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Production cursors of lipopeptides families by some *Bacillus* spp.

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Abstract: The influence of voulme mean power dissipation (P_{VL}) and volumetric oxygen transfer coefficient (k_La) as cursors on lipopeptides production types and levels were estimated for 8 *Bacillus* strains. The optimal oxygenation conditions for synthesis of lipopeptides families (surfactins, kurstakins, iturns and fengycins) by *Bacillus* strains are different. Surfactins and kurstakins production is clearly favoured at good oxygenation of the cells (k_La) more than 0.06 s^{-1}, while optimal fengycin and iturns production could be obtained at moderate oxygen supply (k_La = 0.01 and 0.015 s^{-1} respectively). The low (P_{VL}) could be sufficient for synthesis of each lipopeptide in small flasks. However, it is difficult to obtain similar productivities in higher volume flasks even at the corresponding higher (P_{VL}). The maximum production of *B. amyloliquefaciens* FZB42 were 294, 62 and 210 $mg.L^{-1}$ surfactin, fengycin and mycosubtilin, respectivelly. *B. amyloliquefaciens* S499 were 872, 103 and 103 $mg.L^{-1}$ surfactin, fengycin and bacillomycin respectivelly. *B. subtilis* ATCC 21332 were 1060 and 226 $mg.L^{-1}$ of surfactin and plipastatin respectivelly. While, *B. subtilis* 168 has no production. The production of *B. licheniformis* ATCC 14580 strain was 358 $mg.L^{-1}$ of lichenysin, and *B. pumilus* was produced 642 $mg.L^{-1}$ of pumilacidin. Also, the strains of *B. thuringiensis kurstaki* and *B. thuringiensis israelienne* NRRL HD-522 were produced 102 and 62 $mg.g^{-1}$ cells respectivelly of kurstakin. Therefore, to scale-up production process from Erlenmeyer flasks to the fermentation on large-scal, this two cursors should be scrutinize (k_La and P_{VL}) as indicators for bioreaction direction.

Key words : Lipopeptides, *Bacillus* spp., oxygen supply (k_La), power dissipation (P_{VL}).

Introduction

The Latest years, different families of nonribosomal lipopeptides were identified in *Bacillus* species (surfactin, iturin and fengycin). Surfactin is considered as one of the most potent biosurfactant and shows antiviral properties. Iturin and fengycin are strong antifungal compounds[1]. Also, surfactin and fengycin are able to the elicitation of induced systemic resistance (ISR) in plants and could be used in a next future as biocontrol agent of plant diseases[2,3]. *Bacillus subtilis* produces a wide range of bioactive molecules among which lipopeptides; surfactin, iturin and fengycin families[3]. A new family of lipopeptide (kurstakin) with antifungal activity produced by *Bacillus thuringiensis*, the kurstakin consided as biomarker of these bacterial species[4]. *Bacillus amyloliquefaciens* isolated from some Thai medical plants showed antimicrobial activity to four pathogenic bacteria and *Bacillus pumilus* MAIIIM4a ginger rhizome endophytic bacterial isolates was shown to have antifungal activity against *Rhizoctonia solani*, *Pythium aphanidermatum* and *Sclerotium rolfsii*[5]. Bacteria belonging mainly to the *Bacillus sp.* and the related genera e.g., *Baciluus cereus*, *Bacillus subtilis* and *Bacillus lincheniformis* have been widely used for the commercial production[6]. Lipopeptides production is strongly

affected by oxygen supply conditions and many studies have reported the critical factors that affect microbial fermentation system include cultivation conditions, particle size, substrate, inoculum concentration and moisture level of the substrate and using immobilized cells[7].

The deficiency in dissolved oxygen in flasks and in fermentors has no adverse effect on fengycin production by *Bacillus subtilis* ATCC 21332 strain[8,9]. Also, a negative effect of oxygen transfer rates in flasks and bioreactor was reported on the iturin production[10,11]. While, very few of publications reported the relation betwen the lipopeptides production and k_La or power dissipation parameters used as critical factors on aerobic fermentation[4,8].

