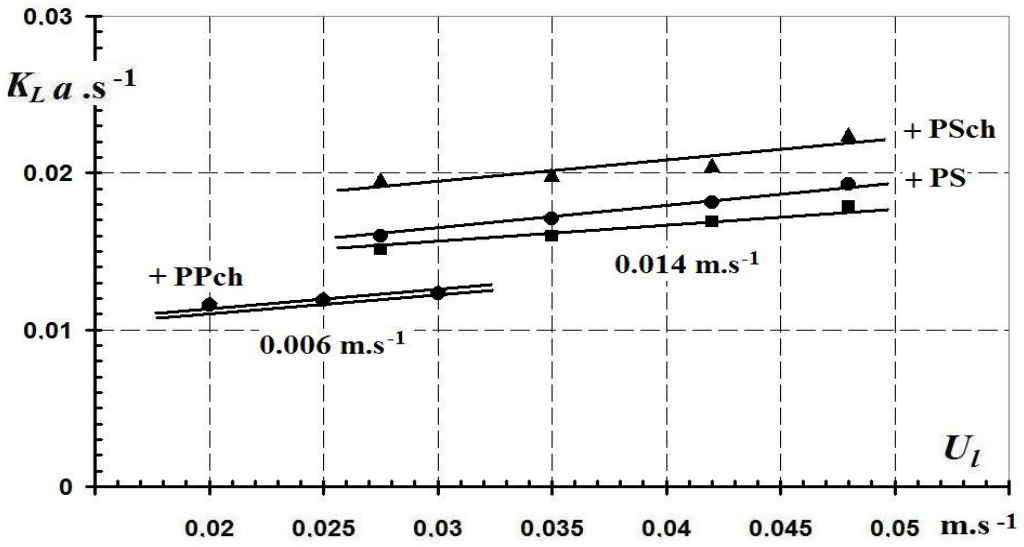


Fig (IV-4): Effect of the presence of carriers and carriers surfaces coating on (k_La) at different (U_g)
Data concern (Ug = 0.006 m.s^{-1}); SD% (k_La ± 4.28: 7.37%)

As it can be seen, the presence of treated polypropylene beads (PPch) did not change or slightly increased k_La values contrary to the results obtained by Sanchez *et al*. (2005) and Dimitrov (2007). However, the k_La values did show big differences with increasing (U_g) in the absence or presence of modified and not modified polystyrene carriers.

A significant reducing of k_La values was observed in the presence of non modified carriers. It can be assumed that this difference is a result of increased surface roughness of the coated carriers (PPch) particles in comparison to that of the non-coated carriers (PS), this surface roughness morphology was already studied by scanning electron microscopy (SEM) by Gancel *et al*. (2009) and Chtioui *et al*. (2010). It can facilitate the break-up of gas bubbles and increase in that manner the gas-liquid contact.

IV.3.1.4. Effect of the liquid surface tension

The volumetric mass transfer coefficient was measured at different gas superficial velocities (U_g) and at the same liquid superficial velocities in aqueous solutions having different surface tensions (σ) due to the addition of sodium dodecylsulfate (SDS) and surfactin. Results are compared to those obtained by (Dimitrov, 2007) and presented in figure (IV-5).

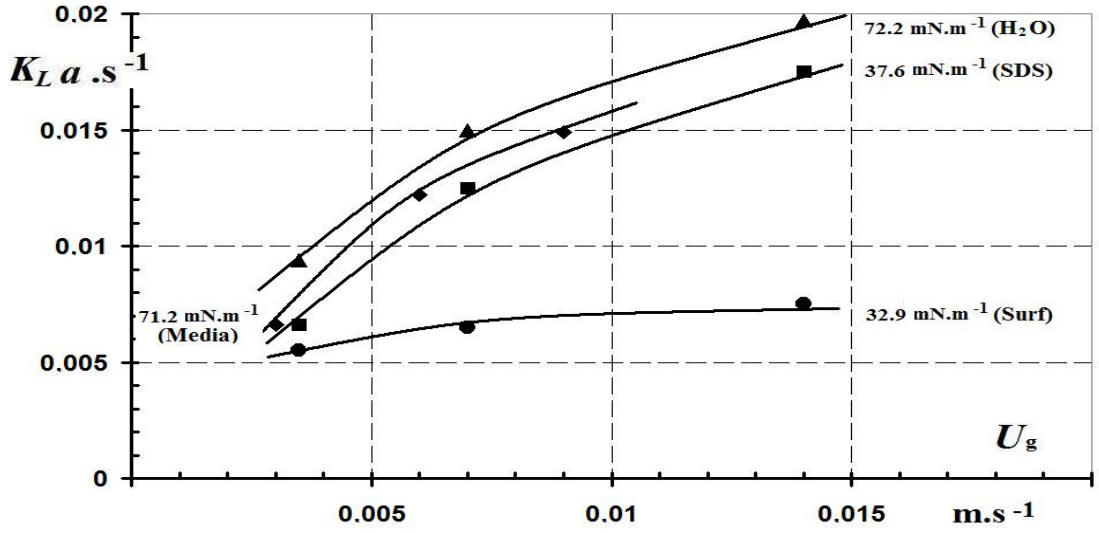


Fig (IV-5): Effect of superficial gas velocity on (k_La) at three different liquid surface tension
Data concern (σ = 71.2. mN.m^{-1}); SD% (k_La ± 4.28: 7.37%)

In the three solutions, an increasing in the k_La values with increasing the (U_g) values was observed but this increase was strongly depending of the surface tension (σ) values.

While, the effect of SDS and surfactin on k_La values was clearly negative, its intensity depends on the tested surfactant; k_La values in the liquid modified by surfactin was more reduced by comparing with those obtained in the liquid modified by SDS. The lowering of the surface tension inhibited the mass transfer because of the surface tension gradients developed around the bubble surface and its immobilizing; surfactants could also modify the bubble growth rate. We can see and confirm an increase in k_La values by increasing the gas velocity; while, effect of the liquid velocity is limited.

IV.3.1.5. Real fermentation conditions

In view of developing an IFB reactor designed to lipopeptide production with adequate hydrodynamic and energy conditions, the effect of culture medium containing lipopeptides on

oxygen transfer coefficient k_La was examined. Different samples were tested and compared to previous results obtained in water in the presence or not of biocarriers.

K_La values were thus measured during two cultures of *Bacillus subtilis* BBG21 of first four hours in Landy medium with and without solid carriers. Three different liquid and gas velocities were applied (U_l = 0.02, 0.025, 0.03) and (U_g = 0.003, 0.006, 0.009). During these four hours, cells grew from an optical density at 600 nm of 0.1 up to 2 and the surfactin and fengycin productions reduced the surface tension from 71.2 to up to 41.7 mN.m^{-1}.

Experiments were first performed at three different liquid superficial velocities and one gas superficial velocity 0.006 m s^{-1}.

Three different cultivation samples were tested, the first one was obtained after 2h of cultivation of strain BBG21 and contained about 3 mg L^{-1} surfactin and the surface tension of this sample was 64.8 mN m^{-1}. The other samples contained biocarriers and were obtained after 4 hours of cultivation, they contained a surfactin concentration near the critical micellar concentration (CMC) of the biosurfactant (10 mg L^{-1}). Their surface tension was 46.3 and 41.7 mN m^{-1}. At higher concentrations, the surfactant tend to keep the average bubble size very small, provoking formation of a fine foam and liquid overflow, in these conditions, the correct measurement of k_La is not possible. We can see in the figure (IV-6) that liquid velocities have a slight effect on oxygen transfer.

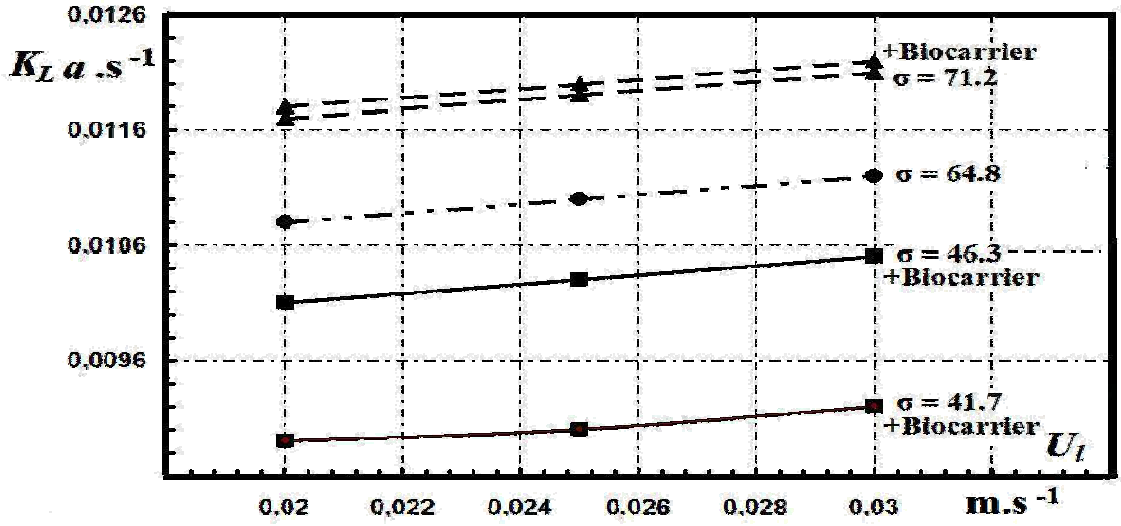


Fig (IV-6): Effect of the culture media containing different concentrations of surfactin on (k_La). Experiments were carried out at different (U_l) 0.02, 0.025 and 0.03 m s^{-1} and one Ug (0.006 m s^{-1}); SD% (k_La ± 4.28: 7.37%).

