UNIVERSIDADE DA BEIRA INTERIOR

Ciências da Saúde

Biosynthesis of small noncoding RNAs in *Rhodovulum sulfidophilum*: optimization of the fermentation process

Jane Risete Pires dos Santos Dias

Dissertação para obtenção do Grau de Mestre em

Ciências Biomédicas

(2º ciclo de estudos)

Orientador: Professor Doutor Luís Passarinha

Co-orientador: Professora Doutora Fani Sousa

Covilhã, outubro de 2018

Aos meus pais, pelo amor.

À minha irmã por sempre acreditar em mim.

Acknowlegments

My academic journey in Covilhã, including this dissertation, has been of intense learning for me, and I would like to express my gratitude to the people who have supported me.

First, to my supervisors, Professor Doctor Luís Passarinha and Professor Doctor Fani Sousa, I would like to acknowledge for the trust placed on me for this work and for the dedication, scientific expertise, continuous guidance, enthusiastic encouragement, as well as the constructive criticisms and suggestions made during the guidance of this work. It was a privilege to work with you.

I would like to express my sincere gratitude to Doctor Patricia Pereira and Doctor Augusto Pedro for the engagement in this project. Thank you for the immediate availability to guide me in this work and for the assistance in keeping my progress on schedule. I really appreciate the way how you shared your knowledge with me.

To my lab colleagues, Inês Rodrigues and Bruno Batista, and to my research group, for the friendship, companionship, and help, specially to Diana Duarte for the enthusiastic way how she shared her knowledge with me. Your passion and dedication for bioreactors are captivating and made my learning process pleasant.

I would also like to extend my thanks to the CICS, to the University of Beira Interior and to Covilhã.

To the friends that CICS gave me, Marina, Dalinda, Carla, Diana, Marta, Rita and Catarina, for always be there for me. Thank you for the “coffee time”, for always being so kind, for the advices, and for all the great moments spent together. I am grateful for having had the opportunity to meet people as adorable and brilliant as you.

To my “friendfamily” in Covilhã for all the support since the first day in Covilhã. You are lit. “Nobody exists truly without friends”.

Finally, and more important to me, I would like to thank my lovely family.

First, to my greater love, my mom Ricardina, for being my daily support and inspiration, and for being always by my side even miles away.

To my dad, José Pedro, for all the love, support, and for be such an example of bravery.

To my little sister and best friend, Alcione, for believing more in me than anyone else and for understanding my absence during these years, and to my stepdad, Alcides, for the love and good advices.

Last but not least, to my older brothers, Dany and Kleidir, to my aunties, Ana Cristina and Filomena, and to my grandmothers for always blessing me with your love.

Resumo Alargado

Durante um longo período de tempo, a contribuição dos ácidos ribonucleicos (RNAs) nas funções celulares foram amplamente subvalorizadas, limitando-se a serem descritos como intermediários no processo celular em que uma sequência de ácido desoxirribonucleico (DNA) codifica uma proteína. A investigação em torno destas moléculas, contudo, permitiu identificar pequenas moléculas de RNA não codificantes (ncRNA) que participam em funções celulares regulatórias. Em muitos casos, a expressão diferencial destes RNAs é reconhecida como *hallmark* de muitas patologias humanas incluindo vários cancros, as doenças neurodegenerativas, as doenças cardiovasculares e a diabetes. Assim, assumem-se como potenciais biomarcadores para diagnóstico e prognóstico, mas também como novos alvos e agentes terapêuticos. O uso de RNAs para fins clínicos tem exigido à comunidade científica uma investigação centrada nestas moléculas por forma a decifrar corretamente a sua estrutura, função e interações. Assim, a necessidade de aceder a grandes quantidades de RNA aumentou e levou à procura de novos métodos para a produção de RNA. A combinação da tecnologia de DNA recombinante e de processos de fermentação de microrganismos, já amplamente utilizada para a produção recombinante de proteínas, tem-se mostrado adequada para a biossíntese de RNA em quantidades que, dificilmente, seriam obtidas por outras metodologias de forma sustentável. Um dos principais objetivos deste método é desenvolver estratégias para expressar o produto alvo de forma mais eficiente, ou seja, maior produtividade a menor custo. Para tal, a indústria biotecnológica tem apostado em fermentações de elevada densidade celular cujo sucesso assenta na otimização do bioprocesso. Inúmeros fatores devem ser considerados incluindo o organismo hospedeiro, as condições de fermentação, mas também as estratégias para maximizar seu crescimento e produção. A *Rhodovulum sulfidophilum* (*R. sulfidophilum*), bactéria marinha fototrófica facultativa, tem-se mostrado um potencial hospedeiro para a biossíntese recombinante de pequenos RNAs regulatórios, em parte, graças ao baixo nível, intracelular e extracelular, de RNAses que permitem manter a integridade dos RNAs recombinantes. Ao longo dos anos, poucos estudos têm sido levados a cabo com o propósito de maximizar o crescimento deste hospedeiro, deixando-a pouco apelativa para a indústria biotecnológica quando comparada com a *Escherichia coli*, cujas estratégias de crescimento já se encontram claramente definidas. Assim o objetivo da dissertação apresentada, centra-se em aumentar a densidade celular das fermentações de *R. sulfidophilum* e consequentemente incrementar a produtividade de ncRNAs. Para tal, foram realizadas fermentações *batch* em mini-bioreatores, nas quais o efeito do tamanho do inóculo, da disponibilidade de oxigénio e da temperatura foram estudadas, tendo como referência valores aplicados em fermentações em *erlenmeyers*. Além disso, tendo em consideração o impacto que a fonte de carbono pode ter na formação de biomassa, a glucose e o glicerol foram comparados em diferentes outputs deste bioprocesso. Relativamente ao inóculo, foram testados quatro tamanhos diferentes (13%,

18%, 24% e 30%) o que permitiu estabelecer 18% como o tamanho de inóculo mais adequado às fermentações em bioreator devido aos maiores níveis de biomassa alcançados. Foram, igualmente, testadas temperaturas diferentes para as fermentações, 25, 30 e 37 ºC, e avaliada a sua influência no perfil de crescimento. A temperatura que favoreceu a obtenção de níveis de biomassa mais elevados foi a de 30 ºC sendo, por isso, selecionada como temperatura ótima para as fermentações de *R. sulfidophilum* em bioreator. A glucose (10 g/L) e o glicerol (20, 10, 5 e 2,5 g/L) foram testados como fonte principal de carbono nas fermentações em bioreator e suas concentrações no meio extracelular foram quantificadas ao longo do tempo por HPLC acoplado com um detetor por índice de refração. Ambas as fontes de carbono, apresentaram um perfil de consumo semelhante, onde começaram a ser efetivamente metabolizados pela bactéria após 40 h de fermentação. Contudo a utilização de glicerol como principal fonte de carbono resultou num aumento considerável de biomassa final (5,94 g/L) quando comparado com a glucose (3,30 g/L). Em termos do produto alvo, foi estudada quantitativamente a produção de RNA total e qualitativamente a produção de ncRNAs. A produção de RNA total, em amostras normalizadas pela massa de células obtida, foi analisada ao longo do tempo, evidenciando-se, para ambas, um pico de produção às 60 horas de fermentação correspondendo a 537 ± 49 µg/mL para a glucose e 446 ± 58 µg/mL para o glicerol. Paralelamente, a análise qualitativa da expressão de ncRNAs demostrou um pico de produção às 60 h para as duas fontes de carbono, sugerindo que a produção recombinante de RNA em *R. sulfidophilum* acompanha o perfil de produção de RNAs homólogos. Considerando todos os resultados obtidos, é possível concluir que a substituição da glucose por glicerol para a produção recombinante de ncRNAs em *R. sulfidophilum* em fermentações *batch* à temperatura de 30 ºC e com 18 % de inóculo é promissora, uma vez que resultou numa maior produção volumétrica graças ao simples incremento da biomassa. Os resultados em *batch* deixam uma porta aberta para o estudo de novas estratégias de fermentações (*fed-batch*) e para otimização das fermentações recorrendo a design experimental no qual os ensaios são planeados estrategicamente analisando o efeito sinergético de *inputs* alvos, para se obter maior informação sobre o efeito de mais do que um parâmetro na produtividade do *biotarget*.

Abstract

Biosynthesis of noncoding RNAs (ncRNAs) in microorganisms has stood out as a cost-effective and favourable method for natural RNA production, and *Rhodovulum sulfidophilum (R. sulfidophilum),* a marine phototrophic bacterium, has been studied as a potential host for this bioprocess. Then, this work intends to optimize, in a mini bioreactor platform, the fermentation process of *R. sulfidophilum* strain DSM 1374 as a recombinant host to produce ncRNAs. So, the effect of the inoculum size, temperature and oxygen availability was studied, and the best outcome was achieved in fermentations at 30 ºC with 18% inoculum where fully aerobic-dark conditions are provided. Under such conditions the effects of the main carbon source were analysed in which glucose (S_i = 10 g/L) was replaced by glycerol in *R. sulfidophilum* fermentation. Glycerol metabolization was analysed when using differents initial concentrations (Si = 20, 10, 5, and 2.5 g/L) and the consumption of both the carbon sources was assessed by HPLC-RID. Briefly, the optimized conditions in bioreactor scale yielded 1.8 times more biomass when glycerol (S_i = 10 g/L) was the main carbon source, and 2.3 times more biomass compared to the optimized conditions in shake flask scale. Both carbon source lead to a maximum of total RNA production at 60 h, being of 537 ± 49 µg/mL in glucose and 446 ± 58 µg/mL in glycerol. Noteworthy, the electrophoretic qualitative analysis of small ncRNAs biosynthesis demonstrated a higher concentration also at 60 h in both glucose and glycerol fermentation. The results from the batch fermentations suggest a more efficient utilization of glycerol by *R. sulfidophilum* which reflects in higher biomass and reasonably higher ncRNA productivity. With a view of increasing the overall biomass and productivity, fed-batch experiments were carried out through constant glycerol feed. The results proved that a feed solution containing only glycerol does not lead to biomass increasing, as is expected, highlighting the interaction of different growth limiting factors. All these results together leave an open door for the study of glycerol as a substrate for recombinant biosynthesis of ncRNAs in *R. sulfidophilum,* specifically for the development of new feed strategies adequate to the nutritional requirements of the bacteria and that allow increasing the biomass and the productivity of ncRNAs.

Keywords

Rhodovulum sulfidophilum, noncoding RNAs, Recombinant biosynthesis, Mini-bioreactors

Table of Contents

List of figures

List of Tables

List of Acronyms

μ	Specific growth rate
μg	Microgram
μL	Microliter
ATP	Adenosine Triphosphate
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSP	Downstream Processes
E. coli	*Escherichia coli*
gDNA	Genomic DNA
GTA	Gene Transfer Agent
h	Hours
HPLC	High Performance Liquid Chromatography
iRNA	RNA interference
k	Reduction rate per carbon
L	Liter
lncRNA	long ncRNAs
mg	milligram
min	Minutes
miRNA	MicroRNAs
mL	milliliter
mRNA	messenger RNA
NaCl	Sodium chloride
ncRNAs	non-coding RNAs
O_2	Oxygen
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
pO_2	Oxygen pressure
R. sulfidophilum	*Rhodovulum sulfidophilum*
RID	Refractive Index Detector
RNA	Ribonucleic acids
RNases	Ribonucleases
rpm	Revolutions per minute
rRNA	ribosomal RNAs
S	Substrate concentration
S_f	Final substrate concentration
S_i	Initial substrate concentration
siRNA	Small interfering RNAs
sRNA	Small RNA
TES	Trace elements solution
t_{max}	Time to achieve the higher biomass
tRNA	transfer RNAs
TRNA	Total RNA
USP	Upstream Processes
UV	Ultraviolet
V	Volt
X	Biomass
X_{max}	Maximum biomass
X_i	Initial Biomass

Work presented in this thesis has resulted in:

Poster presentation at the XIII CICS-UBI Symposium, Covilhã (July 2018): Jane Dias, Diana Duarte, Fani Sousa, Luís Passarinha: Optimization of fermentation conditions of *Rhodovulum sulfidophilum*in mini-bioreactor for the production of noncoding RNAs.