Our latest reports have demonstrated that, the isolated strain of *Bacillus subtilis* 168 and *B. subtilis* ATCC 21332 co-produces of surfactin and plipastatin under several oxygen supply conditions[8]. Also, the studying of bubble-less oxygenation in flasks is a good way to understand the relation between power dissipation, oxygen transfer and lipopeptides biosynthesis to facilitate the scale-up of lipopeptide fermentation process and its selectivity [9]. Such strategy could be very interesting to bioreactor designer and the mathematic model developer to characterize for various fermentation process typical parameters such as: influence of voulme mean power dissipation (P_{VL}) and volumetric oxygen transfer coefficient (k_La) as cursors on microbial cell response[12,13], the information in standard Erlenmeyer flasks could be useful to design a strategy to facilitate working in various bioreactors and improving the studied bioreaction in a large-scale fermentation[14]. Conforming to the above aspects, the aim of this study was to investigate the production capacity of lipopeptides families production and selectivity and the surface aerated planktonic microbial cells of various *Bacillus* strains by measuring the relations between produced lipopeptides families with k_La and P_{VL} cursors of the fermentation process.

Materials and methods

Cultures and production conditions.

The cultures collections of *Bacillus subtilis subsp. subtilis* str 168 and *B. subtilis* ATCC 21332; *Bacillus amyloliquefaciens* FZB42 and *B. amyloliquefaciens* S499; *Bacillus thuringiensis kurstaki* and *B. thuringiensis israelienne* NRRL HD-522; *Bacillus licheniformis* ATCC 14580 in addition to *Bacillus pumilus* were used[15]. Cultures were performed in standard Erlenmeyer flasks of various sizes from 50 to 1000 mL at different shaking frequency conditions from 150 to 250 min^{-1} and various relative filling volumes (*Rv*) from 0.05 to 0.4 $mL.mL^{-1}$ (liquid/flask) were tested in each kind of flask, the cultures were performed during four days of fermentation at 30°C in modifed Landy MOPS medium with glutamic acid, presented results are means of triplicate experiments[8].

Analytical measurement methods.

Quantitative analysis of lipopeptides.

Lipopeptides families concentrations were determined by reverse phase high-performance liquid chromatography (HPLC), the produced lipopeptides were extracted after microbial batch fermentation. The bacterial cells were removed from fermented culture by centrifugation at 15.000 rpm at 5°C for 20 min according to Hussein and Fahim[16], kurstakin produced strains, cells were collected and sonication for 1 min in low temperature at 6 Watt (Ultrasonic processor, Cole-Parmer Instruments, Illinois, USA)[15].

The total yield was collected before analysis by (HPLC) in C_{18} column (5 µm; 250 by 4.6 mm, VYDAC 218 TP, Hesperia, CA), the mobile phase was isocritical acetonitrile-water-trifluoroacetic acid solvent system (80:20:0.5, 55:45:0.5, 45:55:0.5 and 40:60:0.5 [vol/vol/vol] for surfactin, kurstakin famlies and fengycins or plipastatins, iturins families, respectively). (20 µl) of collected samples were injected and then eluted at a flow rate of 1 $ml.min^{-1}$. Surfactin (Sigma), kurstakin, fengycins or plipastatins, iturins were purchased with purity of 98% as standards. The retention time and second derivatives of UV-visible spectra (Waters PDA 996 photodiode array detector; Millenium Software) of each peak were used to identify the eluted molecules[17].

(k_La) and (P_{VL}) quantifications.

To determine volumetric gas$_{(oxygen)}$-liquid mass transfer coefficient k_La in standard flask, the empirical correlation was applicated: $K_La = 6.67 \times 10^{-6} N^{1.16} V_L^{-0.83} d_0^{0.38} d^{1.92}$ .....(1). where (N) is the shaking frequency (min^{-1}), (V_L) the reactional volume (mL), (d_0) the shaking diameter (cm) and (d) the maximum inner Erlenmeyer flask diameter (cm). This correlation number (1) was suggusted by Maier and Büchs[18] for hydrophilic Erlenmeyer flasks, nominal flask volume of 50-1000 mL, shaking diameters of 1.25-10 cm, relative filling volumes of 0.04-0.2 mL.mL^{-1}, and shaking frequencies of 50-500 min^{-1}. The power input (P) was calculated according to the equation proposed by Peter and Büchs *et al.*[19] using the Reynolds number (Re) and modified Newton number (Ne_{mod}): $P = Ne_{mod} N^3 d^4 V_L^{0.33} \rho$ .....(2).