Evaluation of k_La was also performed in these different samples at three different gas velocities. Whatever the tested samples, increasing gas velocities lead to the increase of k_La values; this increasing is however reduced at low surface tension between 0.006 and 0.009 m s^{-1}. This can be attributed to the existence of surface tension gradients tending to immobilize the bubble-liquid interface and therefore limiting the mass transfer.

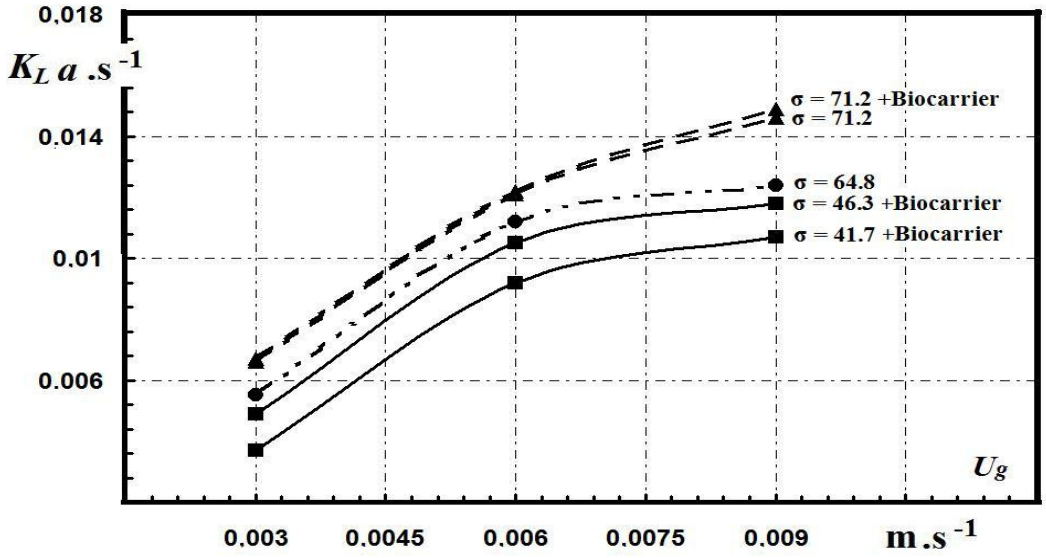


Fig (IV-7): Effect of the culture media containing different concentrations of surfactin on (k_La). Experiments were carried out at different (U_g) (0.003. 0.006 and 0.009 m.s^{-1}) and one U_l (0.03 m S^{-1}); SD% (k_La ± 4.28: 7.37%)

A significant decrease of K_La values was also observed (figure (IV-7)) with decreasing surface tension whatever the gas or liquid velocities while the presence of solid carriers had no significant effect on k_La values. These results agreed with those obtained by Dimitrov (2007) in the model conditions.

IV.3.1.6. Development of an empirical equation for k_La estimation

Sanchez *et al*. (2005) have studied the gas-liquid mass transfer in a laboratory scale inverse turbulent bed reactor and have proposed an equation considering that the volumetric mass transfer coefficient k_La is a function only of air velocity. The results given above, show that the oxygen mass transfer in three-phase fluidisation depends on various parameters.

The aeration efficiency of aerated reactors with respect to Kolmogorov theory is usually reported in terms of power input per unit volume of liquid (Bang *et al*., 1998):

$$P_G/V_L = U_g\, g\, \rho_l \quad \text{inW.m}^{-3} \qquad \text{(IV.3)}$$

where P_G, in Watt is the power input due to aeration, V_L (m^3) the culture volume, g (m. s^{-2}) the gravitational acceleration (gravity), U_g (m. s^{-1}) is the gas velocity and ρ_l (kg m^{-3}) the liquid density.

On the other hand, there are two sources of gassed power delivered to a liquid reactor volume: (1) isothermal expansion of gas as it moves up the vessel and (2) the kinetic energy (ε) of gas imported to the gas-liquid dispersion at the point of gas injection.

When the two sources of gassed power are considered separately; the contribution of (ε) to the total power input is shown obviously to be negligible.

Thus, the specific power input due to the aeration could be calculated using the above equation.

In the TPIFBR, there are two inputs of the power delivered to a reactor: the specific power input due to aeration (P_G/V_L) and the mechanical power input due to the pump (P_M). The mechanical energy provided to the fluid in closed loop is hydraulic power communicated to the liquid of its passage through the pump.

The mechanical power is given by the following formula:

$$P_M = Q\, H_f\, 9810 \qquad \text{(IV.4)}$$

with, P_M (in Watt), the power transmitted to the fluid by the pump, (Q) the flow in $m^3.s^{-1}$ and (H_f) the energy or pressure loss of the hydraulic network expressed (in meter).

$$P/V_L = (P_G + P_M) / V_L \qquad \text{(IV.5)}$$

The influence of the surface tension can be taken into account using a dimensionless group (σ/σ_w). At the same time, during its movement through the liquid - solid pseudo-fluid a bubble can deform and the deformation is opposed to the surface tension.

According to the observed phenomena not only the interface can deform, but it also moves along with the surrounding liquid.

This interfacial mobility, very important for the mass transfer, appears to be opposed by two phenomena: dissipation in the bubble and occurrence of surface tension gradients tending to immobilize the interface. To take into account the effects during the bubble motion in the liquid the Morton number *Mo* can be introduced:

$$Mo = \frac{g(\rho_l - \rho_s)\mu_l^4}{\rho_l^2 \sigma^3} \qquad \text{(IV.6)}$$

The analysis leads to the following dimensionless grouping and variables:

$$k_l a = f\left[\left(\frac{P}{V_{reac}}\right), \frac{\Delta\rho}{\rho}, \mathrm{Re}, \frac{\sigma}{\sigma_w}, Mo\right] \qquad \text{(IV.7)}$$

After evaluating the coefficients by trial and error, the final form of the equation has been proposed by Dimitrov *et al*. (2007):

$$k_l a = 0.0039 + 0.076 \times \lg\left[\left(\frac{P}{V_{reac}}\right)\mathrm{Re}^{0.4}\left(\frac{\Delta\rho}{\rho}\right)^{1.9}\left(\frac{\sigma}{\sigma_w}\right)^{1.75} Mo^{0.2}\right] \qquad \text{(IV.8)}$$

In figure (IV-8) experimental and calculated volumetric coefficients of 100 data sets were compared.

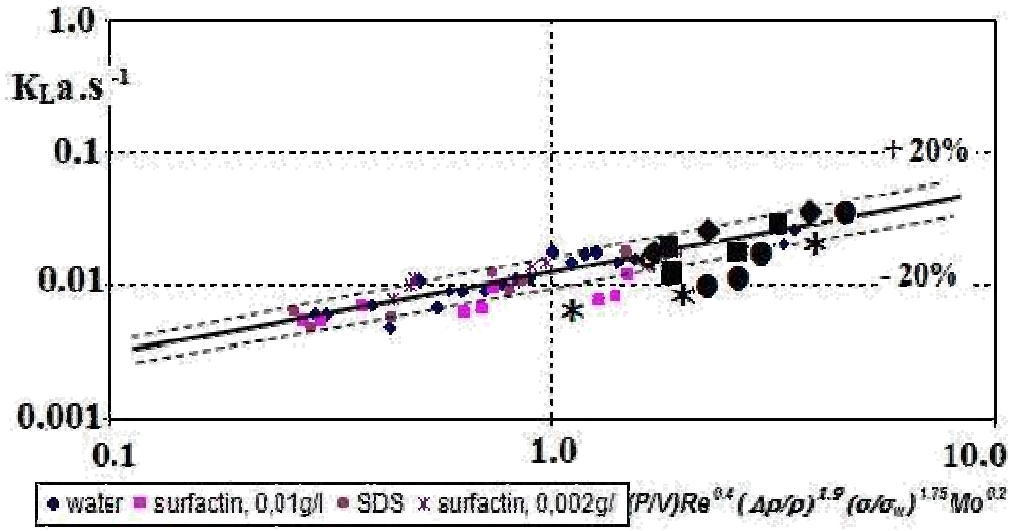


Fig (IV-8): Comparison between the experimental results and calculated k_La
(◆ σ = 71.2; ■ σ = 64.2; ● σ = 46.3; ✱ σ = 41.7) SD% (k_La ± 4.28: 7.37%)

On the graph, we have introduced our experimental values in bold. We can see that these values reasonably fit with the model previously proposed in which k_La is depending of the Morton number, surface tension, densities differences and total volume mean power dissipation.