Chapter 1 - Introduction

1.1 Noncoding RNAs

Unlike the central dogma of molecular biology, recent evidence suggests that the majority of the human genome, and even the genome of other complex organisms, is transcribed into RNAs that do not code for proteins. These RNAs are classified as non-coding RNAs (ncRNA) and include the housekeeping RNAs, also known as transcriptional ncRNAs, and the regulatory ncRNAs [1, 2].

The transcriptional ncRNAs, including transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs), are constitutive RNAs that do not directly yield coding proteins but are infrastructurally involved in the processes of protein expression (Figure 1) [1, 2].

Regulatory ncRNAs, on the other hand, are mostly transcribed in a location and time dependent manner [2]. Based on the size they can be classified into small ncRNAs (shorter than 200 nucleotides) and lncRNAs (200 nucleotides or longer) (Figure 1) [2, 3]. Regulatory RNAs control crucial cell processes such as proliferation, differentiation, apoptosis, development and stress response [4]. Hence, they determine most of our complex characteristics and play a significant role in disease.

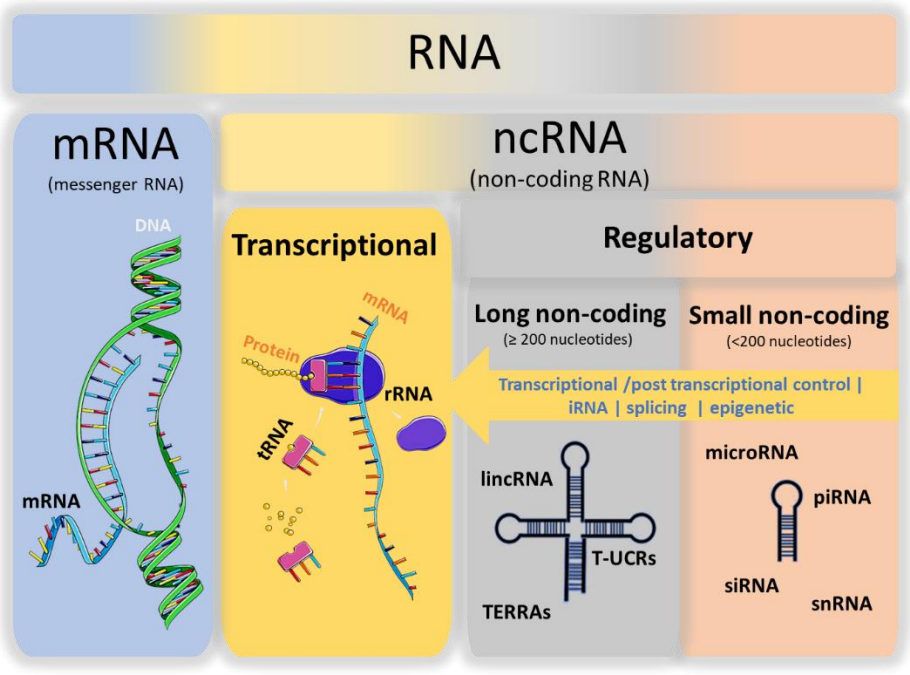


Figure 1 - RNA classification and representative members of RNA classes.

The small ncRNAs group includes well-documented ncRNAs which have triggered the RNA-centered research, such as micro RNAs (microRNA) and small interfering RNAs (siRNAs) but also

small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), and PIWI-interacting RNAs (piRNAs) (Figure 1) [1, 2].

Small ncRNAs are involved, mainly, in post-transcriptional gene regulation by mean of translational repression, RNA interference (iRNA) or DNA methylation, and basically define which genes are turned on and which are turned off [1]. For instance, the subclass of microRNAs itself is estimated to regulate 30% of all human genes [5, 6]. In most of the cases, differential or abnormal expression of small ncRNAs is related to pathology scenarios, including cancer [7–10], neurodegenerative diseases [11–14], cardiovascular diseases [15–17] and diabetes [18]. This is an indicative of their potential usage as novel diagnostic, prognostic, predictive biomarkers and, most recently, as novel therapeutic agents and targets [19–21].

All research behind both the discovery of new RNA drugs and the RNA-based therapy itself requires easy and cost-effective access to large amounts of RNA agents that meet the requirements established by regulatory authorities [21]. In one way or another, all these "requirements" are related to, or even dependent on, the RNA sources.

1.2 Sources of RNA

The rise of the RNA-centered research, mostly justified by the discoveries of novel roles of noncoding RNAs, such as microRNAs and small interfering RNAs, imposes the access to large amounts of affordable ncRNA agents.

Currently, RNAs obtained through synthetic methods, such as *in vitro* transcription and chemical synthesis (Figure 2), account for the major source of RNA [22]. However, in recent years large efforts have been made to develop recombinant methods to produce natural RNAs *in vivo,* using prokaryotes hosts (Figure 2) [21].

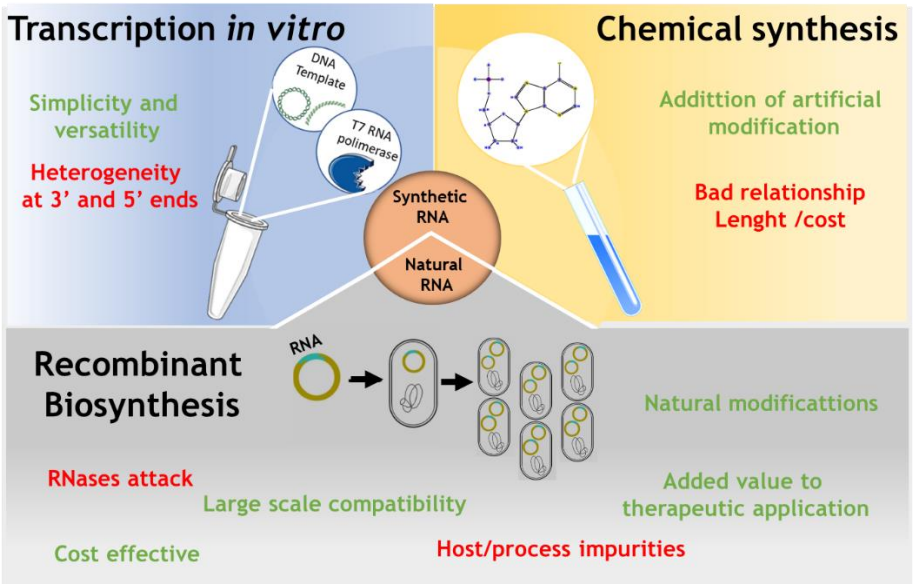


Figure 2 - RNA sources and their main characteristics. The advantages are represented in green and the disadvantages in red.

1.2.1 *In vitro* transcription

In vitro transcription is a widely used enzymatic approach for template-directed synthesis of RNA molecules. It is based on the engineering of a DNA template (oligonucleotides, PCR products or plasmid) that includes a bacteriophage promoter sequence (usually from the T7 bacteriophage) upstream of the sequence of interest, followed by the transcription using the corresponding RNA polymerase [23, 24]. Despite this method has RNA yields in order of milligrams and presents commercial convenience, it is a very laborious and costly approach that usually leads to RNA products with heterogeneous 3' and 5' ends. Such heterogeneity appears as a consequence of transcription termination can occur out of the desired site, for example, few bases before the 3' end or bases beyond the template length, and might be a hindrance to its future applicability [25] (Figure 2).

1.2.2 Chemical synthesis

Chemical synthesis of RNA is a fast RNA production approach mainly based on phosphoramidite chemistry and commonly has a high yield of pure RNA. Yet, the main advantage of this strategy concerning the therapeutic applicability is the possibility to add chemical modifications to enhance metabolic stability and other pharmacokinetic features [14]. Unfortunately, this method has also some limitations in terms of length of the desired RNA that can be synthesized, and in terms of ensuring the synthesis of an RNA with a desired sequence [25]. Furthermore, the cost sharply increases with the adding of chemical modifications or with the increase of the RNA length [25] (Figure 2).

1.2.3 Recombinant biosynthesis

Recombinant RNA biosynthesis is a method in which natural RNAs are produced *in vivo,* mostly in microorganisms, and appears as a strategy to overcome some of the problems faced by the synthetic methods. This method is expected to provide large quantities of biological non-coding RNA agent with proper folding and natural modifications that are critical for RNA higher-order structure, stability, activity, and safety [22] (Figure 2).

When compared with the synthetic methods previously mentioned, we can safely say that the synthetic methods are most expensive and laborious and have their drawbacks with respect to sequence requirements, variations in yield, non-templated nucleotide additions and/or the limitation on the maximum length of the oligonucleotide. Notwithstanding, artificial RNAs have a wide applicability, ranging from structural studies to therapeutic application [23, 26]. However, through the view of therapeutic application, these methods face a problem: they produce "artificial" RNA. These artificial RNAs do not experience the same post-transcriptional modifications by which "natural" RNAs are subject [27].

Actually, while *in vivo* natural RNAs go through modifications mostly on the nitrogenous bases, artificial ones undergo modifications on the backbone, either on the phosphate group or on the sugar ring (Figure 3).

The post-transcriptional modifications that occur in natural RNAs are often related to their biological function and hold biochemical and physiological functions [27]. Thus, the absence of natural modification, or the presence of artificial ones, may interfere with the natural folding and consequently with the right performance and biologic activity. Not to mention the likelihood of alteration in the safety properties offered by artificial RNAs [21]. For this reason, and for the cost-effectiveness advantages, there is a growing interest in the use of biosynthesized RNA based on recombinant methods [22].

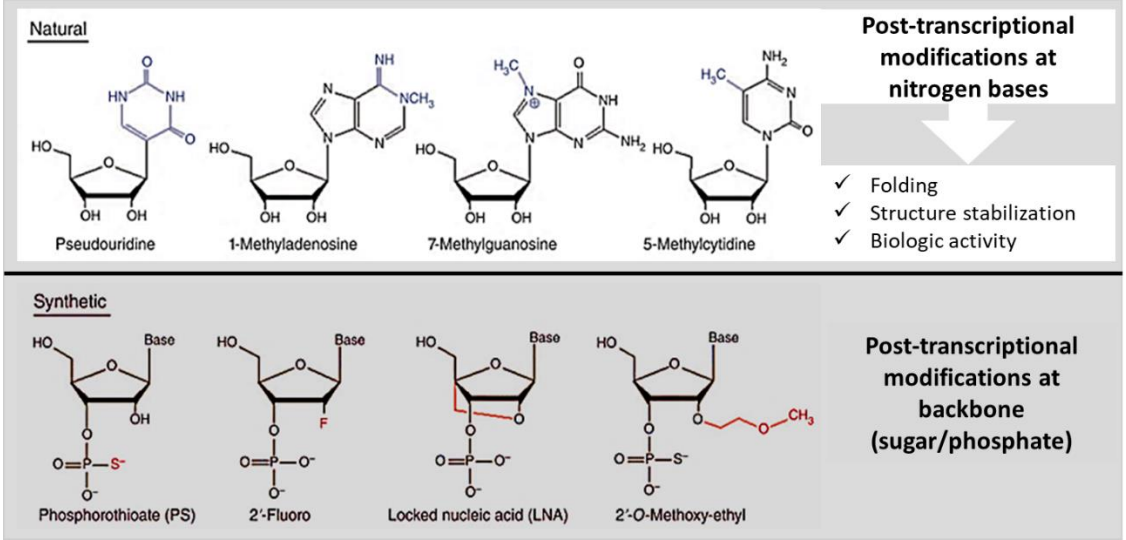


Figure 3 - Natural and synthetic RNA modifications, highlighting the advantages of natural modifications. Adapted from [21].

Recombinant RNA biosynthesis follows almost the same footprints of recombinant protein biosynthesis (Figure 4). Within upstream processes (USP) a vector with strong transcription signals and carrying the gene that codes the desired ncRNA is inserted into a host. The host's cell machinery then promotes the fermentation-based expression of the gene [28, 29].

Within the downstream processes (DSP) the product, in this case, ncRNA, is separated from all types of impurities, either host impurities, process contaminants or even product-related impurities [28, 30, 31]. Together, USP and DSP aim to obtain RNA products that are suitable for analytical and clinical applications [32].