$Ne_{mod} = 70Re^{-1} + 25Re^{-0.6} + 1.5\ Re^{-0.2}$ .....(3). With Reynolds number (Re) = $Nd^2\rho/\mu$ where ρ (kg.m^{-3}) is liquid density, and μ (Pa.s) the dynamic viscosity (water values at 30°C). The mechanical mean power dissipation (P) and volumetric mass transfer coefficient k_La were estimated for each kind of flask, for different relative filling volumes (Rv of 0.05, 0.1, 0.2, 0.3 or 0.4 mL.mL^{-1}) and agitation conditions (150, 200 or 250 min^{-1})[8].

Results and discussions

(K_La) as cursors on lipopeptides productivity.

Kinetics of *Bacillus* strains lipopeptides families production was determined during 96 h of fermentation, the results of experiments revealed that (surfactin, kurstakin) and (fengycin, iturin) had complete different behaviour in oxygen transfer function. The increase of K_La was behind the increase of (surfactin, kurstakin) and decrease of (fengycin, iturin) concentrations levels. The maximal concentrations of lipopeptides families were noted at various period of fermentation depending on the lipopeptide type and cultivation condition, which leading to compare lipopeptides and its family type production at various K_La levels, these levels of K_La were used as cursors on surfactins, kurstakins, fengycins and iturins concentrations from various *Bacillus* strains. Whatever the results at the various study conditions, *Bacillus amyloliquefaciens* strains produced three families of lipopeptides; surfactin, fengycin and iturin (mycosubtilin type). *Bacillus subtilis* strains produced two families of lipopeptides surfactin and fengycin (plipastatin type) or no production. While, strains of *Bacillus licheniformis* and *Bacillus pumilus* produced only surfactin family with two types of lipopeptides (Lichenysin and Pumilacidin, respectively). Also, *Bacillus thuringiensis* produce one lipopeptides family (kurstakin) which illustered in Table (1).

Table 1: *Bacillus* strains produced lipopeptides families with maximum production.

***Bacillus* strains**	**Produced Lipopeptides families**							
	Surfactin types	**Production mg. L^{-1} ± SD**	**Fengycin types**	**Production mg. L^{-1} ± SD**	**Iturin types**	**Production mg. L^{-1} ± SD**	**Kurstakin types**	**Production mg. g^{-1} ± SD**
B. thuringiensis kurstaki	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	Kurstakin	146 ± 18.62
***B. thuringiensis isra.* NRRL HD-522**	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0	Kurstakin	93 ± 14.34
B. pumilus	Pumilacidin	852 ± 8.32	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0
***B. licheniformis* ATCC 14580**	Lichenysin	503 ± 10.17	-	0.0 ± 0.0	-	0.0 ± 0.0	-	0.0 ± 0.0
***B. subtilis str.* 168**	Surfactin	0.0	Plipastatin	0.0	-	0.0 ± 0.0	-	0.0 ± 0.0

***B. subtilis* ATCC 21332**	Surfactin	1431 ± 9.11	Plipastatin	360 ± 17.35	-	0.0 ± 0.0	-	0.0 ± 0.0
***B. amyloliquefaciens* S499**	Surfactin	1146 ± 9.46	Fengycin	228 ± 11.02	Bacillomycin	614 ± 17.21	-	0.0 ± 0.0
***B. amyloliquefaciens* FZB42**	Surfactin	309 ± 13.77	Fengycin	198 ± 16.13	Mycosubtilin	414 ± 9.53	-	0.0 ± 0.0

In all cases, *Bacillus* strains produced much more surfactins types than iturin and fengycin and low amount of kurstakin. The adjusting of shaking frequency and the Erlenmeyer flask size of flasks led to adjuste K_La levels which controlled on the lipopeptide concentration and the selectivity of family type, the most suitable conditions for surfactins types production (surfactin, lichenysin, pumilacidin) and krustakin type increased with flask size minimization and shaking frequency increasing, while the iturins types (mycosubtilin, bacillomycin) and fengycins types (fengycin, plipastatin) was maximized at low shaking frequency and biggest flask size. The obtained maxiumum surfactins types concentrations (surfactin, lichenysin and pumilacidin) in 50 mL flask at 250 min^{-1} and Rv = 0.05 $mL.mL^{-1}$ was about (1431, 503 and 852 $mg.L^{-1}$ respectivelly), while in 1000 mL flask at 150 min^{-1} and Rv = 0.4 $mL.mL^{-1}$ surfactins types weren't practically produced, the kurstakin production was (146 $mg.g^{-1}$) which is due to the same behaviour of surfactins[8].