IV.3.2. Influence of oxygen and energy supply on lipopeptide production in inverse fluidised bed bioreactor

Two major factors particularly spoil the surfactant producing process in TPIFB: (1) Stable foaming evolution causes liquid outflow and changes substrate concentration and mixing intensity, (2) Regulation of the oxygen transfer rate, related to power dissipation and hydrodynamic behaviour. Conforming to the above aspects, the aim of this paragraph is to investigate the oxygen supply and foaming control through modifications of energy supply and structure of a three-phase inverse fluidised bed.

Two kinds of oxygen supply were applied to improve cell density in the bioreactor: a "**bubbling**" regime and an "**alternate**" regime.

In bubble mode only periodically gas stream was applied from the bottom. While, in alternate regime; two gas inputs were used, bubbling in the bottom of the reactor and continuous

distribution of a gas stream in contact with the liquid film formed on the wall in the top section.

It is well known, that this thin liquid film increases the air-liquid mass transfer. In order to avoid a potential oxygen limitation the bubbling regime has been applied periodically in parallel with the continuous "film" transfer regime.

However, the temperature was maintained constant during fermentation at 25 or 30 °C.

IV.3.2.1. Influence of power dissipation and oxygen transfer rate: lipopeptide production in flasks

As shown in figure (IV- 9A), the results in shake flasks obtain a positive correlation between the increasing on volume mean power dissipation and the maximum surfactin concentrations values for all flask volumes. On the contrary, in function of flask volume, the increased values of power dissipation (Fig. IV- 9B) decrease or eliminate the fengycin production.

More precisely, in the first phase of figure (IV- 9B), low power dissipation had a positive effect on fengycin production. For higher power dissipations, a rapid decrease in fengycin concentration was determined, suggesting that the power input might be limiting factor for fengycin selectivity. However this value increased with the total volume of the flasks

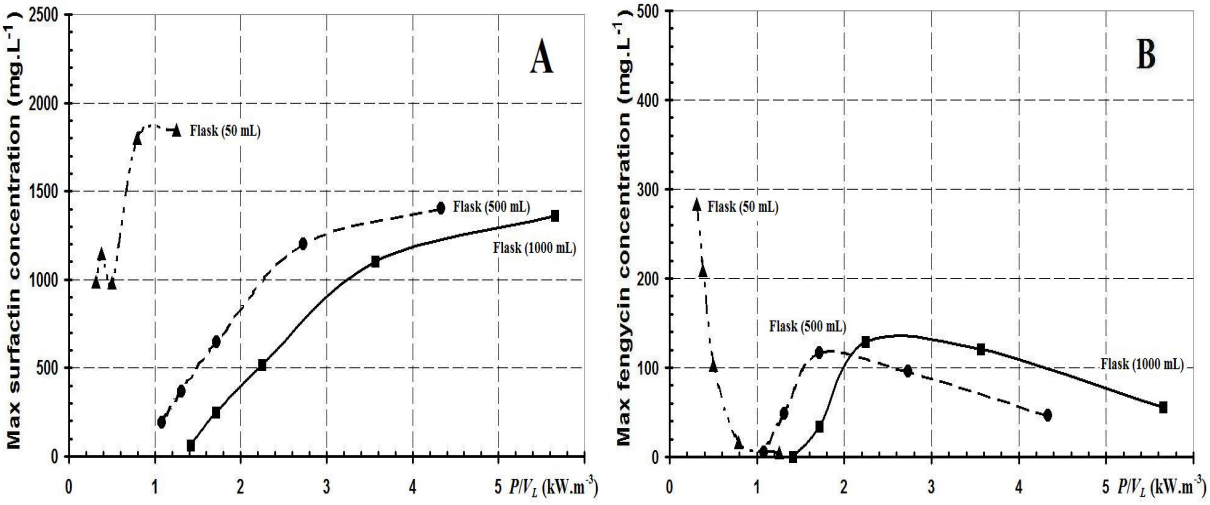


Fig (IV-9): Variation of Power dissipation effect on lipopeptides production. *B. subtilis* BBG 21 was grown in Landy medium; pH 7.0 at 30 C° and 200 rpm with three flasks sizes (50, 500 and 1000 mL); different relative filling volumes were studied (**A**: surf and **B**: feng); SD% (surf ± 3.34: 9.96 and feng ± 2.03: 59.55)

The power dissipation (P) was calculated according to the equation proposed by Büchs *et al*. (2000) using the modified Newton number (Ne_{mod}):

$$P = Ne_{mod}\, \rho\, N^3\, d^4\, V_L^{1/3} \qquad \text{(IV.9)}$$

$$Ne_{mod} = 70\, Re^{-1} + 25\, Re^{-0.6} + 1.5\, Re^{-0.2} \qquad \text{(IV.10)}$$

with the Reynolds number: $Re = \rho\, N\, d^2 / \mu$ (IV.11)

where, ($\boldsymbol{\rho}$) (kg.m^{-3}) is the liquid density; ($\boldsymbol{\mu}$) (Pa.s^{-1}) the dynamic viscosity (1 mPa.s^{-1} water values at 30°C); ($\boldsymbol{d}$) (m) the maximum flask diameter ; ($\boldsymbol{N}$) (s^{-1}) the rotational frequency and ($\boldsymbol{V_L}$) (m^3) the liquid working volume.

All these experiments suggest that power dissipation (P/V) could be one of the main parameters responsible for lipopeptide production.

One hypothesis is that the effect of the power dissipation in flasks could be indirect, through a modification of oxygen transfer.

In fact (for constant shaking frequency), low filling volumes form higher mechanical volumetric power, thus resulting in thin liquid film formation on the flask wall. This one modifies the shear rate or shear-thinning behaviour, the liquid-side mass transfer coefficient (k_L) and interfacial area (a).

It was discussed in Chapter III that, an increase in the power dissipation was expected to entail a higher mass transfer (Fahim *et al*., 2011).

In literature, Mehmood *et al*. (2010) suggest that a volume mean power dissipation from 2.3 to 5.6 kW.m^{-3} was necessary to get pristinamycins production by *Streptomyces pristinaespiralis* in those batch cultures performed in Erlenmeyer flasks of 250, 400 and 1000 ml. As for the volume mean power dissipation, the same authors observe an antibiotics production only for the values of k_La superior to 0.028 s^{-1}, whatever the type of flasks used (practically, no production for values inferior to 0.02 s^{-1}).

IV.3.2.2. Surfactant production in IFBR

For instance, the oxygen transfer rate, the power consumption and the mixing time were shown to provide an engineering basis for the scale-up.

Practically, in TIFBR conditions, the oxygen transfer is affected also by many other parameters but the flasks experiments suggest clearly that k_La could be related to power dissipation (P/V). We thus decided to apply different volume mean power dissipation to TPIFB and to check if we observed similar behavior of lipopeptide biosynthesis obtained in flasks. Two types of production experiments: with and without carriers, were performed and realized in two ranges of oxygen transfer conditions.

The selectivity of processes, for each experimental condition, was monitored up to 48 h of fermentation. Consequently, a specific level of liquid velocity was employed: up to 3 cm/s representing the interval of minimal fluidization velocity of carriers used and low frictional force (low shear stress), respectively (Essadki *et al*., 2005).

In order to asses the influence of power dissipation on the onset of surfactant production, surfactin and fengycin productions was determined in two different liquid working volumes in the 5 liter's reactor: 20 and 50 % (1.0 and 2.5 liter of liquid, cycled by the pump 7 (see Fig. IV- 1). *B. subtilis* BBG21 was cultivated in Landy modified media, pH 7.0 and 30°C with a liquid velocity of 0.03 $m.s^{-1}$ and a gas velocity of 0.006 $m.s^{-1}$.

As shown in flask, a high volume mean power dissipation led to a high surfactin production (more than 1 g. L^{-1}) and no fengycin (Fig. IV-10 and see also Table IV-1). Equivalent but small amounts of both lipopeptides were observed in low volume mean power dissipation.

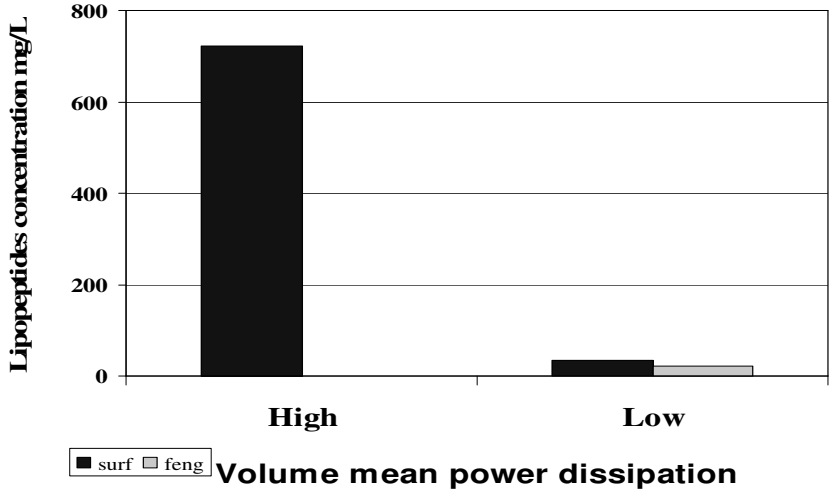


Fig (IV-10): Variation of surfactant production with power dissipation in TPIFBR

In the case of high surfactin production, in "alternate" regime, using the same regime and mechanical power of the pump 7 (see Fig. IV- 1), the 2.5 time less liquid working volume was recycled with greatest frequency. In result, the interfacial "film" contact in the gas-liquid top section was renovated more frequently which increase k_La.