Noteworthy, the design of a biosynthesis process includes the combination of a considerably high number of options. The right combination of options, for example, in terms of the host, vector, fermentation conditions and purification methods determines the success of the

biosynthesis process [33–35]. In the recent methods for RNA biosynthesis, the options in terms of host microorganisms have been narrowed down to two: *Escherichia coli* (*E. coli*) and *Rhodovulum sulfidophilum* (*R. sulfidophilum*). However, the choice of one rather than another influences all the process stages from the plasmid construction to the purification strategies.

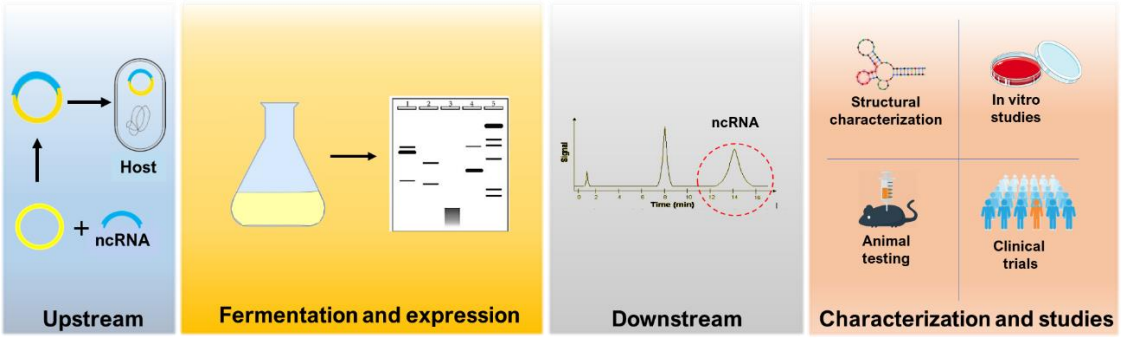


Figure 4- The process of recombinant ncRNA biosynthesis for analytical and clinical applications.

1.3 RNA biosynthesis: the hosts

There are many available production systems for recombinant biomolecules, such as microorganisms, mammalian cells, and insects. Microorganisms, like bacteria or yeast, are considered the most promising systems for this purpose and so, devoting efforts have been made in the field of fermentation-based manufacturing of biomolecules [28]. In fact, microorganisms offer many advantages in the production of recombinant biomolecules since they have well-characterized genomes, there are some versatility of the vectors that can be used, and there is availability of different host strains, and the process usually presents cost-effectiveness when compared with other cell systems [28].

As above mentioned, up until the moment *E. coli* and *R. sulfidophilum* have been the bacteria hosts used for recombinant RNA biosynthesis. Depending on the host chosen so is the plasmid design, the fermentation condition, and the DSP [36].

1.3.1 RNA biosynthesis in *Escherichia coli*

The historical development of microbial physiology studies, molecular genetics, and engineering genetic has always been based on the gamma-proteobacteria *E. coli* resulting in unique accumulation of information about this specie [37].

Then, it is not surprising that *E. coli* was the first-choice in what regards to expression systems for recombinant RNA production. In fact, there are some advantages of using *E. coli* as the host microorganism, since: (i) it has an unparalleled growth rate; (ii) can easily be achieved a high cell density; (iii) the growth conditions are simple and undemanding and (iv) its transformation with exogenous DNA is fast and easy [38]. Table 1 summarizes the RNAs produced in *E. coli*, as well as the strategy used in each case, and the respective RNA yield.

In what concerns to methods for RNA production in *E. coli*, Ponchon and Dardel [35], taking advantage of the natural "anti-degradation" characteristics of tRNA molecules, developed a specific technology to biosynthesize RNAs. In this innovative technology, the RNA to be produced is basically inserted into the anticodon stem of a tRNA scaffold. This biostructure plays a role of protective shielding, in which the recombinant RNA is "disguised as a natural RNA and thus hijacks the host machinery, escaping cellular RNases" [35] (Figure 5A).

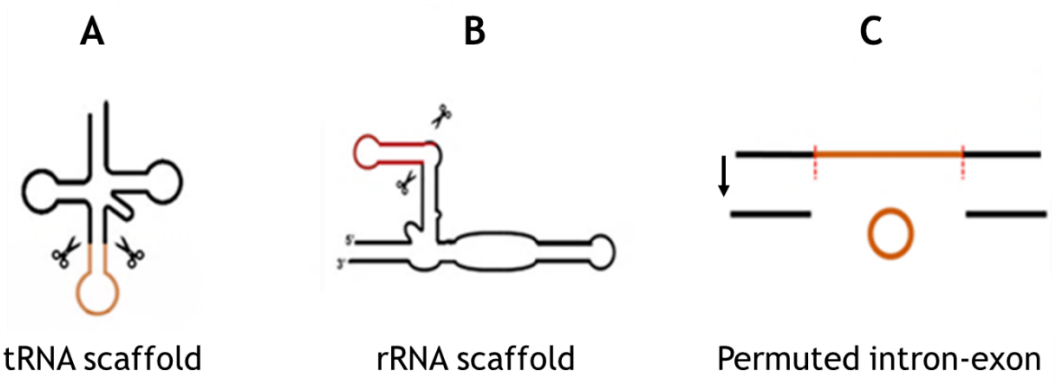


Figure 5- Plasmid design for recombinant RNA production in *E. coli*. (A) and (B): Secondary structure of recombinant RNA chimeric where sequences in black represent the scaffold and sequence in orange represent the inserted RNA. Scissors represent the excision site. (C) Permuted intron exon plasmid where black sequences are the 3´and 5´half intron and the orange sequence is the inserted RNA. Adapted from [29, 39].

This method seems to work well since it allowed to obtain large amounts of RNA yielding up to 50 mg/L of culture of pure RNA [40] (Table 1). Despite all these advantages, it is important to depict two disadvantages that may not be attractive for the large scale biosynthesis and industrial environment: first, this method is only feasible for RNA molecules with stem-loop secondary structures and, second, it is necessary a cleavage step to release the RNA from the chimeric structure, which requires the use of enzymes [40, 41].

Table 1- Examples of Recombinant RNAs typically produced in *E. coli*.

	Plasmid Design	Product	Intracellular RNA per L of culture	Ref.
Epsilon RNA sequence	tRNA scaffold	Chimeric tRNA/RNA	21-50 mg	[35, 40]
Pre-miR-1291	tRNA scaffold	Chimeric tRNA/RNA	2-3 mg	[42]
Hsa-pre-mir-27b	tRNA scaffold	Chimeric tRNA/RNA	0.5 mg	[43]
Streptavidin RNA aptamer	PIE	Circular RNA	21 µg	[39, 44]
Pre-miRNA-29b	Hammerhead ribozymes	Linear RNA	1.64 mg	[36]

Based on the tRNA scaffold strategy, some variants of this method were tested over the years including the co-expression of the tRNA-fused RNA with an interacting protein [45] and the use of a scaffold of tRNA/miRNA [46]. Besides the tRNA-based method, other strategies have been developed for RNA biosynthesis in *E. coli*, especially for ncRNA biosynthesis. Some examples are rRNA scaffolds [47] (Figure 5B) which follows the same principle of tRNA scaffolds, and Permuted intron-exon (PIE) sequence from T4 bacteriophage (Figure 5C) that produces circular target RNAs [44]. All these methods have advantages and disadvantages and despite they are all different strategies, all aim to prevent the *E. coli* nucleases attack to the recombinant RNA.

1.3.2 RNA biosynthesis in *Rhodovulum sulfidophilum*

During the last years, the peculiar characteristics of the bacterium *Rhodovulum sulfidophilum* have aroused the interest on its applicability as a host for recombinant RNA production. For instance, Suzuki and co-workers proposed a method for recombinant RNA biosynthesis in *R. sulfidophilum* [48]. Among all the characteristics of this bacterium, two of them stood out in what concerns to its feasibility for recombinant RNA production. The first one is that it secretes nucleic acids into the extracellular medium and, the second one, is that it does not produce RNAses to the culture medium and even the intracellular level of RNases is also low [48].

In this method, the RNA sequence is flanked on both sides by hammerhead ribozymes (Figure 6) that catalyze the cleaving of its own phosphodiester backbone after transcription without the intervention of co-factors or enzymes [49].

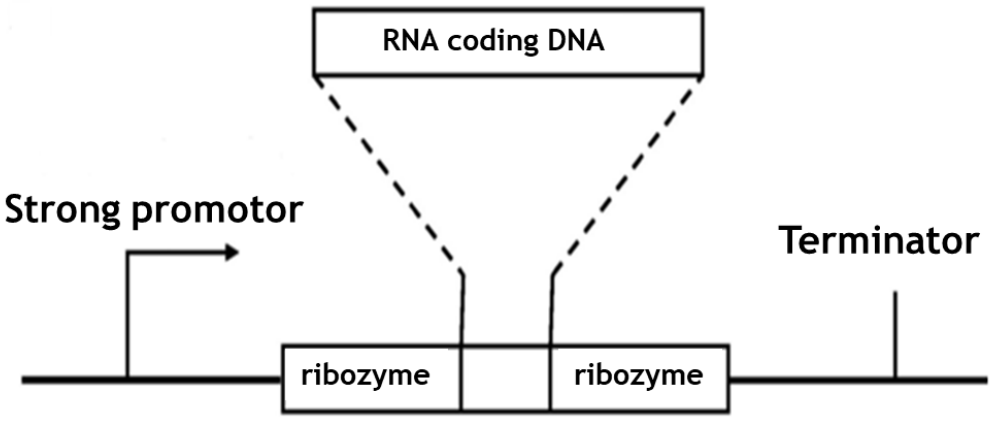


Figure 6 - Plasmid design for recombinant RNA production in *R. sulfidophilum*. Adapted from [48].

Unlike what happens when *E. coli* is the host, herein recombinant RNAs are produced intracellularly and extracellularly without any modification such as circularization or chimeric structures [48].

Under such transcription signal (Figure 6), the maximum level of extracellular production found for a Streptavidin RNA aptamer, was 45 ng/L of culture after 16h of anaerobic-light cultivation

(which corresponds to the last lag phase) and the total RNA production (intracellular and extracellular) was nearly 7 ug/L of culture. Despite cultivation under aerobic-dark conditions provided best results, the values still unpractical for industrial scale-up or laboratory applications [48].

In order to improve the productivity, modifications were carried out in the promotor and allowed a 4 times increase of the extracellular Streptavidin RNA aptamer production and about a 3 times increase on the amount of total RNA production (intracellular and extracellular) when compared to the wild-type promoter [50, 51] (Table 2). With such modifications the time course of RNA production is maintained until the late stationary phase, contrarily of what happens with the wild-type promoter where the maximum extracellular RNA production occurs in the last lag phase [50].

With the purpose of generalizing this method, another type of RNA, short hairpin RNA (shRNA), with a different secondary structure, was produced under the same conditions and the result in terms of RNA productivity was quite similar [51] (Table 2).

Table 2- Recombinant RNA produced in *R. sulfidophilum* through Hammerhead ribozymes plasmid design

	Intracellular RNA per L of culture	**Extracellular RNA per L of culture**	**Ref.**
Streptavidin RNA aptamer	20 µg	0.195 µg	[50]
shRNA	-	0.200 µg	[51]
Pre-miR-29b	358 µg	181.0 µg	[52]

More recently, Pereira and co-workers described the successful biosynthesis of hsa-pre-miR-29b in *R. sulfidophilum*, also based on recombinant RNA technology with the hammerhead ribozymes and under the same transcription signals [52]. The target RNA productivity was considerably higher when compared with the previously described (Table 2). Noteworthy, the growth conditions were different, as in this case aerobic conditions were used [52]. The RNA secretion, together with the high yields of the target RNA, made this method a promisor methodology for ncRNA production in *R. sulfidophilum* [52]. Therefore, it is important to extend and consolidate the knowledge of the characteristics and growth conditions of *R. sulfidophilum* to optimize the process.

1.4 *Rhodovulum sulfidophilum*

1.4.1 Taxonomic and morphologic characterization

Rhodovulum *sulfidophilum*, (basionym *Rho*dopseudomonas *sulfidophila* and *Rhodobacter sulfidophila*) whose name means sulfide loving small red egg [53], is a purple non-sulfur gram negative alpha-proteobacteria [54]. As all species of the *Rho*dovulum genus, they are morphologically characterized by being rod-shaped cells with 0.6 - 0.9 μm in width and 0.9 - 2.0 μm in length, usually movable by polar flagellum [55].