A different behaviour was observed for iturins types (mycosubtilin and bacillomycin) and fengycins types (fengycin, plipastatin), the concentrations levels weren't the same with adjusted relative filling volume and shaking frequency for the various flasks size. The smaller ones (50 mL) enhanced the maximum production of (mycosubtilin, bacillomycin, fengycin and plipastatin) types with Rv increasing from 0.05 to 0.4 $mL.mL^{-1}$ which was (414, 614, 228 and 360 $mg.L^{-1}$ respectivelly). The shaking frequencies effect on fengycin and iturin production is very complicated since the increasing of oxygen supply of the culture media which is favored for iturins and fengycins production could be observed by combined the agitation , filling voulme and the size of flask. Many authors reported that, *Bacillus subtilis* is known to produce lipopeptides aerobically and able to grow under microaerobic conditions, the oxygen transfer can thus have a strong influence of *Bacillus* metabolism which have a complex regulatory system[8]. Also, lipopeptide production by *B. subtilis* ATCC 6633 was determined based on the oxygenation levels[20].

Influence of (K_La) on lipopeptides selectivity.

Selective production has been observed from multiple lipopeptides families produced strains which was very correlated with oxygen transfer condition. Thus, (K_La) values were higher at small relative filling volumes with fast shaking frequencies conditions, small volumes of liquid bulk led to liquid film formation in flask wall which was very important to increase the specific contact area (a) and consequently the coefficient (K_L) liquid mass transfer with air and make a good oxygenation[21]. The increasing of (K_La) values are favorable for surfactin and kurstakin production but on the contrary with iturin and fengycin production.

The very poor oxygenation condition (k_La less than 0.003 s^{-1}) weren't proper in the circumstances for all types of lipopeptides production because of the low rates of pH values, the increasing of k_La values towards 0.08 s^{-1}, a net extrusive correlation between the concentration levels of produced surfactins types (surfactin, lichenysin and pumilacidin) and k_La values were established. The concentration levels of surfactin increased strongly with k_La values increase, especially for k_La values ranged from 0.003 to 0.015 s^{-1} (Fig. 1-A). A similar positive correlation of k_La values on surfactin production by *Bacillus subtilis* was reported but in smaller ranges of k_La values studied (k_La = 0.004 to 0.014 s^{-1})[16]. The maximal surfactins types productivity was observed at k_La more than 0.04 s^{-1} (surfactin, lichenysin and pumilacidin), the same behaviour was observed for maxiumum kurstakin production which is due to the indirect effect of the planktonic cells growth (Fig. 1-B).

In fact, the studies in Erlenmeyer flasks, there were two types of liquid hydrodynamic behaviours were observed: bulk and film liquid. The bulk liquid usually rotates in the centrifugal acceleration direction, while, the film liquid is distributed on the Erlenmeyer flask wall as the rotating sickle within the flask. With this

behaviour, the oxygen is primarily absorbed in the thin film of liquid thrown up on the flask walls of Erlenmeyer flasks[19].

On the other hand, the oxygen supply at high relative filling volume and slow shaking frequency is so poor since, practically there is no liquid film formation on the inner surface of the Erlenmeyer flasks by this condition and the oxygen is only transferred on very slow rates from the bulk surface to the voluminous liquid core[20].