In conclusion, when both k_La and mean power dissipation values were increased, the surfactin production increases dramatically.

In fact, in "film" or alternate regime the k_La values appeared to be higher more 0.05 s^{-1}. However, in the same conditions (Fig. IV- 11), for high volume mean power dissipation, the presence of modified solid carriers improves the surfactin concentration with little effect in fengycin one.

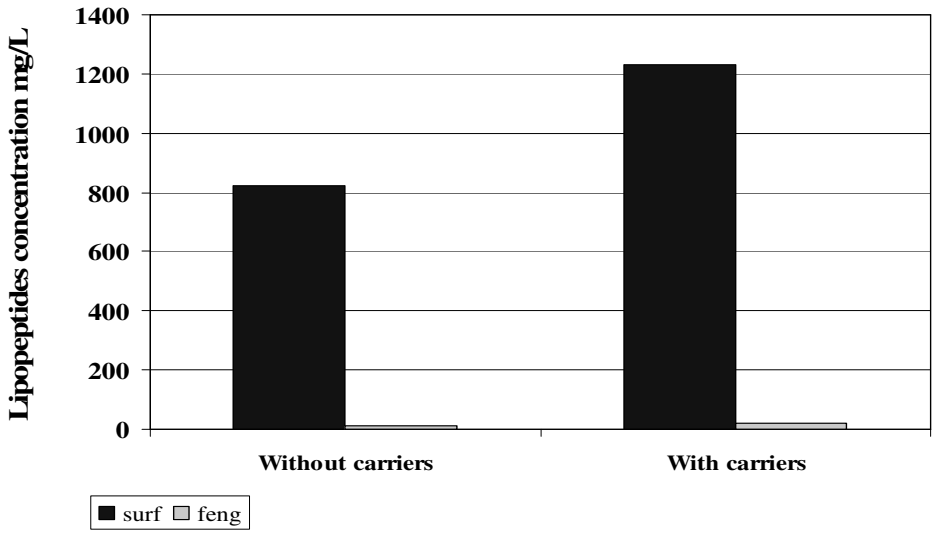


Fig (IV-11): Influence of solid carriers and immobilized cells

In addition, the results obtained (see Table IV-1) suggest that by using immobilized cells the selectivity of the bioreaction concerning the fengycin or surfactin production could be modified.

Table (IV-1): Lipopeptide selectivity conditions (*B. subtilis* growth at 30 °C, Landy modified medium, pH 7.00; U_l = 0.03 m.s^{-1} and U_g = 0.006 m.s^{-1}); with and without carrier
SD% (surf ± 5.11: 41.59% and feng±37.22: 42.76%)

Time/ h	Volume V_L/V_t	Without carriers						With carriers (PP_{ch})					
		OD 600 nm	pH	DO mg.L^{-1}	σ mN.m^{-1}	Surf mg.L^{-1}	Feng mg.L^{-1}	OD 600 nm	pH	DO mg.L^{-1}	σ mN m^{-1}	Surf mg.L^{-1}	Feng mg.L^{-1}
0	0.5	0.10	7.00	7.49	71.2	0	0	0.10	7.00	7.48	71.2	0	0
4	0.5	0.58	7.00	7.44	64.8	3	0	1.36	7.00	7.10	41.7	6	13
8	0.5	3.32	6.91	5.62	36.8	5	18	4.96	6.86	4.92	36.2	11	47
24	0.5	9.10	5.80	0.74	35.4	**34**	**21**	ND	ND	ND	ND	ND	ND
0	0.2	0.10	7.00	7.49	71.2	0	0	0.10	7.00	7.48	71.2	0	0
4	0.2	0.88	6.97	7.29	64.2	7	0	1.84	6.97	6.81	39.2	24	7
8	0.2	8.80	6.80	4.73	36.3	22	2	9.76	6.76	3.25	34.7	67	16
24	0.2	9.60	7.61	0.11	33.3	**723**	**1**	9.48	7.72	0.34	33.1	919	15
48	0.2	10.86	7.60	2.51	33.1	824	11	10.06	7.53	1.09	32.9	1232	19

The Table (IV-1) summarizes some results concerning the surfactant production in the fluidised bed bioreactor at different levels of power input per unit volume of liquid and oxygen transfer. For low k_La values and volume mean power dissipation (liquid working volume fraction of 50%, k_La values inferior to 0.01, s^{-1}) surfactin production was very limited (inferior to 100 mg/l).

Without carriers, only planktonic culture is observed, whereas the carbon (PP) carriers promoted also biofilm formation. When cells were immobilized on (PP) particles with

activated carbon (PP_{ch}), the surfactin and fengycin productions were increased in comparison to the free cells culture.

IV.3.2.3. Foaming control in IFBR

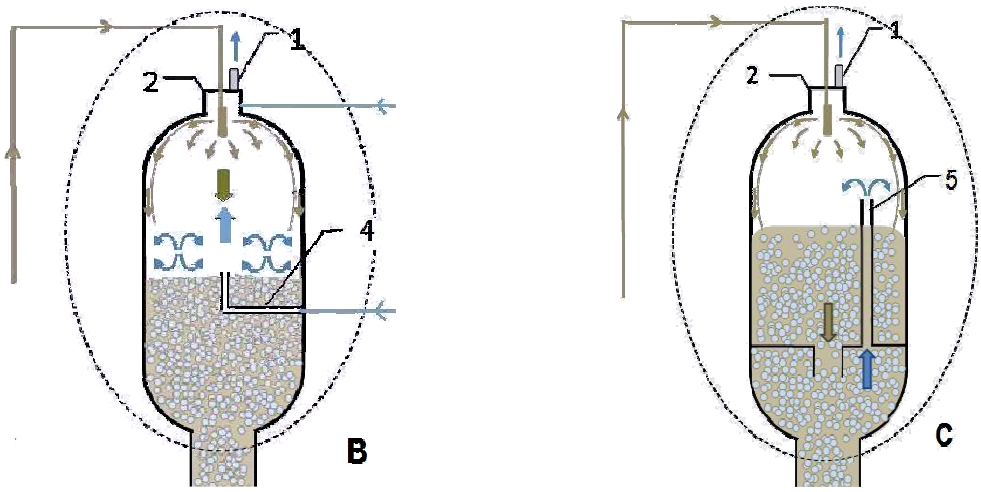
In the IFB fermentor studied, under the aerated conditions, surfactin accumulation generates foam that is apt to flow out of the reactor.

In fact, follows 4-6 hours of fermentation the formed fine foam makes the process difficult to control and decreases the valid reactor volume.

To foaming control of the reactor, three major interventions were adapted to the producing process: (1) modification of the reactor construction by incorporation a calmed gas-liquid section of the top of reactor equipped with a gas inlet and a specific liquid distributor; (2) distribution of all recycled liquid by forming a down stream liquid film at wall of the top section; (3) combination of two types of aeration of culture systems. In particular, in the top section it was distributed the liquid as a film to increase the shear rate and air-liquid mass transfer and also for foam regulation (no contact between the wall and foam flow). Practically, only an interfacial contact between opposite liquid and foam flow was adapted.

The proposed procedure carried out to continuous steady oxygen flux from the continuous gas phase to the liquid film formed on the wall of calmed section with stable control of the foaming and high reactor performances.

The modifications of the top section were presented in figure (IV-12). The left design of the top section maximizes surfactin production and increases the $\boldsymbol{R}_{surf/feng}$ (filling volumes not less than 20% , highest K_La). The second design accelerates the fengycine production and decreases the $\boldsymbol{R}_{surf/feng}$ (filling volume no more than 50%, lowest K_La).



Design suitable for surfactin production

Design suitable for fengycin production

Fig (IV-12): Modification of the top-section of the reactor: producing regimes and foam controlling

IV.4. Conclusions

A lipopeptide production based on "alternate regime" of oxygen transfer in IFB and a new design for long-term foam control has been developed:

-The procedure proposed leads to a steady oxygen flux from the gas phase to the liquid with stable control of the foaming and high reactor performance (biosurfactant concentrations up to 1.2 g. L^{-1}). In particular, following the end of initial fast cells development in bubbling regime (after 4 hours) the continuous oxygen transfer from injected bubbles train (stream) at liquid-solid bed bottom is replaced by continuous oxygen transfer from a gaseous phase to liquid film formed at the wall in the top section of the reactor accompanied periodically with bubble injection in the bottom,

-The power dissipation and oxygen supply controls the onset, productivity and selectivity of produced surfactants.

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CHAPTER V

Discussion and perspectives

The actual level of knowledge of the biosynthesis of lipopeptides from *Bacillus subtilis* such as surfactin and fengycin and its regulation mechanism allows developing different techniques to overproduce the main active compounds and to reach yields that are compatible with industrial development of these bioactive surfactants (Jacques, 2011).

The first aim of this study was to investigate the lipopeptide (surfactin and fengycin) production capacity of surface aerated culture in shake flasks with immobilised and planktonic cells of *B. subtilis* BBG21 in order to establish the relationship between the power dissipation and k_La and to analyse the selectivity during the production process in function of several environmental parameters.