*Rho*dovulum species are metabolically versatile since they are facultative anaerobic photoautotrophic. Therefore, in addition to growing anaerobically using light as an energy source, they also have the ability to use organic compounds aerobically in the dark as an energy source [54]. Depending on the growing condition, anaerobic or aerobic, they can be yellowish-brown to pinkish-red, respectively (figure 7). They are also known as "anoxygenic bacteria" since it is unable to produce O_2 during photosynthesis due to their inability to use water as an electron donor. Instead, they are capable of assimilating nitrogen, carbon dioxide and other organic compounds [56].

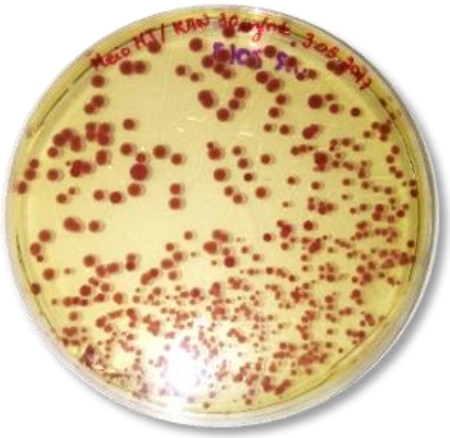


Figure 7- Morphology of *R. sulfidophilum* colonies in agar plate under aerobic conditions after 96 h incubation at 30 ºC. Photo acquired during the course of this experimental work.

Interestingly, unlike other facultative photosynthetic species, *R. sulfidophilum* synthesize a complete photosynthetic apparatus even under fully aerobic growth conditions, as well as under anaerobic growth conditions in the light, conflicting with the patterns of expression regulation in bacteria [56]. The photosynthetic membrane system is of the vesicular type and the pigments consist in bacteriochlorophyll α and carotenoids, most probably due to the spheroidene group [53, 54]. Table 3 summarize typical *R. sulfidophilum* characteristics.

*R*hodovulum *sulfidophilum*, as the name implies, are cells that are tolerant to large concentrations of sulfide, and unlike all other related species of purple non-sulfur bacteria, converts sulfide and thiosulfate to sulfate without the intermediate accumulation of sulfur [54].

In many aspects, *R. sulfidophilum* show high similarity with species of *Rho*dobacter genus and one of the most similar species is *Rho*dobacter *Capsulatus* which is used in many studies as a model to understand *R. sulfidophilum* features [53, 57]. Although *R. sulfidophilum* shares lots of features with *Rhodobacter* family, they can be undoubtedly distinguished based on their natural habitat. While *Rhodobacter* bacteria are found in freshwater and terrestrial environments, *R. sulfidophilum* is found only in marine and hypersaline environments [53].

Table 3- Summary of taxonomic and morphologic characteristis of *R. sulfidophilum* [54, 55]

Characteristic	Description
Cell shape	Ovoid to rod-shaped
Cell size	0.6-0.9 μm wide and 0.9-2.0 μm long
Cell Motility	Motile or non-motile
Flagellum type	Polar, varying from 0 to 4 in number
Gram classification	Gram-negative
Intra-cytoplasmatic membrane system	Vesicular type
Natural habitat	Marine and hypersaline environments
Photopigments	Bacteriochlorophyll α and carotenoids
Colonies color	Yellowish-brown to pinkish-red
Aerobic growth in the dark	Present
Anaerobic growth in the light	Present

During the last years, three distinct strains have been identified: W4, the type strain (DSM 1374, ATCC® Number 35886™, Taxonomy ID 1188256) which was first isolated from marine mud flats, W12 (DSM 2351) [53, 54, 58], PS88 which is well recognized by their high flocculation ability [58, 59] and P5 recognized as an hydrogen-producing photosynthetic strain [60].

This bacterium has been used in many fields by taking advantage of their metabolic characteristics. For example, it can be used as a cheap option of live feed at the aquaculture industry [61, 62], or as a eco-friendly means for hydrogen production [63, 64] or even as cheap tool for bioremediation including sardine wastewater treatment [65], crude oil degradation [62], among others [66]. Its peculiarity also leads to its use as a model for the study of mechanisms underlying anoxygenic photosynthesis [56, 57] and oxidative sulfur metabolism [67]. Furthermore, diversity in metabolic activities and the production of valuable products such as enzymes, proteins, and nucleic acids are great advantages of this bacterium in biotechnology [66].

1.4.2 Growth and flocculation

Multiplication of *Rhodovulum sulfidophilum* cells occurs by binary fission and the increase in cell density is accompanied by active secretion of polymers, including sugars, proteins and nucleic acids, and the establishment of structured communities of cells called flocs [58].

Over the years, many efforts have been made to understand the mechanisms underlying nucleic acids secretion in *R. sulfidophilum* and although the mechanism is not yet unveiled, many cases of nucleic acid secretion in bacteria have been used as comparative models [68–71].

The first supposition to explain the presence of nucleic acids in the extracellular medium of a cell culture is autolysis. However, the presence of nucleic acids in *R. sulfidophilum* extracellular medium does not appear to be linked to autolysis since cell viability seems to be maintained [68, 69]. But even if the presence of nucleic acids in the extracellular medium was a consequence of autolysis, it would not be a common autolysis like the one that occurs in the stationary phase of bacterial growth but rather a programmed autolysis where partial cell death might be induced by a high cell density, as happens in the case of Streptococcus *pneumoniae* [68, 69]. The cell lysis of *Streptococcus pneumoniae* is induced by the extracellular concentration of a secreted peptide pheromone by a quorum sensing mechanism [68, 69]. Quorum sensing describes one particular form of cell-to-cell communication, in which genes are expressed in a cell-density-dependent manner after a critical concentration of signal molecules (autoinducers) has been reached [70].

Another possible explanation for the existence of nucleic acids in the culture medium could be a horizontal exchange of genetic information between prokaryotes [71]. A Gene Transfer Agent (GTA), was discovered in *Rhodobacter capsulatus* as a system of genetic exchange [71]. The GTA resembles bacteriophages and works in a process similar to the phage-mediated gene transfer in which the nucleic acids are packed within the GTA [71]. In many other species of bacteria, including *R. sulfidophilum*, clusters of genes homologous to the GTA genes of *Rhodobacter capsulatus* have been found [71]. In studies aiming to unveil the mechanism of nucleic acid secretion on *R. sulfidophilum* it was found that this bacterium produces a GTA-like particle that indeed packs nucleic acids but lacks the gene transfer activity [71]. Like *Streptococcus pneumoniae,* the release of GTA in *Rhodobacter capsulatus* was reported to be a consequence of a cell density dependent-lysis of a subpopulation of cells in a process controlled by quorum sensing [71]. It is rational to risk that this is the mechanism underlying the presence of nucleic acids in the extracellular medium of *R. sulfidophilum*. However, there are evidences that do not corroborate this hypothesis since the nucleic acids present in the extracellular medium of *R. sulfidophilum* are not packed and the existent GTA-like particles have not a transfer activity [71].

Another example of a bacterial nucleic acid secretion system is from Pseudomonas *aeruginosa* in which the active DNA secretion has the main purpose of forming biofilms [69]. Similarly, in

the case of *R. sulfidophilum*, the nucleic acids secretion plays a key role in the establishment of biofilms [69]. Some studies had shown that secreted nucleic acids are essential not only for the maintenance of flocculated cells but also to the establishment of cell to cell contact, existing in the extracellular medium as a cell-to-cell interconnecting compound [69]. Furthermore, enzymatic deflocculation experiments demonstrated that despite flocculated cell are made of a miscellany of exopolymers (proteins, nucleic acids, and others), only nucleases can truly lead to deflocculation. This finding elucidated the role of nucleic acids on the flocculation of *R. sulfidophilum*. Actually, RNA and DNA were determined to be the major component in the extracellular polymers mix [72, 73]. Noteworthy, the flocculation mechanism is affected by the growth conditions since in rich mediums the flocculation appears to be decreased or even absent [68, 69].

In terms of characterization of the extracellular exopolymers and focusing on nucleic acids, the RNA fraction released into the culture medium contains mainly non-aminoacylated fully mature-sized tRNAs, what suggests an intracellular processing before secretion, and fragments of 16S and 23S rRNAs. The sequence of extracellular DNA fragments showed no differences from the intracellular genomic DNA sequence [68, 69].

In terms of stability of the extracellular nucleic acids, it is easy to understand the fact that they can maintain the stability on the extracellular medium as part of the floc. However, even in the soluble form they maintain the stability which is a consequence of the very low, or even undetectable, level of nucleases on the culture medium of *R. sulfidophilum* [74].

1.4.3 Fermentation conditions and metabolism

Three important characteristics of this bacterium must be considered regarding *R. sulfidophilum* cultivation. It is a halophilic, mesophilic and facultative aerobic photoautotrophic system [53]. The first two characteristics are clearly a consequence of the natural habitat. As marine bacteria, the presence of sodium chloride (NaCl) and mild temperatures are imperative for optimal growth. When first isolated it was settle down that it grows in 0.5 to 7.5 % of sodium chloride with 30 to 35 ºC as the optimal temperature [53, 54]. The pH of the culture could vary from 5 to 9 [55]. However, the optimal pH must be adapted according to the electron donor and the substrates used which can be exemplified by the reported shift in the optimal pH range from 6.5 to 8.0 on sulfide and from 5.0 to 7.5 on malate [54].

As mentioned before, *R. sulfidophilum* can be cultivated under both anaerobic light conditions and aerobic dark conditions. In both cases, extracellular nucleic acids are produced. The growth in anaerobic light conditions can be photolithotrophic or photoorganotrophic. For these two phototrophic conditions, the method of Hiraishi and Ueda [53] is commonly applied with or without slight modifications. In this method, the bacteria grow anaerobically at 30°C in screw-

cap test tubes or bottles filled with the medium under incandescent illumination (ca. 5,000 lux) [53]. Under such conditions, the culture is yellowish-brown [53].

The growth in aerobic dark condition is chemotrophic and the aerobic condition is commonly provided by rotary shaking. Under such conditions, the culture turns into red [58]. The set of organic compounds used by *R. sulfidophilum* as electron donor or carbon source depends both on the growth conditions, being anaerobic or aerobic, and on the strain. Methanol, citrate, mannitol, benzoate and tartrate are examples of organic compounds not used by the bacteria [53, 54, 58]. On the other hand, glucose, fumarate, lactate, pyruvate and glycerol are examples of organic compounds used by the bacteria in both aerobic and anaerobic conditions and by all the strains [53, 54, 58]. In terms of nitrogen sources, the ammonium salts are documented as the best source [54]. Growth factors requirements include B-complex vitamins such as biotin, niacin, and thiamin, however, the need for these growth factors can be well satisfied with yeast extract. Besides this, casamino acids and peptone are recognized as essential to its growth [53, 54, 58]. Table 4 summarizes some of the growth requirements.

Table 4- Summary of carbon sources, electron donor and other substrates for R. sulfidophilum cultivation [54, 55].

Category	
Sulfur sources	Sulphate, Thiosulphate, Cysteine
Vitamins requirement	Biotin, Niacin and Thiamin / Yeast extract
Nitrogen and amino acids source	Ammonium salts, peptone, casamino acids,
Carbon source (some examples)	Glucose, Fumarate, Lactate, Pyruvate, Glycerol

Pereira and coworkers [52] studied the best conditions for *R. sulfidophilum* growth and overexpression of hsa-pre-miR-29b. A semi-defined medium was tested (Table 5) with different sodium chloride concentrations (170, 342, 513, and 850 mM) and the ideal temperature for growth (25, 28, 30, 32, and 35 °C) was also tested. The study demonstrated that 513 mM is the ideal sodium chloride concentration and that under such salinity the ideal temperature is 30 ºC. The growth conditions established by Pereira and coworkers (2016) for the growth of *R. sulfidophilum* in shake flask experiments are illustrated in Figure 8.

In recombinant biosynthesis the growth environment in terms of growth medium composition and physical parameters (pH, temperature, and agitation) is decisive not only to achieve high cell densities but also to guarantee high productivity of the recombinant product [75]. As example, some nutrients may enhance the productivity by acting as precursors for a product or even by preventing the degradation of the product [75, 76]. Along this same line of thinking,

the growth environment must thus be developed concomitantly with the production outcomes since they will affect each other.