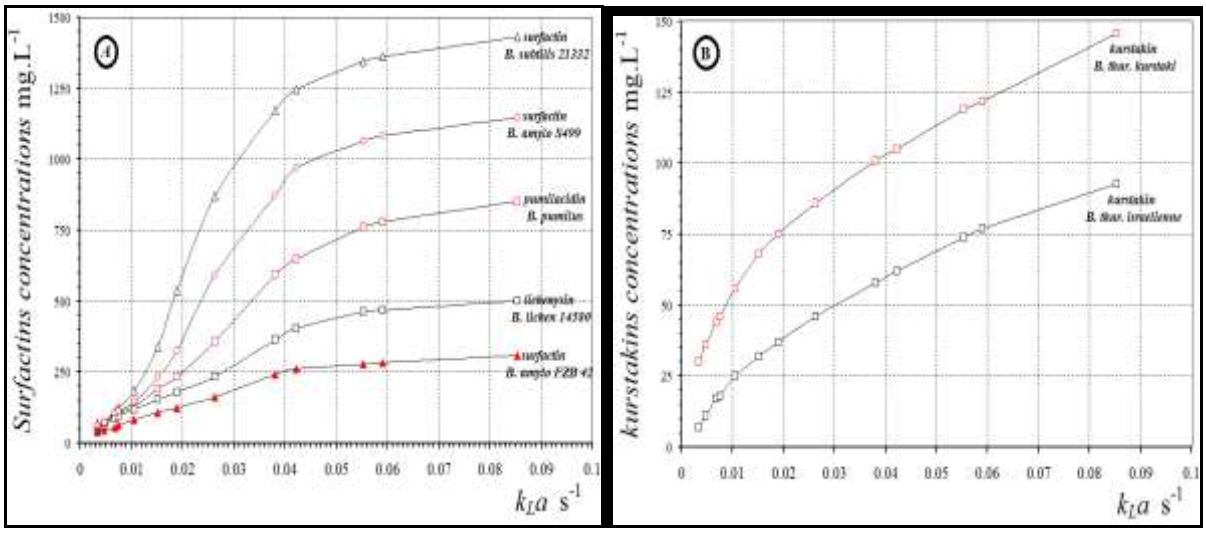

Fig. 1: Relation betwen k_La and lipopetides (A) surfactins, (B) Kurstakins production.

On the other hand, The production of iturins was maximal at relatively low oxygen transfer coefficient k_La egal 0.015 s^{-1} and k_La egal 0.01 s^{-1} for fengycins (Fig. 2).

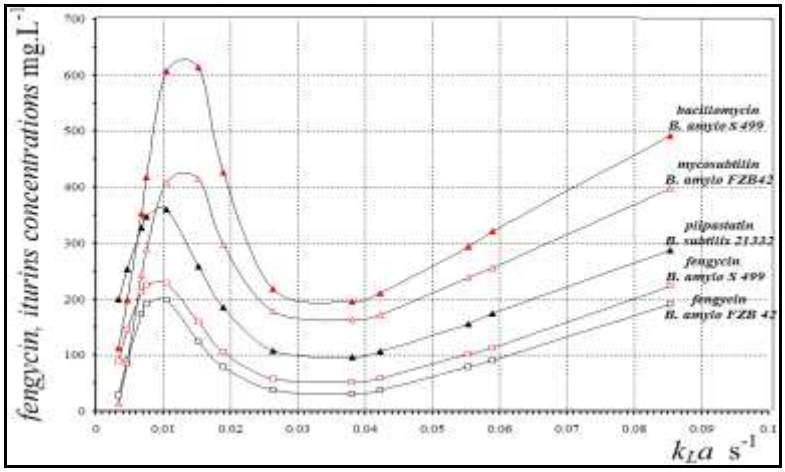

Fig. 2: Relation betwen k_La and maximum lipopetides (iturns, fengycins) production.

Further oxygen supply amelioration wasn't suitable for both lipopeptides production and, at k_La 0.04 s^{-1}, the concentrations of iturins and fengycins were extremely low, the optimal conditions for the mixed lipopeptides production are not identical. Therefore, vary the terms of oxygen transfer, the production could be directed to surfactin mono-production at k_La less than 0.04 s^{-1} or to mixed production at k_La more than 0.045 s^{-1}. Frequently, at all oxygen supply paraphernalia (different flasks volumes, relative filling volumes and shaking frequencies), except the excessive oxygen limitation conditions of apparent (k_La less than 0.006 s^{-1}), the clear positive effect of k_La on selectivity of the lipopeptides types production. Taking into account that, all studied *Bacillus* strains are able to produce much more surfactins than other lipopeptides. Thus, to expand surfactins production especially and the other types of lipopeptides by *Bacillus* species from Erlenmeyer flasks to vraious bioreactors models.

Obviously, it appears sufficient to control k_La values as production cursors in large-scale fermentors, and certainly, the high k_La values necessary for surfactins, kurstakins production, but taking into consideration the surface tension forces generated from the produced lipopeptides, it may represent a complicated problem is the potential excessive foam formation which very difficult to get rid of them in traditional bioreactors model, should be considered that, other bioreactors designs are strongly required to get rid the excessive foam formation stalemate and magnification of k_La values in the same time[23].

Integration of (K_La) and power dissipation on lipopeptides synthesis.