V. 1. The parameters which influence lipopopetide bioproduction

Lipopeptide production was thus studied in the following different conditions:

- different type of shake flasks (Pallone, cylinder, Erlenmeyer),
- different culture medium volumes (50 to 1000 ml),
- various relative filling volumes (0.05 to 0.4),
- different agitation rates (150 to 250 rpm),
- presence or not of solid particles
- various temperatures (20 to 40 °C)
- different initial pH (6.8 to 7.2)

Kinetics of growth, pH evolution and lipopeptide production were established in these different conditions. A comparison between the behaviour of *B. subtilis* ATCC21332 and its spontaneous derivative BBG21 known to produce higher concentrations of lipopeptides especially fengycin was also done.

V.1.1. Effect of power dissipation and oxygen transfer rate

All the obtained results suggest that the volume mean power dissipation (P/V) could be one of the main parameters which influence lipopeptide production.

One hypothesis was that the effect of the power dissipation in flasks and reactor could be indirect through a modification of oxygen transfer.

The structure of the free surface and thus the interfacial area was expected to change dramatically with filling volume or, for constant shaking frequency with power dissipation. This one modifies both the liquid-side mass transfer coefficient (k_L) and interfacial area (a).

The experiments obtained in different working volumes of flask confirm clearly that power dissipation changes via interfacial gas-liquid contact surface the surfactant production.

From the collected data on the surfactin and fengycin concentrations three zones of conditions for lipopeptide production could be established.

On one hand, when both mechanical power per unit working volume and gas-liquid mass transfer coefficient k_La values remained very high (more 0.07 s^{-1}…) only surfactins were produced.

On the other hand, when both k_La (less to 0.003 s^{-1}…) and volume mean power dissipation values reminded very low surfactins, as well as fengycins, were not produced.

A mixed production was observed when low values of k_La were compensated by a relatively high value of power dissipation and *vice versa*.

Three different regimes covering the range of tested oxygen transfer conditions and leading to three different patterns of lipopeptide produced can be thus defined:

Regime 1:

Culture performed in flask size of 50 mL with relative filling volume of 0.05 mL/mL flask and an agitation rate of 250 r.p.m. In these conditions, k_La is higher than 0.06 s^{-1} and the ratio between the surfactin and fengycin concentrations ($R_{surf/feng}$) reached 50.

Regime 2:

Culture performed in flask size of 50 mL with a relative filling volume of 0.2 mL/mL flask and an agitation rate of 150 r.p.m. In these conditions k_La is less than 0.003 s^{-1} and ($R_{surf/feng}$) is about 5.

Regime 3:

Culture performed in flask size of 1000 mL with a relative filling volume of 0.4 mL/mL flask and an agitation rate of 250 r.p.m. In these conditions k_La is about 0.015 s^{-1} and ($R_{surf/feng}$) is about 0.5.

V. 1.2. Effect of cultivation conditions

The effect of four different cultivations parameters was tested: cell immobilization on solid carrier, partial replacement of glutamic acid by ammonium sulphate, temperature and initial pH. The choice of these parameters has been determined by previous results obtained in literature and in ProBioGEM laboratory and showing an influence of these parameters on lipopeptide production.

These experiments were carried out in different oxygen transfer conditions. Cell immobilization on solid carrier and used of ammonium sulphate appeared favorable in most of the oxygen transfer conditions to the production of surfactin and fengycin, this set of results confirmed the different regimes previously described.

On continuing investigation and regime optimization, we can say that the optimum pH values and incubated temperature were, respectively, 7.0 and 30 °C in all the production regimes but increasing the temperature to 35 °C in the first regime and reducing it to 20 °C in the second and third regime are very preferable.

The comparison of the behavior of *B. subtilis* BBG 21 and the original wild type *B. subtilis* ATCC 21332 showed that there was a big difference between the two strains in the maximum lipopeptides yield. The kinetic and production profiles of the two *B. subtilis* strains were observed in the different production regimes.

The two strains showed positive correlation between the microbial growth and increasing of (OTR) in the different regimes.

Reduced pH values were observed with the decreasing of (OTR) in these regimes and the pH values were more reduced for the BBG strain comparing to the strain ATCC, these results confirm that strain BBG21 is probably modified in a pleitropic regulator gene which is directly or indirectly involved in lipopeptide regulation biosynthesis.

V. 2. Bioreactor appropriation and oxygenation supply

In the second part of the thesis, we focused on reaction parameters affecting the volumetric oxygen transfer coefficient in the inverse fluidized bed (IFB).

We first modelized in water the effect of the gas and liquid superficial velocities, the presence of carriers, the surface carrier coating and the surface tension on the k_La.

A correlation was observed between volumetric oxygen transfer coefficient and the gas superficial velocity, the presence of modified carriers and the surface tension of the culture medium, these results were confirmed when tests are performed in culture media instead of water. This work led to the redetermination of the correlation obtained by Dimitrov (2007):

$$k_l a = 0.0039 + 0.076 \times \lg\left[\left(\frac{P}{V_{reac}}\right)\mathrm{Re}^{0.4}\left(\frac{\Delta\rho}{\rho}\right)^{1.9}\left(\frac{\sigma}{\sigma_w}\right)^{1.75} Mo^{0.2}\right] \qquad \text{(V.1)}$$

To foaming control of the reactor, three major interventions were adapted to the producing process: (1) modification of the reactor construction by incorporation a calmed gas-liquid section of the top of reactor equipped with a gas inlet and a specific liquid distributor; (2) distribution of all recycled liquid by forming a down stream liquid film at wall of the top section; (3) combination of two types of aeration of culture systems. The proposed procedure leads to sufficient oxygen transfer and foaming control.

V. 3. Production and proceed economization

Using the procedure previously described to control foaming; four different experiments were performed in the TPIFB bioreactor.

For low $k_L a$ values and mean volume power dissipation, surfactin production was very limited. When cells were immobilized on (PPch) carriers, the surfactin and fengycin productions were increased in comparison to the free cells culture.

On the other hand, at conditions of higher power, surfactin production was drastically promoted. Obviously, the conditions used in present study in TPIFB bioreactor were much more favorable for surfactin than for fengycin production.

Generally, a lipopeptide production based on “alternate regime” of oxygen transfer in TPIFB bioreactor and a new design bioreactor for long-term foam control has been developed.

The procedure proposed leads to a steady oxygen flux from the gas phase to the liquid with stable control of the foaming and high reactor performance (lipopeptide concentrations up to 1.2 g L^{-1}).

V. 4. Perspectives

The results obtained in this thesis open the opportunity of several perspectives at cellular or bioprocess level. The different behaviors observed between strain *Bacillus subtilis* ATCC21332 and its derivatives BBG21 should be analysed at molecular level to understand the genetic modifications and their role in the lipopeptide production. In addition the differences observed between the production of surfactin and fengycin probably mean that these compounds are differently regulated into the cells. An analysis of these modes of regulation should be carried out in the future.

The original bioprocess set up in this study should be tested with strains overptroducing one family of lipopeptides. In addition, continuous process should be developed.

To end with an optimization of the support for cell immobilization could be considered.

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Appendixes

Appendix 1

Results valorization

The results of this study led to the participation in several national and international congresses as following:

Participation in national and international congresses with poster presentations

Fahim S, Montastruc L, Gancel F, Dimitrov K and Nikov I. Development of Inverse Fluidized Bioreactor for Surfactant Production: Foam Controlling. 2009. XII^{eme} congrès de Société Française de Génie des Procédés (SFGP). Marseille, France.

Fahim S, Gancel F, Dimitrov K, Nikov I and Jacques P. Influence of environmental factors on lipopeptide production by a spontaneous mutant strain of *Bacillus subtilis* ATCC21332. 2009. 2eme Congrès du Génie des Procédés appliqué aux bio-industries (GEPROC). Gembloux, Belgium.

Nikov I, **Fahim S**, Dimitrov K, Gancel F and Jacques P. 2010. Volumetric Oxygen Transfer Coefficient and Foaming control in Inverse Fluidized Bed Recycle reactor for biosurfactant production. 8th Conference of European Symposium on Biochemical Engineering Science (ESBES). Bologne, Italy.

Fahim S, Gancel F, Dimitrov K, Jacques P and Nikov I. Optimization of lipopeptide production bioprocess in modified inverse fluidized bed bioreactor. 2011. XIIIeme congrès de Société Française de Génie des Procédés (SFGP). Lille, France.