Table 5- Semi-defined medium composition for *R. sulfidophilum* DSM 1374 growth in aerobic dark condition [52].

Medium Component	Concentration	TES composition	Concentration
Triptone	10.0 g/L	$FeSO_4 \cdot 7H_2O$	20.0 mM
Polypeptone	5.0 g/L	$MnCl_2 \cdot 4H_2O$	20.0 mM
Yeast extract	0.5 g/L	$CoSO_4 \cdot 7H_2O$	20.0 mM
NaCl	513.0 mM	$CuCl_2 \cdot 2H_2O$	2.0 mM
K_2HPO_4	23.0 mM	$ZnSO_4 \cdot 7H_2O$	2.0 mM
KH_2PO_4	7.0 mM	H_3BO_3	9.7 mM
Glucose	278.0 mM	$NiCl_2 \cdot 6H_2O$	0.2mM
$CaCl_2$	0.3 mM	$Na_2MoO_4 \cdot 2H_2O$	0.2 mM
$MgSO_4$	0.8 mM		
$(NH_4)_2SO_4$	7.6 mM		
Trace elements solution	1 mL		

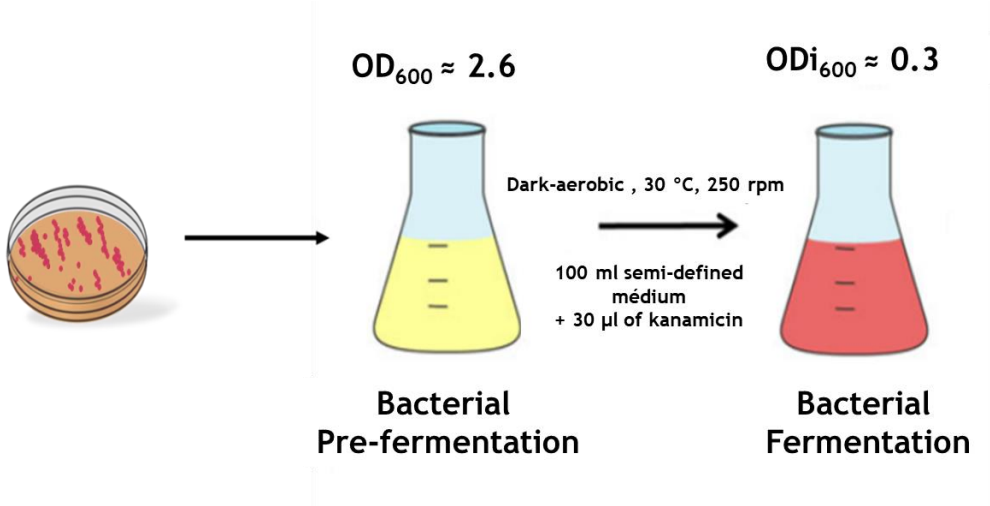


Figure 8 - Fermentation process of *R. sulfidophilum* in shake flask experiments. Adapted from [44].

1.4.4 RNA biosynthesis: *Rhodovulum sulfidophilum* versus *Escherichia coli*

Based on the data from Tables 2 and 3 it is safe to say that, in terms of recombinant RNA productivity, higher values are achieved when *E. coli* is the host. However, in a manufacturing bioprocess, productivity is not the only parameter to be evaluated. In fact, the host choice affects not only the productivity but all the other stages of the production process, including the USP and DSP. Therefore, the host choice may also depend on plasmid design, fermentation

conditions, and purification methodologies, which can be more adequate to the target recovery.

In terms of plasmid design, *R. sulfidophilum* offers more advantages since *E. coli* requires a plasmid design capable to avoid RNases degradation. In addition, the strategies for RNA protection from RNases either require a post-production procedure, as enzymatic cleavage or biosynthesize the RNA product in a nonlinear form. On the other hand, considering the fermentation time, medium and conditions, *E. coli* offer advantages since it grows faster and the growth medium and conditions are undemanding when compared with *R. sulfidophilum* [48, 50–52].

Considering the isolation and recovery of the target product, *R. sulfidophilum* offer much more advantages. Beside the isolation of intracellular RNAs, *R. sulfidophilum* offers the possibility to recover the secreted RNAs without the need of lysis procedures that usually involve hazardous chemicals and affect the RNA integrity and stability [36, 52].

Table 6 specifies the manufacturing process and the host that offer more advantages in each stage. The two stages in which *R. sulfidophilum* loses out to *E. coli* are closely related to the growth characteristics of this bacteria that, when compared with *E. coli*, is slow and fastidious. This slow growth implies that fermentations reaches low cell density or take longer to reach considerable cell density which in turn affects the RNA productivity. Hence, an optimization in the fermentation process of *R. sulfidophilum* could overcome this drawback and level up the biosynthesis of ncRNA.

Table 6- Comparison of *E. coli* and *R. sulfidophilum* through all the stages of recombinant RNA biosynthesis. Green tag represents the host with the best rating.

Manufacturing parameters	***E. coli***	***R. sulfidophilum***
Plasmid design		
Fermentation requirements		
RNA Isolation and recovery		
Contaminants		
RNA Productivity		
RNA integrity and stability		
TOTAL score	**2**	**4**

Based on this comparison, so far *E. coli* should be the choice when the aim is to obtain large amounts of RNA but *R. sulfidophilum* should be the choice when the RNA stability and integrity are a major concern [36].

1.5 Biosynthesis in bioreactors

Many high-value products, including therapeutic agents, are microbial fermentation by-products. Thus, microbial fermentation is an important practice for biotechnology and for the development of bioprocesses [28].

In a laboratory environment, shake flasks fermentations are the most common system for bacterial cultivation. However, it is limited in that it lacks the control of many parameters that play a key role in microbial growth and production. On the other hand, bioreactors fermentation technology is a more complex system that aims to control growth environments. Bioreactors control many parameters that affect the fermentation process like temperature, pH, dissolved oxygen, stirring among others [77].

1.5.1 Factors affecting biosynthesis

Nutrients, temperature, and pH are factors of extreme importance to the cell growth and to the biosynthesis and must be a concern when running an optimization of the fermentation process of a microorganism.

Nutrients must satisfy the elemental requirements in terms of energy supply for cell maintenance and biosynthesis. In an aerobic fermentation, this process is represented by Figure 9 [78].

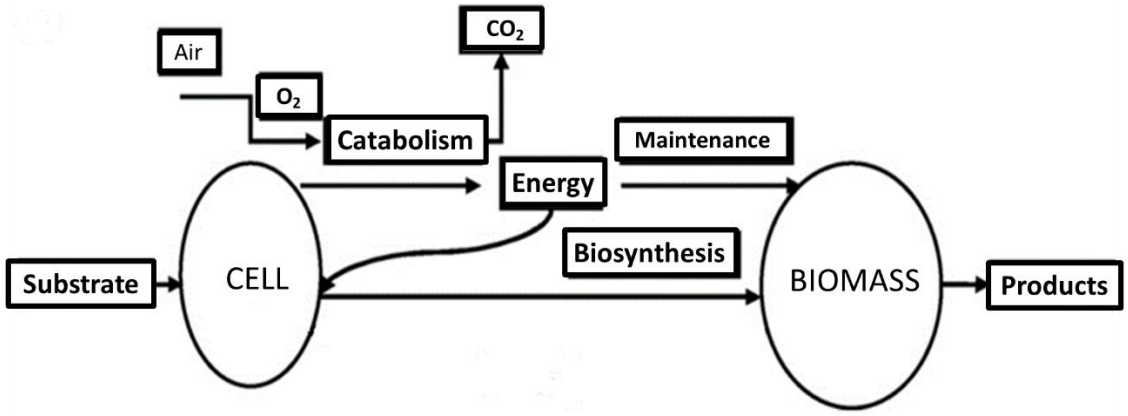


Figure 9- Schematic mechanism of the aerobic process of energy generation, biomass formation and product synthesis in microbial cultures. Adapted from [79].

The conversion of a carbon source into biomass and energy consists of its transport to the cell and subsequent degradation in simpler molecules by central metabolic pathways [80, 81]. Carbohydrates, such as glucose, can be catabolized by different pathways (such as Glycolytic, Entner-Doudorof, and Pentose-Phosphate). These pathways lead not only to energy production in the form of Adenosine Triphosphate (ATP) and reduced coenzymes but also to the biosynthesis of monomers of different macromolecules constituting the cells (proteins, nucleic acids, lipids and polysaccharides) [80, 81]. On the other hand, lipids, proteins, and other

carbohydrates can be converted to several intermediates of carbohydrates catabolic pathways and slip into the catabolic pathway through a multitude of side doors [82].

A good example is glycerol (1,2,3-propanetriol, glycerin), a by-product of transesterification of vegetable oils and animal fats, which share with glucose the same metabolic pathway from glyceraldehyde-3-phophate to pyruvate and the respective derived products. Yet, likewise with glucose, other pathways can be used by bacteria to its metabolization. Its degree of reduction per carbon, *k*, is significantly higher ($C_3H_8O_3$: k = 4.67) than for sugars such as glucose ($C_6H_{12}O_6$: k = 4) or xylose ($C_5H_{10}O_5$: k = 4) [76, 83, 84]. The high *k* provides a distinct advantage over more oxidized carbohydrate-based carbon sources in the production of reduced chemicals. Actually, by the view of industrial microbiology, glycerol has been considered a promising carbon source. Besides being cheap, many microorganisms can naturally utilize glycerol thanks to its abundant occurrence in nature. One of the promising applications for the use of glycerol is its bioconversion to high value compounds through microbial fermentation assuming as a promising carbon source to the industrial microbiology [76, 83, 84].

Oxygen is a crucial substrate of aerobic fermentations which is needed for growth, maintenance and metabolic pathways, including product synthesis. Therefore, oxygen must be continuously provided by a gas phase to assure the correct oxygen supply to the cells. The concentration of dissolved oxygen in the culture medium depends on the oxygen transfer rate from the gas to the liquid phase, and on the oxygen uptake rate which represents oxygen consumption by the microorganism [79]. Agitation is required to ensure homogeneous distribution of the nutrients. In shake flasks scale, oxygen transport by aeration and agitation are accomplished by the action of the shaker apparatus. In bioreactors, oxygen is commonly supplied as compressed air and distributed by a gas distributor, and mechanical devices are used to improve mixing of the culture medium [79]. The oxygen is transferred from a suspended gas bubble into a liquid phase, where it is taken up by the microorganism and finally transported to the site of reaction inside the cell where it is used to energy generation, biomass production and biosynthesis (Figure 9). During the fermentation process, the oxygen requirements depend on many factors such as the metabolic activity, the changes in the medium viscosity and with the foam formation [78]. For example, oxygen uptake rate commonly presents an increase during the exponential stage and a decrease during the stationary stage as a function of the metabolic activity [79].

Temperature, like essential chemical elements and organic substrates, play a key role in the bacterial growth and thus in the productivity in terms of natural and recombinant by-products. Since it affects all chemical and biochemical process it is a potential limiting factor for bacterial growth and, at some point, govern substrates consuming kinetic. However, while the temperature is always a factor in microbial growth, respiratory rate, and organic carbon assimilation, it is not always the only factor or even a dominant one [85].

Likewise, each microorganism has an optimal pH in which they experience ideal growth. However, pH optimization in recombinant product-formation should be a compromise between the microorganism optimal pH and the pH that assure the stability of the target recombinant products [76].

1.5.2 Fermentation strategies

Basically, fermentation can follow three different strategies: Batch, fed-batch and continuous fermentation. Batch fermentation represents a simple and robust strategy characterized by no addition or removal of nutrients (Figure 10). The initial medium composition is not altered during fermentation resulting in the interruption of growth, product formation, and substrate utilization after a certain period. After the initial nutrients are consumed, the culture stops growing or, in some cases, microorganism metabolism may shift and begin to consume other metabolites in the culture broth, what in turn may alter the patterns of biomass formation and productivity [76, 86].

Otherwise, continuous fermentation guarantee that growth and product formation last more, since continuous nutrient medium is added to the fermentation while cells and products are also withdrawn (Figure 10) until a moment that the system reaches a steady state where substrates, products, and cells concentration are constant. The idea of continuous fermentation seems promisor for bioprocess intending to obtain a large quantity of a fermentation by-product. However, besides the higher probability of mutation and contamination, in cases of expression systems based on plasmids more instability is reported [76, 86].