Despite the lack of a direct relationship between mechanical power dissipation and the productivity of lipopeptide. It's clear that, the increase of k_La values was correlated with the increase of mechanical power dissipation, which was due to the effect of the Erlenmeyer flask diameter, reactional liquid voulme and rotational speed. In Erlenmeyer flasks, there was not air injection or gas flow rate likes fermentors and so, the mechanical power dissipation due to the liquid movement was only estimated. The motion of the liquid media in the Erlenmeyer flask is intersection between two partial movements, the liquid distribution opposite movement around the wall of the flask and the rotational paraboloid movement around the flask eccentric axis[20].

At all studied fermentation process, the effectivity and the selectivity of lipopeptide were correlated with k_La values. The shaking frequency increase and reducing of realtive filling voulme coupled with smaller flasks led to directly increase of k_La values. Therefore, k_La is the most appropriate parameter for the lipopeptide productivity and selectivity cursors.

The influence of the k_La/P_{VL} fraction by (kw.m^{-3}.s) of producers strains towards the concentrations of lipopeptides in volume mean power dissepation C_{lipo}/P_{VL} (g.kw.m^{-3}) results are mentioned in terms of mechanical power dissipation (gas power input and mechanical power per unit liquid reactional volume). The influence of k_La/P_{VL} fraction on the production of lipopeptide in various Erlenmeyer flasks size at various shaking frequencies, the k_La/P_{VL} fraction data showed that relatively low power dissipation could be sufficient for each lipopeptides production in smallest flasks. However, it is difficult to obtain the same productivity in the biggest flasks even with the high increase of power dissipation (Fig 3).

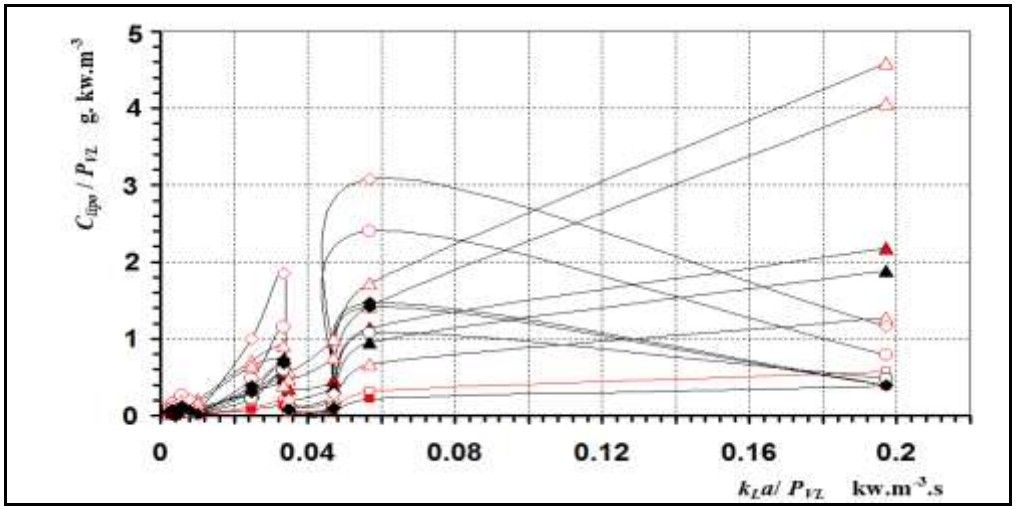

Fig. 3: Relation betwen k_La/P_{VL} fraction and C_{lipo}/P_{VL} (Δ surfactins, ◊ iturins, ○ fengycins, □ kurstakins)

The favoured (k_La) value for surfactins, kurstakins, iturns and fengycins production by *Bacillus* strains were different. Surfactins and kurstakins production is clearly optimal at high oxygen transfer (k_La) more than 0.06 s^{-1}, while optimal fengycin and iturins production could be observed at moderate oxygen transfer (k_La) egal 0.01 and 0.015 s^{-1} respectively.

Generally, the influence of fraction of the two parameters k_La/P_{VL} is very necessary for the scale-up of the lipopeptides production process and the (k_La) is real cursor of productivity and the selectivity of lipopeptide to *Bacillus* cells[15]. Therefore, to scale-up production process from Erlenmeyer flasks to the fermentation on large-scal, this two cursors should be scrutinize (k_La and P_{VL}) as indicators for bioreaction direction.

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