Participation in international congresses with oral presentations

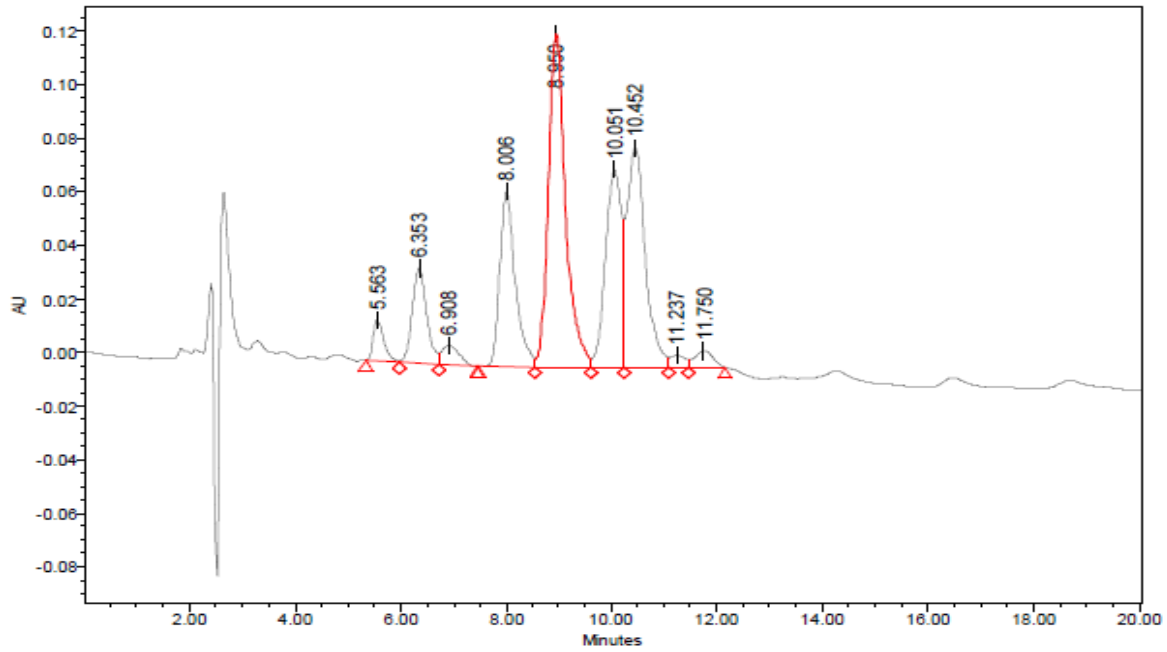
Fahim S, Dimitrov K, Gancel F, Nikov I and Jacques P. 2010. Influence of environmental factors on lipopeptide production in three phase inverse fluidized bed bioreactor. 1st Conference of Bio-Processing and Application of Microbial Biotechnology in Agriculture, N.R.C, Cairo, Egypt.

Fahim S, Dimitrov K, Gancel F, Nikov I and Jacques P. 2010. Volumetric Oxygen Transfer Coefficient and Foaming control in Inverse Fluidized Bed Recycle reactor for biosurfactant production. 1st Conference Bio-Processing and Application of Microbial Biotechnology in Agriculture, N.R.C, Cairo, Egypt.

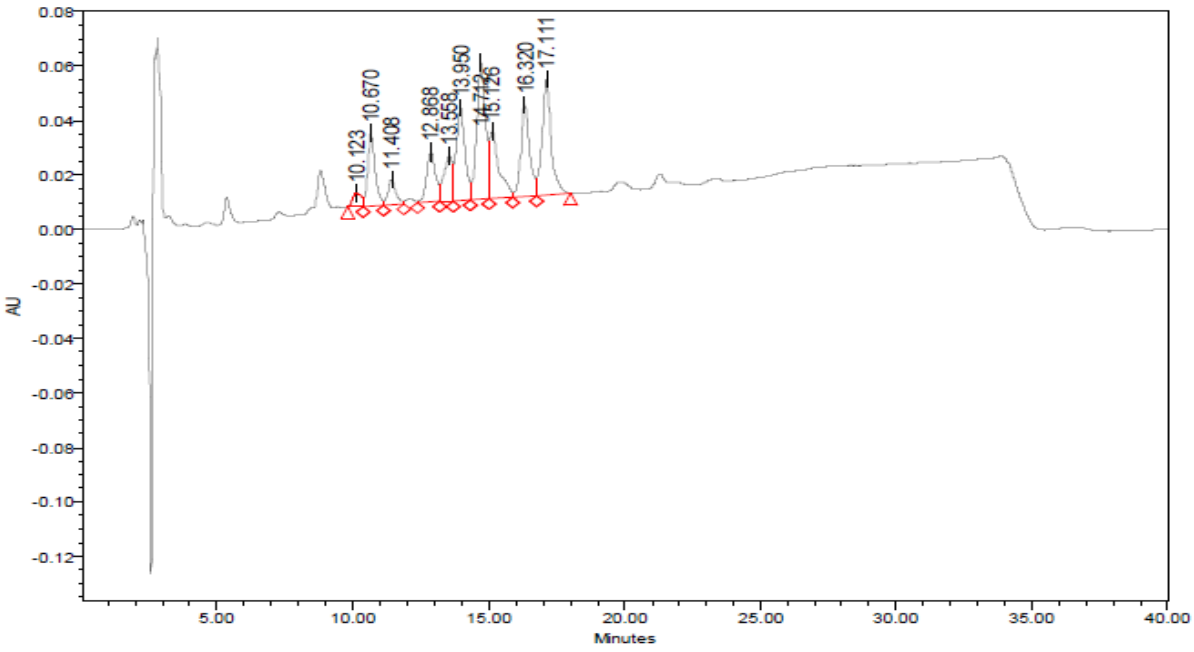
Appendix 2

Standards

1. Surfactin standard spectrum



2. Fengycin standard spectrum



AU: Arbitrary unit

Appendix 3

Values and concentrations

P/V_L kw.m^{-3}	k_La s^{-1}	r.p.m	Flask size mL	Rv mL/mL flask	Surf mg. L^{-1} ± SD%		Feng mg. L^{-1} ± SD%	
0.214351	0.04224	150	50	0.05	1472	± 6.82%	46	± 15.37%
1.283012	0.04534	150	100	0.05	1289	± 9.55%	61	± 9.27%
1.188244	0.02738	150	250	0.05	1102	± 9.37%	82	± 14.69%
1.827506	0.02364	150	500	0.05	976	± 8.98%	94	± 10.53%
2.387251	0.01888	150	1000	0.05	918	± 9.71%	106	± 6.67%
0.333979	0.02376	150	50	0.1	1298	± 6.86%	52	± 10.88%
0.808248	0.0255	150	100	0.1	1043	± 8.54%	102	± 9.71%
0.748547	0.0154	150	250	0.1	895	± 9.96%	119	± 5.94%
1.151259	0.0133	150	500	0.1	783	± 9.57%	134	± 7.39%
1.503872	0.01062	150	1000	0.1	721	± 8.43%	146	± 5.85%
0.210394	0.01337	150	50	0.2	758	± 8.77%	96	± 13.26%
0.509164	0.01435	150	100	0.2	684	± 9.72%	108	± 7.86%
0.471556	0.00867	150	250	0.2	553	± 6.91%	137	± 8.26%
0.725247	0.00748	150	500	0.2	417	± 7.46%	157	± 5.41%
0.947381	0.00597	150	1000	0.2	302	± 3.28%	168	± 5.90%
0.160561	0.00955	150	50	0.3	637	± 9.77%	148	± 3.82%
0.388566	0.01025	150	100	0.3	441	± 5.45%	120	± 9.43%
0.359864	0.00619	150	250	0.3	367	± 5.40%	30	± 28.28%
0.553468	0.00534	150	500	0.3	207	± 6.15%	30	± 33.00%
0.722987	0.00427	150	1000	0.3	55	± 7.71%	16	± 17.68%
0.13254	0.00752	150	50	0.4	403	± 6.67%	170	± 2.50%
0.320754	0.00807	150	100	0.4	ND	ND	ND	ND
0.297061	0.00487	150	250	0.4	ND	ND	ND	ND
0.456877	0.00421	150	500	0.4	73	± 7.75%	0	± 0.00%
0.596813	0.00336	150	1000	0.4	0	± 0.00%	0	± 0.00%
1.256428	0.05897	200	50	0.05	1848	± 4.06%	5	± 28.28%
3.040635	0.0633	200	100	0.05	1705	± 4.40%	21	± 47.14%
2.816044	0.03823	200	250	0.05	1527	± 4.91%	38	± 37.22%
4.331044	0.03301	200	500	0.05	1401	± 5.35%	46	± 33.82%