The fed-batch strategy appears as an intermediate between batch and continuous fermentation. Fed-batch starts as a batch approach and then the metabolism is controlled by a feed (Figure 10) that can follow different strategies. The feed can be either the initial medium or a concentrated solution of the growth limiting substrate(s) and can also follow different feed profiles (constant, exponential and stepwise). Besides the increase in the fermentation duration, the overall reactor productivity is increased [76, 86].

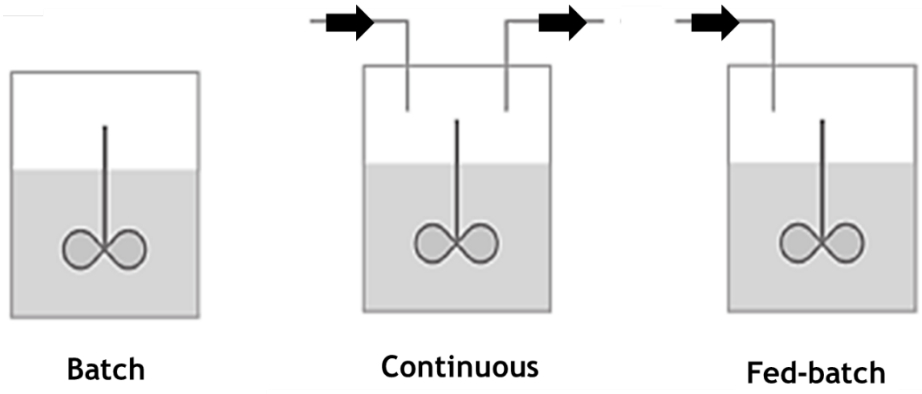


Figure 10 - Fermentation strategies in bioreactor. Arrows represent addictions and removals to the system.

The decision towards a fermentation strategy must take in account many factors such as economic balances, type of product, desired productivity, type of microorganism and its vulnerability to mutation, stress exposure and metabolic shifts [87].

1.5.3 Microbial growth

The typical growth of a bacterial culture usually follows a pattern like the growth curve shown in Figure 11. The curve can be divided into six well defined phases [88, 89]: The lag phase indicates the time that the bacteria require to adapt to the new environment. It is characterized by long generation time, zero growth rate and maximum rate of metabolic activity. The acceleration phase indicates the end of the adaptation period and the beginning of cell generation. It is characterized by decreasing generation time and increasing growth rate. The exponential phase, also known as log phase, is represented by minimal but constant generation time and maximum rate of substrate utilization. Declining phase represents a slowing in the growth because of gradual decrease in substrate concentration as well as increased accumulation of toxic metabolites. This phase is characterized by increased generation time and decreased growth rate. The stationary phase indicates stagnation of the microbial population generally because of depletion of the substrate, maximum physical crowding, a higher concentration of toxic metabolites and/or balance between growth and death rate of biological cells. The endogenous decay is the phase in which death rate exceeds growth rate. The phase is characterized by endogenous metabolism and cell lysis and is usually the inverse of the exponential growth phase.

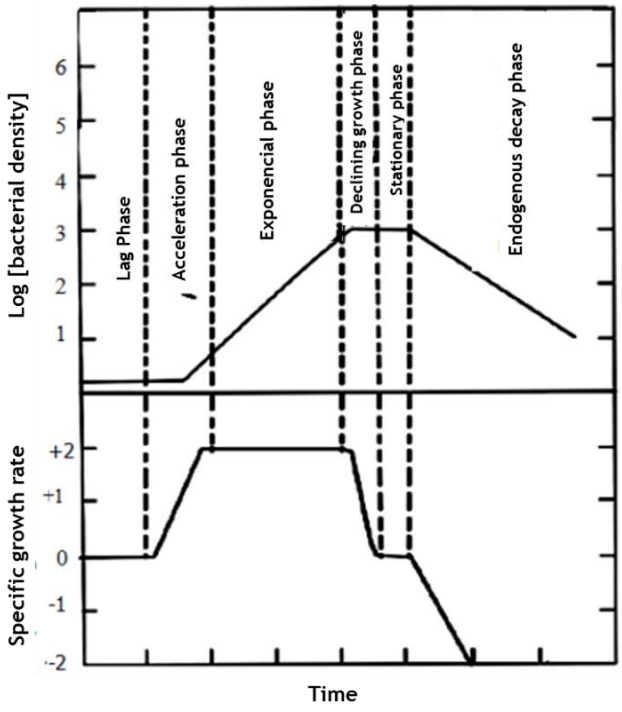


Figure 11 - Typical bacterial growth curve: the phases and its respective specific growth rate. Adapted from [89].

The growth rate of a batch culture under the exponential phase is generally believed to follow the first order kinetic model in which the growth rate is proportional to the microbial mass in the system. Mathematically expressed by Equation 1 where μ represents the specific growth rate (h^{-1}), t is the time (h), and X is the biomass concentration (g/L) [88–90].

$$\frac{dX}{dt} = \mu X \qquad (1)$$

Summing up, the main goal of the biosynthesis processes in microorganism is the transformation of substrates into the desired metabolic products. This requires well-controlled conditions, which can be supplied by bioreactors platforms, where several factors should be considered such as nutritional control, the type of process (batch, fed-batch, continuous), temperature, pH, and oxygen supply control. The process outcomes, in terms of growth and productivity should suggest a direction for the process optimization [28, 76].

Chapter 2 - Motivation and Objectives

Given the important role of small ncRNAs in many cellular processes and the relationship between their deregulation pattern and pathology scenarios, there is an increasing interest to find new biomethods for RNA synthesis. Recombinant biosynthesis of RNA has been shown to be an economical and easy alternative to produce RNAs in adequate amounts to support this increasingly RNA-centered research. The biotechnological process of RNA biosynthesis in microorganisms includes several steps being all of them important to the final yield of the target product.

The fermentation step is of extreme importance to ensure a high yield of the target product since the product formation can be seen as a function of biomass formation and substrate consumption. As these last two conditions depend on the performance of the fermentation process, its optimization will guarantee greater productivity and reproducibility.

The performance of a fermentative process requires a good knowledge of the host microorganism as well as the factors that affect its growth and biosynthesis pathways. *Rhodovulum sulfidophilum* DSM 1374, due to its peculiarities has been used for the biosynthesis of RNAs and has been highlighted as a new opportunity for the industrial production of RNAs. The bioprocess of recombinant RNA production in this strain has been increasingly explored by the scientific community.

The ultimate goal in bioprocess development is commercial production. The optimization of bioprocesses in commercial scale is usually unaffordable, so optimization is often performed in smaller scale bioreactors. An optimized small-scale process can then be transferred to pilot scale following established scale-up strategies.

Therefore, the overall aim of the present thesis was to study the recombinant biosynthesis of small ncRNAs in *R. sulfidophilum* in bench-top mini-bioreactors.

The specific aims of this study were:

- To optimize the growth conditions of *R. sulfidophilum* in bioreactor;
- To identify physical and nutritional factors that may affect the growth of *R. sulfidophilum* in bioreactor and consequently the biosynthesis of ncRNAs;
- To analyze the biosynthesis of ncRNAs in bioreactor through eletrophoretic and spectrophotometric methods.

Chapter 3 - Materials and Methods

3.1 Materials

Ultrapure reagent-grade water was obtained from a Mili-Q system (Milipore/Waters). For *Rhodovulum sulfidophilum* DSM 1374 culture, it was used tryptone and yeast extract from Biokar (Beauvais, France), $CaCl_2.2H_2O$, NaCl and K_2HPO_4 from Panreac (Barcelona, Spain), $MgSO_4$, glucose, KH_2PO_4 and $FeSO_4.7H_2O$ from Sigma-Aldrich (St Louis, MO, USA), kanamycin and H_3BO_3 obtained from Thermo Fisher Scientific Inc. (Waltham,USA), Glycerol from VWR and agar from Pronalab (Mérida, Yucatán). For RNA methodologies it was used isopropanol from Thermo Fisher Scientific Inc. (Walthan, EUA), Chloroform from Merk (Darmstadt, German) Trizol Reagent from Sigma-Aldrich (St Louis, MO, USA) and green safe from NZYTech (Lisbon, Portugal). Solutions were freshly prepared using 0.05 % Diethyl pyrocarbonate (DEPC) treated water. DEPC was purchased in Fluka (St Louis, MO, USA). All the materials used in the RNA methodologies were RNase-free. H_2SO_4 was acquired from PancReac AppliChem (Darmstadt, Germany) acetic acid glacial and citric acid both analytic reagent grade from Thermo Fisher Scientific Inc. (Walthan, EUA), formic acid from Chem-Lab NV (Zedelgem, Bélgica), Lactic acid from Sigma-Aldrich (St Louis, MO, USA).

3.2 Bacterial cultivation

3.2.1 Bacterial strain and culture media

R. sulfidophilum, strain DSM 1374, was used in this study. This bacterium was previously transformed by heat-shock with pBHSR1-RM vector (genetically modified with pre-miR29b-1 sequence) [50, 52]. The selection of this strain transformed with a small ncRNA sequence is in accordance with this study objectives since it allows to evaluate the biosynthesis of the ncRNA in the target bacterium.

Unless otherwise stated, cultivation of *R. sulfidophilum* cells in liquid media was made in a semi-defined medium, developed by Pereira and co-workers [52], containing 10.0 g/L tryptone, 5.0 g/L polypeptone, 0.5 g/L yeast extract, 10 g/L glucose, 513.0 mM NaCl, 23.0 mM K_2HPO_4, 7.0mM KH_2PO_4, 0.8 mM $MgSO_4{\cdot}7H_2O$, 0.3 mM $CaCl_2{\cdot}\ 2H_2O$, 7.6 mM $(NH_4)_2SO_4$ and 1 mL of TES. The TES is composed by 20.0 mM $FeSO_4{\cdot}7H_2O$, 20.0 mM $MnCl_2{\cdot}4H_2O$, 20.0 mM $CoSO_4{\cdot}\ 7H_2O$, 2.0 mM $CuCl_2{\cdot}2H_2O$, 2.0 mM $ZnSO_4{\cdot}7H_2O$, 9.7 mM H_3BO_3, 0.2mM $NiCl_2{\cdot}6H_2O$, and 0.2 mM $Na_2MoO_4{\cdot}2H_2O$ in 0.5 N HCl.

During this work, specific adjustments in relation to the carbon source of the medium above described were tested. Therefore, each medium variation tested in terms of carbon source

receives a designation according to Table 7. Noteworthy, GLU10 correspond to the medium above described.

Table 7 – Liquid media designations according to the carbon source type and concentration

Medium designation	Carbon source	Concentration (g/L)
GLU10	Glucose	10.0
GLY10	Glycerol	10.0
GLY20	Glycerol	20.0
GLY5	Glycerol	5.0
GLY2.5	Glycerol	2.5

For the growth in solid media, a selective agar plate (5 g/L yeast extract, 10 g/L glucose, 20 g/L NaCl, 4.1 g/L $MgCl_2$, 10 g/L polypeptone and 15 g/L agar supplemented with 1 mg/L $ZnSO_4.H_2O$, 10 mg/L $MnO_4S.4H_2O$, 10 mg/L $FeSO_4 \cdot 7H_2O$ and 30 µg/mL kanamycin) was used. At 30 ºC, colonies formation takes about 84-96 h.

3.2.2 Fermentation conditions

For the inoculum, a stride from a selective plate was inoculated into 100 mL of the medium in a 0.5 L shake flask which was incubated at 30 ºC and 250 rpm under aerobic-dark conditions until the cell growth reached the exponential phase – Optical Density at 600 nm (OD_{600}) of about 2.6 (Figure 12B).

Bioreactor fermentation was operated in 0.75 L bench-top parallel mini-bioreactors (Infors HT, Switzerland) with strictly controlled parameters including pH, temperature, airflow, agitation and dissolved oxygen (Figure 12C). The working volume was set as 0.25 L. Unless otherwise stated the reactor was operated at 30 ºC under dark-aerobic conditions, pH was maintained at 7.0 by automatic addition of 0.75 M H_2SO_4 and 0.75 M NaOH. The volume of inoculum was set based on Equation 2 in which $V_{inoculum}$ is the inoculum volume to be added to the fermentation medium accordingly to the OD of the inoculum ($OD_{inoculum}$) to achieve a specific initial OD in the fermentation ($ODi_{fermentation}$).

$$OD_{inoculum} \times V_{inoculum} = ODi_{fermentation}\left[V_{inoculum} + V_{fermentation}\right] \qquad \textbf{(2)}$$

The pO_2 (dissolved oxygen percentage) was controlled by a two-level cascade of stirring (between 250 and 600 rpm) and air flow (between 0.2 and 2 vvm). Foaming was controlled manually by the addition of the antifoam agent Antifoam A (DOW CORNING® Q7-2587, 30% Simethicone Emulsion USP).

