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Impact of gas–liquid mass transfer on organic acids production by *Corynebacterium glutamicum* in unbaffled shake flasks

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ABSTRACT

Corynebacterium glutamicum is widely used for industrial production of amino acids. Recently, its ability to produce organic acids in oxygen-deprived or anaerobic conditions has also been demonstrated. In this study, the effects of oxygenation on *C. glutamicum* growth and organic acids production were investigated through cultures in shake flask at different k_La conditions of 0.6, 3, 11, 19, 33, 36, 48, 77, 120 and 164 h^{-1}. These values were experimentally determined in unbaffled glass shake flasks patched with oxygen probe by using the PreSens system. The results showed that increasing k_La values systematically increased growth performance. Our study also showed k_La ranges where *C. glutamicum* growth was altered while organic acids production was improved. In fact, the different experiments showed that *(i)* k_La between 10 and 19 h^{-1} resulted in optimal succinic acid production (63 mM), *(ii)* k_La values between 19 and 33 h^{-1} favored high acetic acid concentrations (135 mM) and *(iii)* k_La values higher than 164 h^{-1} led to biomass accumulation.

1. Introduction

Butanedioic acid, also known as succinic acid, is a dicarboxylic acid that is a common metabolite in microorganisms, plants and animals. It can be used as building-block for the production of molecules such as tetrahydrofuran, maleic anhydride and 1,4-butanediol, for its tensioactive properties or as additive in the food or chemical industries [1]. Due to the economic and ecological necessity of using renewable carbon sources and less polluting industrial processes, white biotechnology are becoming valuable solutions for the production of molecules which were traditionally synthetized using oil-derived resources [2–4]. For instance, it was shown that the wild-type strain of *Corynebacterium glutamicum* R was able to synthetize succinate under oxygen-limited conditions [5]. However, to be competitive, the succinate production process using this microorganism has to reach the best compromise between an efficient and rapid growth, implying the use of fully aerobic culture conditions (meaning a sufficiently high concentration of dissolved oxygen) and succinate production in oxygen-limited culture conditions. Besides the use of more and more sophisticated genetically modified strains of *C. glutamicum* [7,8] the optimization of the production process, from a chemical engineering point of view, appears as a valuable complementary approach for the intensification of succinate production. For instance, to increase the production of succinate, Okino et al. [7] used a two-step process: during the first step, a sufficient quantity of biomass was produced in fully aerobic conditions. The cell suspension was then centrifuged and the harvested biomass was placed in non-aerated conditions, promoting the production of organic acids and, notably, succinate (step 2). Another strategy consisted in a one-step process in which growth and production were simultaneously managed under aerobic conditions [9]. For both culture strategies, the management of the transition from fully aerobic to anaerobic culture conditions was suggested to be a key step to optimize succinate production [1]. This was confirmed by Blombach and Eikmanns [10] and Blombach et al. [11] during the iso-butanol production process by *C. glutamicum* or during the succinate production process by *Escherichia coli* [12]. In this last study, it was shown that the transition from fully aerobic to anaerobic phase had a strong impact on the ability of the microorganism to efficiently switch its enzymatic machinery from one metabolism to another. On the basis of these previous observations, it is thus worthwhile studying more intensively the impact of oxygenation conditions on the production of succinate by *C. glutamicum*.

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Nomenclature

List of symbols

ATP	Adenosine Triphosphate (mol)
d	Flask diameter (m)
d_0	Shaking diameter (m)
cdw	Cell dry weight (g)
[Gluc]	Glucose concentration (M)
GUR	Glucose uptake rate (mol L^{-1} h^{-1})
k_La	Gas–liquid mass transfer coefficient (h^{-1})
μ	Specific growth rate (h^{-1})
μ_{max}	Maximal specific growth rate h^{-1}
N	Shaking frequency (s^{-1})
$[O_2]$	Dissolved oxygen concentration (mol L^{-1})
$[O_2]^*$	Dissolved oxygen concentration at saturation (mol L^{-1})
OUR	Oxygen Uptake Rate (mol L^{-1} h^{-1})
q_{max}^{OA}	Maximal specific organic acids production rate (g (g cdw h)$^{-1}$)
q_{max}^{gluc}	Maximal specific glucose uptake rate (g (g cdw)$^{-1}$ h^{-1})
t	time (s)
V_L	Filling volume (L)
V_T	Flask maximal volume (L)
X	Biomass concentration g L^{-1}
X_{max}	Maximal biomass concentration g L^{-1}
Y_{max}^{OA}	Maximal production yield of organic acids from substrate (mol mol^{-1})
$Y_{ATP/gluc}$	Yield of ATP production from substrate (mol mol^{-1})

For strict aerobic microorganisms, oxygen limitation and/or anaerobiosis may result in drastic effects on cell physiology and fermentation kinetics, similarly to nutrient limitation. When oxygen becomes limiting in the culture broth, microorganisms may respond by modifying their internal metabolism [13,14]. In general, the central metabolism is known to display important differences under fully aerobic and oxygen-limited conditions. In fact, high activity in the respiratory chain is associated with high glucose uptake and utilization in aerobic bacteria. Besides, oxygen limitation results in cell respiration reduction and in intracellular pyruvate accumulation which will be further converted to anaerobic fermentative byproducts [15,16]. As organic acids production by *C. glutamicum* was related to oxygen limited conditions in recent studies [5,6,17,18], the physiological maximum oxygen consumption of the microorganisms should exceed the maximum amount of oxygen delivered by the gas–liquid mass transfer, promoting this production.