5.657595	0.02635	200	1000	0.05	1360	± 5.51%	56	± 20.20%
P/V_L kw.m^{-3}	k_La s^{-1}	**r.p.m**	**Flask size** mL	***Rv*** mL/mL flask	**Surf** mg. L^{-1} ± **SD%**		**Feng** mg. L^{-1} ± **SD%**	
0.791502	0.03317	200	50	0.1	1803	± 3.37%	16	± 17.68%
1.915483	0.03561	200	100	0.1	1510	± 4.96%	27	± 41.90%
1.773995	0.02151	200	250	0.1	1309	± 6.70%	59	± 19.18%
2.728393	0.01857	200	500	0.1	1200	± 7.31%	96	± 7.37%
3.564056	0.01482	200	1000	0.1	1100	± 7.97%	121	± 7.01%
0.498616	0.01866	200	50	0.2	985	± 8.90%	103	± 9.61%
1.206678	0.02003	200	100	0.2	886	± 9.90%	107	± 10.57%
1.11755	0.0121	200	250	0.2	763	± 7.79%	111	± 8.92%
1.718777	0.01045	200	500	0.2	647	± 9.18%	116	± 7.32%
2.245217	0.00834	200	1000	0.2	520	± 8.70%	129	± 7.67%
0.380516	0.01333	200	50	0.3	1151	± 7.62%	209	± 2.03%
0.920869	0.01431	200	100	0.3	ND	ND	190	± 2.23%
0.85285	0.00864	200	250	0.3	ND	ND	180	± 4.71%
1.311674	0.00746	200	500	0.3	365	± 6.20%	48	± 29.46%
1.71342	0.00596	200	1000	0.3	250	± 4.53%	34	± 41.60%
0.314108	0.0105	200	50	0.4	987	± 8.88%	284	± 1.99%
0.760161	0.01127	200	100	0.4	ND	ND	ND	ND
0.704011	0.00681	200	250	0.4	ND	ND	ND	ND
1.082763	0.00588	200	500	0.4	192	± 8.10%	5	± 56.57%
1.414399	0.00469	200	1000	0.4	64	± 3.34%	0	± 0.00%
2.454496	0.08532	250	50	0.05	1971	± 4.81%	3	± 47.14%
5.940037	0.09157	250	100	0.05	1842	± 5.14%	14	± 30.31%
5.501287	0.05531	250	250	0.05	1693	± 5.60%	25	± 39.60%
8.460915	0.04775	250	500	0.05	1527	± 6.21%	32	± 48.61%
11.0524	0.03812	250	1000	0.05	1483	± 6.39%	37	± 49.69%
1.54624	0.04799	250	50	0.1	1949	± 4.86%	9	± 47.14%
3.741994	0.05151	250	100	0.1	1697	± 5.58%	19	± 59.55%
3.465591	0.03111	250	250	0.1	1486	± 6.38%	51	± 33.28%
5.330055	0.02686	250	500	0.1	1309	± 7.24%	99	± 12.86%
6.962566	0.02145	250	1000	0.1	1221	± 7.76%	103	± 9.61%

0.974072	0.027	250	50	0.2	1305	± 7.26%	107	± 7.93%
P/V_L kw.m^{-3}	***k_La*** s^{-1}	**r.p.m**	**Flask size** mL	***Rv*** mL/mL flask	**Surf** mg. L^{-1} ± **SD%**		**Feng** mg. L^{-1} ± **SD%**	
2.357307	0.02898	250	100	0.2	1223	± 8.67%	113	± 8.76%
2.18319	0.0175	250	250	0.2	1020	± 7.63%	109	± 7.79%
3.357719	0.01511	250	500	0.2	809	± 9.62%	117	± 10.88%
4.386146	0.01206	250	1000	0.2	697	± 9.13%	123	± 6.90%
0.743357	0.01928	250	50	0.3	1253	± 8.47%	227	± 4.98%
1.798965	0.0207	250	100	0.3	ND	ND	200	± 3.54%
1.666085	0.0125	250	250	0.3	ND	ND	100	± 12.73%
2.562423	0.01079	250	500	0.3	583	± 4.61%	124	± 7.98%
3.347254	0.00862	250	1000	0.3	481	± 7.35%	123	± 9.20%
0.613626	0.01519	250	50	0.4	1072	± 9.89%	286	± 2.47%
1.485014	0.0163	250	100	0.4	ND	ND	ND	ND
1.375322	0.00985	250	250	0.4	ND	ND	ND	ND
2.115232	0.0085	250	500	0.4	421	± 8.40%	128	± 9.94%
2.7631	0.00679	250	1000	0.4	276	± 9.74%	123	± 11.50%

Appendix 4

k_La calculation

Calculation of k_La in shaken flasks.

$k_La = 6.67 \times 10^{-6} \times N^{1.16} \times V_L^{-0.83} \times d_0^{0.38} \times d^{1.92}$
In all cases $d_0 = 5$ cm
$d_0^{0.38} = 1.8433534$
$k_La = 12.295167 \times 10^{-6} \times N^{1.16} \times V_L^{-0.83} \times d^{1.92}$
Different flask size (50, 100, 250, 500 and 1000 mL)
With different diameters (5, 7, 8, 10 and 12 cm) frequently

Case 1: 150 r.p.m
N = 150 r.p.m
$N^{1.16} = 334.39969$
$k_La = 4111.5 \times 10^{-6} \times V_L^{-0.83} \times d^{1.92}$

d = 5 cm
$d^{1.92} = 21.979733$
$k_La = 0.0903696 \times V_L^{-0.83}$
d = 7 cm
$d^{1.92} = 41.936119$
$k_La = 0.1724203 \times V_L^{-0.83}$
d = 8 cm
$d^{1.92} = 54.1917$
$k_La = 0.2228091 \times V_L^{-0.83}$
d = 10 cm
$d^{1.92} = 83.176377$
$k_La = 0.3419796 \times V_L^{-0.83}$
d = 12 cm
$d^{1.92} = 118.03967$
$k_La = 0.4853201 \times V_L^{-0.83}$

Case 2: 200 r.p.m
N = 200 r.p.m
$N^{1.16} = 466.86874$
$k_La = 5740.2291 \times 10^{-6} \times V_L^{-0.83} \times d^{1.92}$

d = 5 cm
$d^{1.92} = 21.979733$
$k_La = 0.1261687 \times V_L^{-0.83}$
d = 7 cm
$d^{1.92} = 41.936119$
$k_La = 0.2407229 \times V_L^{-0.83}$
d = 8 cm
$d^{1.92} = 54.1917$
$k_La = 0.3110727 \times V_L^{-0.83}$
d = 10 cm
$d^{1.92} = 83.176377$
$k_La = 0.4774514 \times V_L^{-0.83}$
d = 12 cm
$d^{1.92} = 118.03967$
$k_La = 0.6775747 \times V_L^{-0.83}$

Case 3: 250 r.p.m

N = 250 r.p.m

$N^{1.16}$ = 675.41268

k_La= 8304.3117 x 10^{-6} x $V_L^{-0.83}$ x $d^{1.92}$

d = 5 cm

$d^{1.92}$ = 21.979733

k_La= 0.1825265 x $V_L^{-0.83}$

d = 7 cm

$d^{1.92}$ = 41.936119

k_La= 0.3482506 x $V_L^{-0.83}$

d = 8 cm

$d^{1.92}$ = 54.1917

k_La= 0.4500247 x $V_L^{-0.83}$

d = 10 cm

$d^{1.92}$ = 83.176377

k_La= 0.690722 x $V_L^{-0.83}$

d = 12 cm

$d^{1.92}$ = 118.03967

k_La= 0.9802382 x $V_L^{-0.83}$

However, $V_L^{-0.83}$ is different with different flask size and diameter.

Flask size & diameter	$V_L^{-0.83}$				
	0.05	**0.1**	**0.2**	**0.3**	**0.4**
5 cm (50 mL)	0.467423	0.262939	0.147911	0.105644	0.083204
7 cm (100 mL)	0.262939	0.147911	0.083204	0.059428	0.046805
8 cm (250 mL)	0.122904	0.069137	0.038892	0.027778	0.021878
10 cm (500 mL)	0.069137	0.038892	0.021878	0.015626	0.012307
12 cm (1000 mL)	0.038892	0.021878	0.012307	0.00879	0.006923

- The k_La values in case 1 it will be:

Flask size & diameter	K_La (s^{-1}) for different $V_L^{-0.83}$				
	0.05	**0.1**	**0.2**	**0.3**	**0.4**
5 cm (50 mL)	0.042241	0.023762	0.013367	0.009547	0.007519
7 cm (100 mL)	0.045336	0.025503	0.014346	0.010247	0.00807
8 cm (250 mL)	0.027384	0.015404	0.008665	0.006189	0.004875
10 cm (500 mL)	0.023643	0.0133	0.007482	0.005344	0.004209
12 cm (1000 mL)	0.018875	0.010618	0.005973	0.004266	0.00336

- The k_La values in case 2 it will be:

Flask size & diameter	K_La (s^{-1}) for different $V_L^{-0.83}$				
	0.05	**0.1**	**0.2**	**0.3**	**0.4**
5 cm (50 mL)	0.058974	0.033175	0.018662	0.013329	0.010498
7 cm (100 mL)	0.063295	0.035606	0.020029	0.014306	0.011267
8 cm (250 mL)	0.038232	0.021507	0.012098	0.008641	0.006806
10 cm (500 mL)	0.033009	0.018569	0.010445	0.007461	0.005876
12 cm (1000 mL)	0.026352	0.014824	0.008339	0.005956	0.004691

- The k_La values in case 3 it will be:

Flask size & diameter	K_La (s^{-1}) for different $V_L^{-0.83}$				
	0.05	**0.1**	**0.2**	**0.3**	**0.4**
5 cm (50 mL)	0.085317	0.047993	0.026998	0.019283	0.015187
7 cm (100 mL)	0.091569	0.05151	0.028976	0.020696	0.0163
8 cm (250 mL)	0.05531	0.031113	0.017502	0.012501	0.009845
10 cm (500 mL)	0.047754	0.026863	0.015111	0.010793	0.008501
12 cm (1000 mL)	0.038123	0.021445	0.012063	0.008616	0.006786

Calculation of k_La in TPIFB bioreactor.

$$\frac{P}{V_l} = \rho_l g U_g \text{ , W.m}^{-3}$$

$\rho_l = 1000$ kg.m^{-3}

$g = 9.82$ m.s^{-2}

With three different U_g (0.003, 0.006 and 0.009 m.s^{-1}) and V_l (0.001 m^3)