For fed-batch fermentations, a concentrated solution of glycerol (Glycerol 100 g/L) was added in a rate of 1 g/L/h. The velocity needed to meet the desired glycerol feeding rate was set accordingly to the peristaltic pumps' capacities (mL/h).

Samples from the fermentation broth were collected at specific fermentation periods and treated in accordance with the method used for RNA extraction, HPLC assessment or other analysis. At the end of the fermentation, all cells were harvested, recovered by centrifugation and stored at -20 ºC.

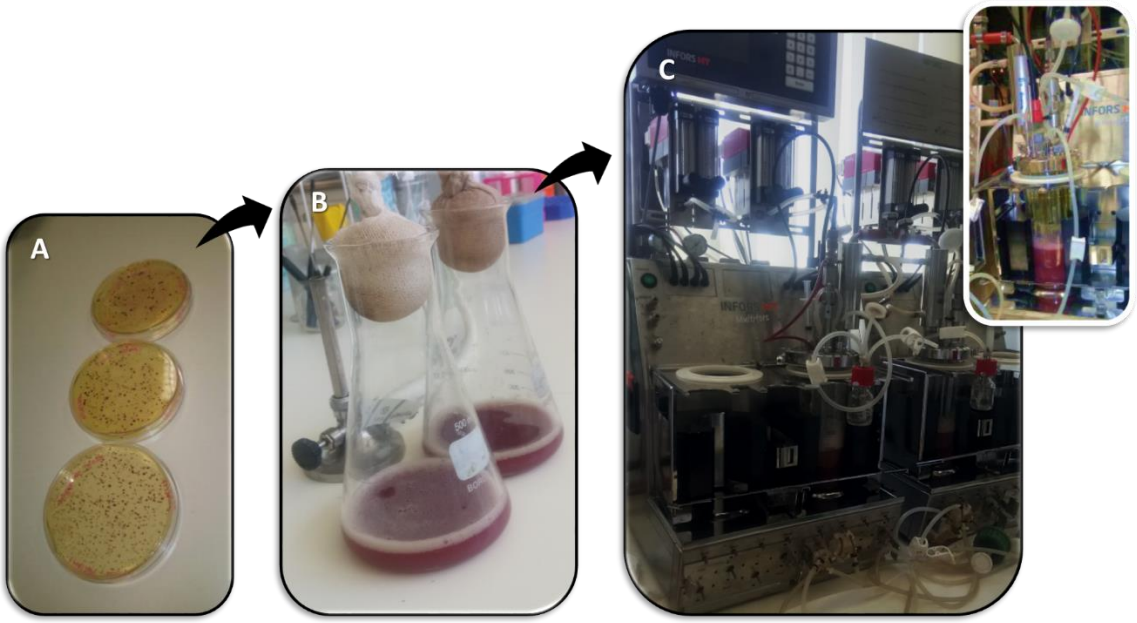


Figure 12 – Cultivation of *R. sulfidophilum*. (A) Cultivation in solid media; (B) Inoculum in shake flask; (C) Fermentation in bench-top mini-bioreactor.

3.3 Bioprocess analysis

3.3.1 Cell growth

Cell growth was measured by monitoring the OD_{600} of the culture. The cell dry weight (CDW) was determined according to [91], where biomass was collected from 1 mL of culture broth by centrifugation at 10,000 rpm for 10 minutes (min) in pre-weighed tubes. Then the pellet was washed three times with an equal volume of deionized water. Pellets were dried at 85 ºC for 2 h and maintained in a desiccator until constant weight. The CDW was calculated from the average of two independent samples. CDW was defined by the dry weight of cells per L of culture broth.

The OD_{600} was converted into DCW using a standard curve (Figure 13) in which 1 unit of OD_{600} corresponds to 0.31 g/L of CDW.

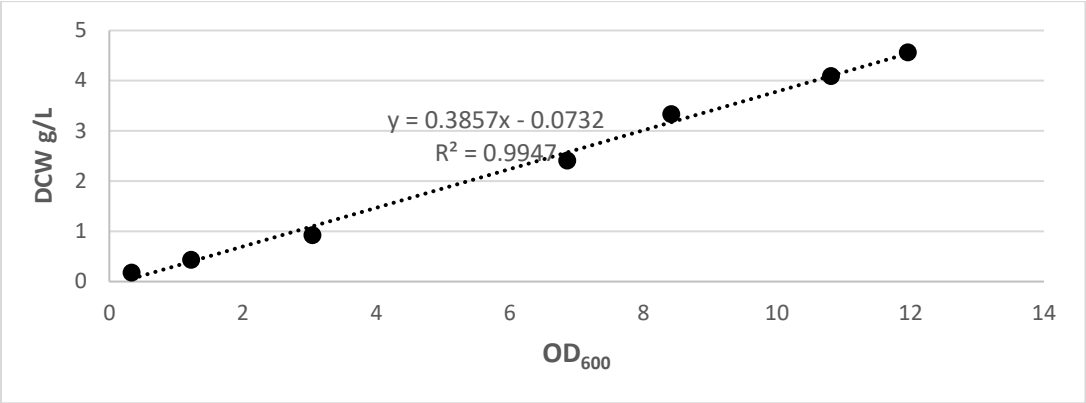


Figure 13 - Standard curve for the relationship between DCW of *R. sulfidophilum* DSM 1374 versus OD_{600}.

Specific growth rates (μ) were determined by equation 3 [92], where N_t is the OD_{600} (or DCW) at *t* hours (h), N_{t0} is the OD_{600} (or DCW) at the beginning of the exponential phase, t_0, and Δt is the time variation $t - t_0$ (in hours).

$$\mu\,(h^{-1}) = \frac{\ln(N_t) - \ln(N_0)}{\Delta t} \qquad (3)$$

3.3.2 Glucose, glycerol, and organic acids assessment

The quantity of the main carbon source over the fermentation, as well as the formation of organic acids and ethanol along the fermentation, was assessed by High Performance Liquid Chromatography (HPLC). For the assessments, samples were retrieved at specific times and centrifuged at 12000 g for 5 min at 4 ºC. The resulting supernatant was then filtered through a 0.22 μm filter for HPLC injections [93, 94].

Quantification was carried out using an Agilent 1290 Infinity LC HPLC system (Waldbronn, Germany) coupled with a Refractive Index Detector (RID) (Agilent 1260 Infinity). Compound separation was done using a Hi-Plex H ion-exchange analytical column (Agilent, Santa Clara, CA, USA) with a 7.7 x 300 mm and 8 μm pore size. The mobile phase consisted of a 5 mM H_2SO_4 solution prepared with ultrapure water, filtered through a 0.2 μm pore membrane and degassed for 15 min before use. The flow rate was set to 0.6 mL/min and the column temperature was set to 55 ºC with an injection of 20 μL. The autosampler of RID was maintained at 4ºC to minimize the degradation of any compound in solutions.

The detection of compounds was ranged between 1 and 12 g/L for glucose (Figure 14A), between 0.02 and 15 g/L for glycerol (Figure 14B), and between 0.001 and 5 g/L for acetic acid (Figure 14C). Time retention through the method above described for glucose, glycerol and acetic acid was 9.6, 13.3 and 15.4 respectively.

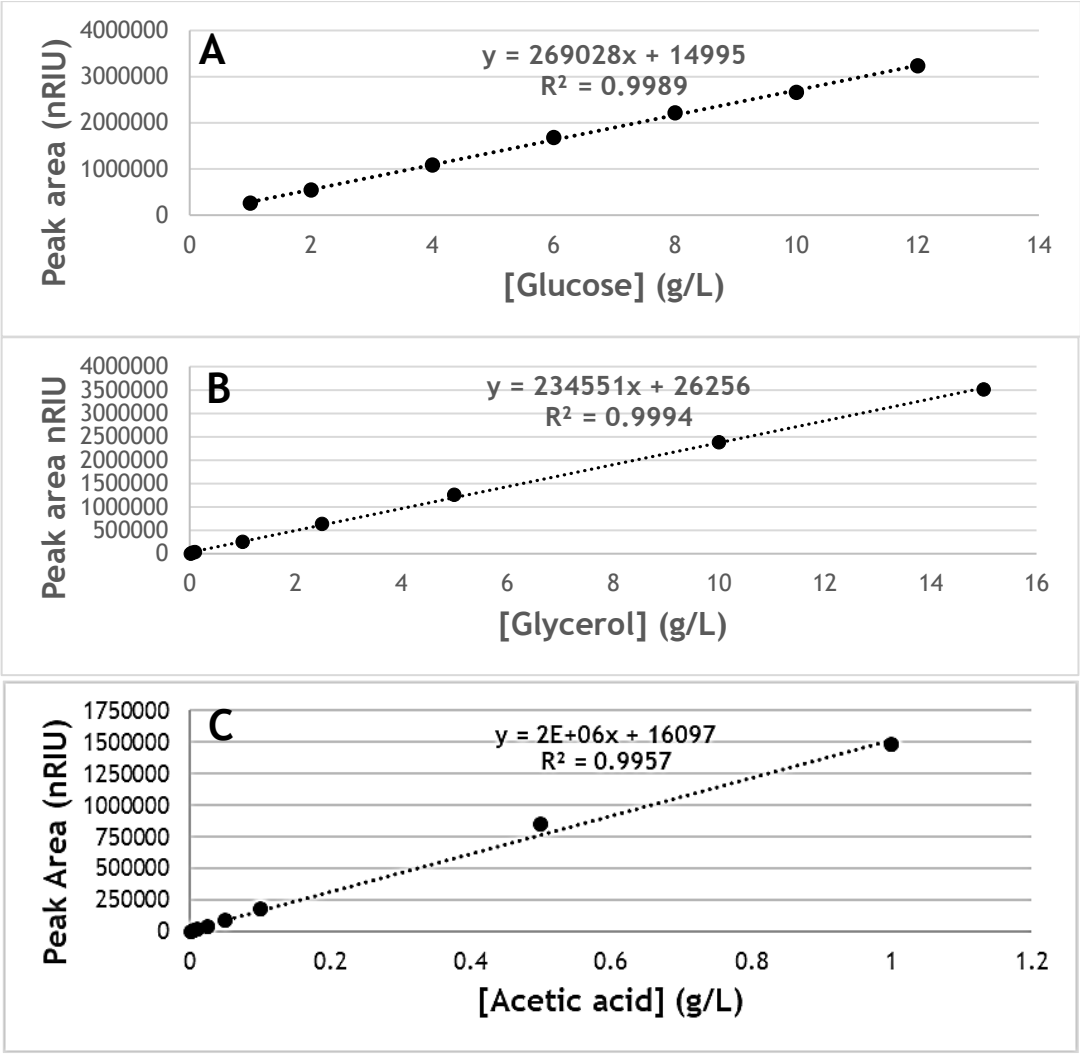


Figure 14 - Standard curve for HPLC-RID quantification of (A) glucose, ranged from 1 to 12 g/L, (B) glycerol, ranged from 0.02 to 15 g/L, and (C) acetic acid, ranged from 0.001 and 5 g/L.

3.3.3 Nucleic acids methodologies

3.3.3.1 Intracellular RNA isolation and quantification

RNA isolation from bacterial pellets was performed with TRIzol® Reagent which is a monophasic solution of phenol and guanidinium isothiocyanate that simultaneously solubilizes biological material and denatures proteins. The first step was to homogenize the sample (pellet) with TRIzol™ Reagent until the formation of a viscous homogenate. In order to quantify RNA over the fermentation period, the samples were retrieved at specific time-points and normalized to the same number of cells based on the OD_{600}. After homogenizing the sample with TRIzol™ Reagent chloroform is added, and after a vigorous shake, homogenate starts to separate in different layers. The distinct separation of the layers occurs after centrifugation at 15000 g for 15 min at 4 ºC, resulting in a clear upper aqueous layer (containing RNA), a creamy thin interphase, and a red lower organic layer (containing the DNA and proteins and Trizol). The

aqueous phase is then carefully transferred to new tubes without disturbing the layers separation. The RNA in the tubes was precipitated with isopropanol and washed with 75% of ethanol. The air-dried total RNA pellet was solubilized in 0.05% DEPC-treated water and the quantity was estimated spectrophotometrically using a NANOPhotometer (Implen, GmbH, Germany). TRIzol extraction is known as an effective method for isolating small RNAs such as microRNAs, piwi-associated RNAs, or small interfering RNAs [95].