To get further insight in the impact of oxygenation conditions on succinate production by *C. glutamicum*, a one-step culture process, undertaken in shake flasks, was chosen in the present study. Indeed, shake flasks show numerous advantages for rapid screening of bioprocess conditions, specific activities of strains, mutant selection, medium characterization, metabolic pathway studies [19–22]. Interestingly, insufficient oxygen supply remains a frequent problem associated with the application of shake flasks [22,23]; this drawback should be thus advantageously used in the present study. Indeed, during their culture in shake flask, microorganisms should switch progressively from an aerobic to an anaerobic metabolism, according to the oxygen transfer rate and to the oxygen uptake rate expected. This oxygen limited environment could lead to byproducts excretion which could, in return, affect the cells metabolism.

In shake flasks, the rotary and reciprocating action of the shaker apparatus achieve oxygen transfer through the gas–liquid free surface and then transport in the culture medium. The oxygen transfer process in the flask strongly depends on the liquid volume, flask size, shaking diameter and agitation rate of the table. As in mechanically stirred and sparged bioreactors, the quantification of oxygenation capacities relies on the determination of gas–liquid mass transfer coefficient (k_La), which relates the driving force ($[O_2]^*$–$[O_2]$) to the oxygen transfer rate (OTR) [24,25]. This coefficient has been measured in shake flasks using conventional systems equipped with sensor spots for online dissolved oxygen monitoring or using the sulfites reaction method, [21,26–28]. Moreover, mathematical models and empirical correlations have also been used for gas–liquid mass transfer coefficient modeling [13,24,29,30]. However, no literature data linking organic acids production by *C. glutamicum* and oxygen transfer in shake flasks could be found.

It is well known that limiting conditions of oxygen transfer may promote succinic acid production while high oxygen transfer capacities rather promote biomass growth. However, literature data are not explicit concerning (*i*) how the transition from biomass to organic acids production occurs when oxygenation capacities of the bioreactor change, (*ii*) the range of adequate oxygen transfer conditions allowing high succinate production with *C. glutamicum*. To bring more detailed information concerning the impact of gas–liquid mass transfer on process performance, this work aimed to study the balance between growth and organic acids production according to the k_La imposed in the culture. Oxygenation was quantified by the determination of the gas–liquid mass transfer coefficient (k_La) imposed to the shake flasks cultures while bioprocess efficiency was notably characterized in terms of growth rate, organic acids production yields and specific consumption rates. Original relationships coupling the physiological response of *C. glutamicum* and k_La were then proposed and discussed in detail.

2. Material and methods

2.1. Bacterial strain, culture media and protocol

Batch cultures of *Corynebacterium glutamicum* 2262 lactate dehydrogenase deleted mutant (Δ*ldhA*) were performed in a modified MCGC mineral salt medium in which citrate was replaced by deferoxamine. Final concentrations of nutrients are indicated in Table 1. Basis salts A and B and urea were prepared separately and autoclaved for 20 min at 121 °C. Vitamins solution was sterilized by filtration through 0.22 μm filter (Millex, Millipore, France). After sterilization, the different solutions were mixed together in accordance to the final concentrations mentioned in Table 1. The mutant strain was constructed via a two-step homologous recombination procedure using the vector pK19*mobsacB*Δ*ldh* [8]. This plasmid was constructed using the suicide vector pK19*mobsacB* as described by Schäfer et al. [31]. The culture medium was supplemented with 155 mM of glucose which was thus the sole carbon and energy source initially present in the medium. A first preculture (A) of *C. glutamicum* 2262 Δ*ldhA* was performed in a 500 mL flask containing 50 mL of MCGC. Temperature and shaking rate of precultures and cultures were respectively 33 °C and 200 rpm. When the optical density at 570 nm reached the value of 60 approximately, 5 mL of preculture (A) were used as inoculum for the second preculture (B) performed with the same culture medium than preculture A. When the optical density at 570 nm of preculture (B) reached a value of 30, bacterial growth was stopped by immersing the flask in 70% v/v ethanol, at a temperature of −20 °C; fermentation broth was then kept at a temperature of 4 °C for 24 h maximum. Preculture B was used for the whole culture experiments. All the cultures were then performed in glass unbaffled shake flasks placed in a temperature-controlled (T° = 33 °C) shaker during the cultures. A more efficient gas–liquid mass transfer should be expected in baffled shake flasks and thus should better prevent from oxygen limitations in the