For $V_L = 0.001$ m^3

U_g	0.003 m.s^{-1}	0.006 m.s^{-1}	0.009 m.s^{-1}
P_G/V_L W.m^{-3}	29.466 W.m^{-3}	58.932 W.m^{-3}	88.398 W.m^{-3}

$Re = \rho_l U_l D_t / \mu$

$D_t = 0.05$ m

$\nu = \rho_l / \mu = 10^6$

With three different U_l (0.02, 0.025 and 0.03 m.s^{-1})

$\nu = \rho_l / \mu = 10^6$	0.02 m.s^{-1}	0.025 m.s^{-1}	0.03 m.s^{-1}
Re	1250	1375	1500
$Re^{0.4}$	17.328621	18.002013	18.639596

$$\frac{\Delta\rho}{\rho} = (\rho_l - \rho_S)/\rho_l = (1000 - 350)/1000 = 0.65$$

$$\left(\frac{\Delta\rho}{\rho}\right)^{1.9} = 0.4410982$$

$$k_l a = 0.0039 + 0.076 \times \lg\left[\left(\frac{P}{V_{reac}}\right) \mathrm{Re}^{0.4} \left(\frac{\Delta\rho}{\rho}\right)^{1.9} \left(\frac{\sigma}{\sigma_w}\right)^{1.75} Mo^{0.2}\right]$$

Different σ values were used

$\sigma_w = 72.2$ mN. m^{-1}	**72.2**	**71.2**	**64.8**	**46.3**	**41.7**
σ^3	376367.05	360944.13	272097.79	99252.847	72511.713
$\frac{\sigma}{\sigma_w}$	1.0	0.9861495	0.8975069	0.6412742	0.5775623
$\left(\frac{\sigma}{\sigma_w}\right)^{1.7}$	1.0	0.9765685	0.8320784	0.4698664	0.3932946
$Mo = \frac{g(\rho_l - \rho_s)\mu_l^4}{\rho_l^2 \sigma^3}$	0.0884252 x 10^{-6}	0.0896671 x 10^{-6}	0.0985231 x 10^{-6}	0.1378898 x 10^{-6}	0.1531007 x 10^{-6}
$Mo^{0.2}$	0.0388432	0.0389517	0.0396924	0.0424528	0.0433506

Appendix 5

P/V_L calculation

$$Re = \frac{\rho N d^2}{\mu}$$

In all cases

ρ = 1000 kg.m^{-3}

μ = 0.001 Pa.s^{-1}

Re **= 10^6 x N x d^2 ,**

Different agitation rate (150, 200 and 250 r.p.m)

N (min $^{-1}$) = N/60 (s^{-1})

N 150 = 2.50 N^3 = 15.625 s^{-3}

N 200 = 3.33 N^3 = 37.03 s^{-3}

N 250 = 4.17 N^3 = 72.34 s^{-3}

With different flask diameters (5, 7, 8, 10 and 12 cm) frequently

Flask size & diameter	Diameter (d)^2 m^2	*Re* (N) 2.5 s^{-1}	*Re* (N) 3.33 s^{-1}	*Re* (N) 4.17 s^{-1}
5 cm (50 mL)	0.0025	6250	8333.333	10416.67
7 cm (100 mL)	0.0049	12250	16333.33	20416.67
8 cm (250 mL)	0.0064	16000	21333.33	26666.67
10 cm (500 mL)	0.01	25000	33333.33	41666.67
12 cm (1000 mL)	0.0144	36000	48000	60000

$$Ne' = 70Re^{-1} + 25Re^{-0.6} + 1.5Re^{-0.2}$$

Flask size & diameter	Diameter (d)^2 m^2	*Né* (*Re*) 2.5 s^{-1}	*Né* (*Re*) 3.33 s^{-1}	*Né* (*Re*) 4.17 s^{-1}
5 cm (50 mL)	0.0025	0.404	0.366	0.340
7 cm (100 mL)	0.0049	0.322	0.294	0.274
8 cm (250 mL)	0.0064	0.278	0.271	0.253
10 cm (500 mL)	0.01	0.258	0.237	0.223
12 cm (1000 mL)	0.0144	0.232	0.214	0.201

$$Ne' = \frac{P}{\rho N^3 d^4 V_L^{1/3}}$$

So we can calculate the volume mean power dissipation (P in W with V_L in m^3) as:

P = (Né x ρ x N^3 x d^4) x $V_L^{1/3}$

Flask size & diameter	Diameter $(d)^4 \times 10^{-6}$ m^4	$P = Né\rho N^3 d^4 V_L^{1/3}$ (W)		
		(N^3) 15.63 s^{-3}	(N^3) 37.03 s^{-3}	(N^3) 72.34 s^{-3}
5 cm (50 mL)	6.25	0.00053588	0.00314107	0.00613624
7 cm (100 mL)	24.01	0.00641506	0.01520318	0.02970019
8 cm (250 mL)	40.96	0.01485306	0.03520055	0.06876608
10 cm (500 mL)	100.0	0.04568766	0.10827609	0.21152288
12 cm (1000 mL)	207.36	0.11936257	0.28287975	0.55262006
5 cm (50 mL)	6.25	0.00166989	0.00395751	0.0077312
7 cm (100 mL)	24.01	0.00808248	0.01915483	0.03741994
8 cm (250 mL)	40.96	0.01871366	0.04434989	0.08663977
10 cm (500 mL)	100.0	0.05756297	0.13641963	0.26650273
12 cm (1000 mL)	207.36	0.15038719	0.35640561	0.69625661
5 cm (50 mL)	6.25	0.00210394	0.00498616	0.00974072
7 cm (100 mL)	24.01	0.01018328	0.02413356	0.04714614
8 cm (250 mL)	40.96	0.02357779	0.05587748	0.10915952
10 cm (500 mL)	100.0	0.07252469	0.17187771	0.3357719
12 cm (1000 mL)	207.36	0.18947617	0.44904337	0.8772292
5 cm (50 mL)	6.25	0.00240841	0.00570773	0.01115035
7 cm (100 mL)	24.01	0.01165697	0.02762608	0.05396896
8 cm (250 mL)	40.96	0.02698982	0.06396372	0.12495641
10 cm (500 mL)	100.0	0.08302016	0.19675113	0.3843634
12 cm (1000 mL)	207.36	0.21689597	0.51402609	1.00417628
5 cm (50 mL)	6.25	0.00265079	0.00628216	0.01227253
7 cm (100 mL)	24.01	0.01283016	0.03040644	0.05940054
8 cm (250 mL)	40.96	0.02970611	0.07040111	0.13753217
10 cm (500 mL)	100.0	0.09137547	0.21655255	0.42304649
12 cm (1000 mL)	207.36	0.23872514	0.56575949	1.10524012

Flask size & diameter	Diameter $(d)^4 \times 10^{-6}$ m^4	P/V_L (kW. m^{-3})		
		(N^3) 15.63 s^{-3}	(N^3) 37.03 s^{-3}	(N^3) 72.34 s^{-3}
5 cm (50 mL)	**6.25**	0.214351	1.256428	2.454496
7 cm (100 mL)	**24.01**	1.283012	3.040635	5.940037
8 cm (250 mL)	**40.96**	1.188244	2.816044	5.501287
10 cm (500 mL)	**100.0**	1.827506	4.331044	8.460915
12 cm (1000 mL)	**207.36**	2.387251	5.657595	11.0524
5 cm (50 mL)	**6.25**	0.333979	0.791502	1.54624
7 cm (100 mL)	**24.01**	0.808248	1.915483	3.741994
8 cm (250 mL)	**40.96**	0.748547	1.773995	3.465591
10 cm (500 mL)	**100.0**	1.151259	2.728393	5.330055
12 cm (1000 mL)	**207.36**	1.503872	3.564056	6.962566
5 cm (50 mL)	**6.25**	0.210394	0.498616	0.974072
7 cm (100 mL)	**24.01**	0.509164	1.206678	2.357307
8 cm (250 mL)	**40.96**	0.471556	1.11755	2.18319
10 cm (500 mL)	**100.0**	0.725247	1.718777	3.357719
12 cm (1000 mL)	**207.36**	0.947381	2.245217	4.386146
5 cm (50 mL)	**6.25**	0.160561	0.380516	0.743357
7 cm (100 mL)	**24.01**	0.388566	0.920869	1.798965
8 cm (250 mL)	**40.96**	0.359864	0.85285	1.666085
10 cm (500 mL)	**100.0**	0.553468	1.311674	2.562423
12 cm (1000 mL)	**207.36**	0.722987	1.71342	3.347254
5 cm (50 mL)	**6.25**	0.13254	0.314108	0.613626
7 cm (100 mL)	**24.01**	0.320754	0.760161	1.485014
8 cm (250 mL)	**40.96**	0.297061	0.704011	1.375322
10 cm (500 mL)	**100.0**	0.456877	1.082763	2.115232
12 cm (1000 mL)	**207.36**	0.596813	1.414399	2.7631

Appendix 6

Bioreactor photos

Original (TPIFB) bioreactor

Modified (TPIFB) bioreactor