3.3.3.2 Genomic DNA quantification

The concentration of genomic DNA (gDNA) was obtained by real-time qPCR in an iQ5 Multicolor Real-Time PCR Detection System (BioRad). The primers used in these experiments were 5′ - ACACGGTCCAGAACTCCTACG-3′ (forward) and 5′-CCGGTGCTTCTTCTGCGGGTAACGTCA-3′ (reverse) for the amplification of a 181-bp fragment of the 16S rRNA gene. PCR amplicons were quantified by following changes in fluorescence of the DNA binding dye Maxima® SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher Scientific Inc.). The calibration curve to achieve the gDNA concentration was constructed accordingly to [52]. Each sample was run in triplicate, and CT values were averaged from the triplicate. The final data were averaged from three independent experiments.

3.3.3.3 Agarose gel electrophoresis

Nucleic acids evaluation was carried out by horizontal agarose gel electrophoresis in which separation was accomplished by a 15-cm-long 1% agarose gel, stained with 0.012 µL/mL greensafe premium (NZYTech, Lda. – Genes and Enzymes, Lisbon, Portugal). The gel was running in TAE buffer for 30 min at 110 Volts. The nucleic acids bands were visualized under UV light in a Vilber Lourmat system (ILC Lda, Lisbon, Portugal).

3.3.3.4 Urea-PAGE

The quality and integrity of oligonucleotides were assessed by vertical denaturing electrophoresis with a 12.5 % polyacrylamide gel with urea as a denaturant agent accordingly to [52]. The denaturing gel is prepared with urea (8M), 10x TBE buffer, deionized water, and 30% polyacrylamide solution (29:1) and the polymerization was provided by ammonium persulfate and Tetramethylethylenediamine. The complete gel polymerization occurs in 20-30 min. Before the samples loading it was imperative to pre-run the gel for 20-30 min and rinse the wells. The samples for Urea-PAGE analysis were prepared by heat denaturation at 55 °C for 5 min and by adding 10 ul of loading mix. The loading mix contains 90% formamide (v/v) with 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanole. The run is performed at 130 volts for 100-120 min or until the dye front reached the end of the gel. Before bands visualization, the gel is stained on 1x TBE solution with 10 % green-safe (v/v) by 15 min and the bands are visualized under UV light. The vertical electrophoresis system and the powerpac are from Bio-Rad and the band visualization was performed on a Vilber Lourmat system (ILC Lda, Lisbon, Portugal).

Chapter 4 - Results and discussion

To design the strategies for recombinant biosynthesis involves knowledge of the behavior of the host strain but also of the recombinant product. It has been shown that even subtle changes at the fermentation process can powerfully interfere with productivity [96, 97]. Therefore, there is a trend to standardize as much as possible the fermentation processes. The optimization of the fermentation aims to achieve higher cell density always considering the productivity and quality of the target product.

4.1 Batch fermentation of *R. sulfidophilum*

Bioreactor cultivation offers many advantages for recombinant biosynthesis as long as contamination is avoided, and the stability of the strain is guaranteed. The advantages include simplicity of culture control, homogeneity of the production, constancy of culture conditions and the possibility to collect samples without compromise the sterility. Initially, to better evaluate the performance of the strain in bioreactor scale and determine the growth parameters, the strain was first grown in the controlled batch reactor at aerobic-dark conditions.

To fresh-start the fermentation experiments of this bacterium in a bench-top parallel mini-bioreactors it was important to determine the work volume. The working volume was set at 250 mL as a compromise between the work volume range of the mini-bioreactors (180 to 500 mL) and the typical low growth rate of the bacteria. Then, the first observations from bioreactor experiments were the visual parameter control like foam formation, flocculation, and broth color. During the fermentation of *R. sulfidophilum* in bioreactor, it was necessary to add antifoam at least once, but the foam formation was not really a concern since it was not dense and fade easily with small antifoam adding. At the end of fermentations, it was not observed flocculated cells since the broth was homogeneous without deposited cells. The broth color was pink likewise at the shake flask. Noteworthy, the pinkish coloration follows the cell growth even getting red-pinkish at the end of the fermentations.

4.1.1 Effect of inoculum size, temperature and oxygen supply

Previously, Pereira and coworkers [52] optimized nutritional and physical conditions for the aerobic dark cultivation of *R. sulfidophilum* in 100 mL shake flask scale. More precisely, the medium composition, the temperature and the salinity were optimized. These best conditions that allowed to achieve a maximum biomass (X_{max}) in small scale, were the starting point for batch fermentation of this bacteria in a mini-bioreactor platform.

Herein, the effect of inoculum size, temperature, and oxygen supply on both the X_{max}, time to achieve the maximum biomass (t_{max}) and μ were tested changing one factor at a time.

The inoculum size determines the initial biomass (X_i). For the shake flask scale, the inoculum size for this strain was set to 13% (v/v) in order to start the fermentation with an OD_{600} of 0.3 [52]. However, the ideal X_i can vary at a different scale. Therefore, fermentations with different inoculum sizes were tested (Table 8, black tags). Besides the one used in shake flask scale, three other inoculum sizes were tested, 18%, 24% and 30 %. Figure 15 illustrates the growth at these different inoculum sizes. With the intend to monitor and interpret the influence of the inoculum size, it was important to grow the inoculum cultures consistently assuring similar physiological state of the cells in the inoculum.

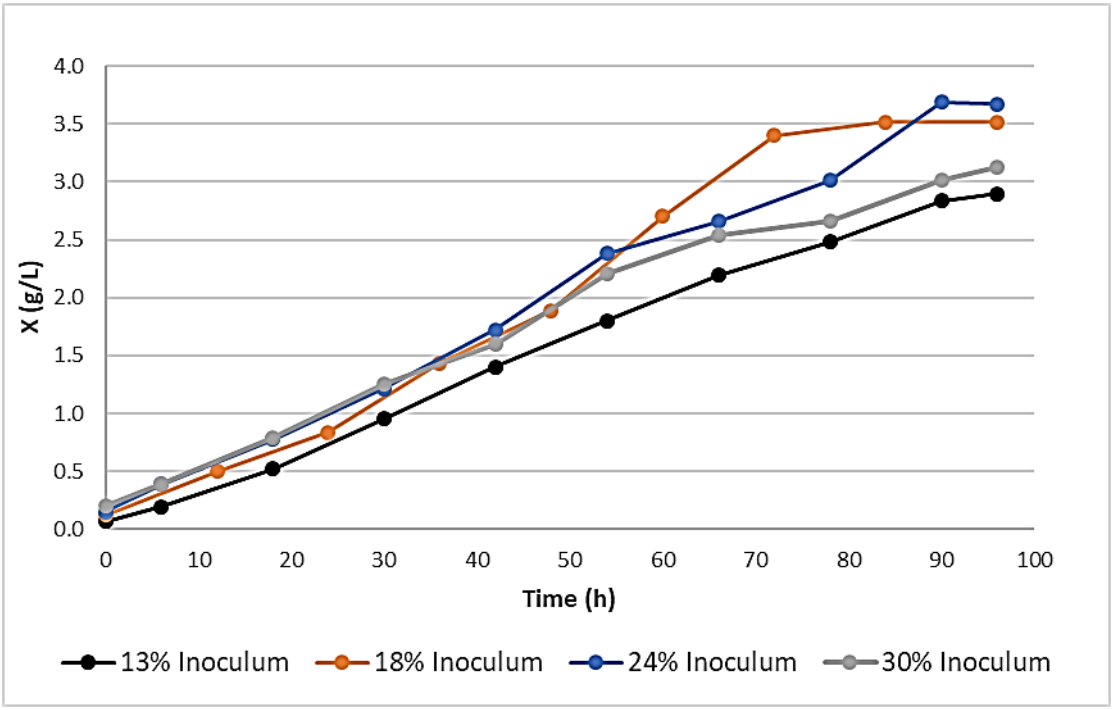


Figure 15 – Effect of the inoculum size on growth of *R. sulfidophilum* DSM 1374 in mini-bioreactors cultivation in GLU10 medium, under aerobic conditions, in the dark at 30 °C.

Considering the specific growth rate, the results between the different inoculum sizes are similar and in the order of hundredths, mostly due to the characteristic long periods of fermentation of these bacteria (Table 8). Examples of slow growth are both the study of Watanabe and co-workers [58, 98] where after 100 hours of fermentation the higher biomass obtained was around 1 g/L of culture broth.

Despite the growth profile and μ are quite similar between the different inoculum sizes, there is a slight difference in the X_{max} (Table 8). Interestingly, the inoculum size used in the shake flask scale, 13%, lead to the lower X_{max} and higher inoculum size, 30%, does not lead to higher X_{max} (Table 8). This result is in accordance with results achieved in other studies describing the

effect of inoculum size in the bacterial growth profile in which higher inoculum size not necessarily lead to higher X_{max} and μ (Table 8) [99].

There are some published studies which suggest that the inoculum size do not has a direct influence on the prediction of growth [100]. Pin and Barancy [101] reported that very small inoculum sizes are usually reflected in a longer lag phase on *E. coli* cultivation and in a more distribution of injury to the small population of cells (known as "low inoculum size effect"). On the other hand, Miller and Bassler demonstrated that from a specific inoculum size threshold a "high inoculum effect" [100] is observed in *E. coli*, *Salmonella typhimurium,* and *Vibrio harveyi*. Quorum sensing is one of the mechanisms suggested to support such effect [100]. It is a particular form of cell-to-cell communication that enables the entire population, or a particular subset of cells, to take advantage of the opportunities arising from a certain population density or to overcome the problems related with it [102]. Some studies demonstrated that *R. sulfidophilum* growth are controlled by a quorum sensing system [69, 71, 103] which suggest that the lower biomass obtained from 30% inoculum could be explained by this mechanism.

Table 8 - Summary of fermentations outcomes of *R. sulfidophilum* DSM 1374 from cultivation in mini-bioreactors in GLU10 medium under aerobic conditions in the dark. Condition 1 to 4: (Black tags) effect of inoculum size; Conditions 5 and 6 (blue tags): effect of temperature; Condition 7 (grey tags): effect of oxygen availability.

Condition Run	Temperature (ºC)			Oxygen supply		Inoculum size (%)				μ (h^{-1})	X_{max} (g/L)	t_{max}(h)
	25	**30**	**37**	**Yes**	**No**	**13**	**18**	**24**	**30**			
1										0.026	2.89	96
2										0.029	3.51	96
3										0.026	3.69	90
4										0.024	3.13	96
5										0.020	2.34	90
6										0.022	2.57	78
7										0.021	2.23	84

The best results were achieved from fermentations with 18% and 24% of inoculum. They presented similar results in terms of X_{max}. However, considering the growth profile, the exponential phase in 18% presents higher biomass values and allows to achieve X_{max} with a lower t_{max}. Based on this, 18% of inoculum was set to start the next fermentations.

The inoculum size experiments were performed with the optimal temperature previously optimized for the shake flask scale, which was 30 ºC. Herein, two other temperatures were tested, starting the fermentation with 18% of inoculum (Table 8, blue tags), namely 37 ºC and 25 ºC. Figure 16 illustrates the effect of temperature on the growth profile.

The results suggest that lowering the temperature from 30 ºC to 25 ºC slows down the growth and leads to lower $X_{max.}$ On the other hand, the growth profiles at 37 ºC is quite similar to the growth profile at 30 ºC up until about 60 h of fermentation. However, after that time the growth at 37 ºC slows down and reaches, even earlier, a X_{max} similar to the one reached at 25 ºC. These findings are in accordance with experiments carried out to determine the effects of temperature on the growth of *R. sulfidophilum* at shake flask scale by Pereira and co-workers in which cultivations at a range of temperature between 25 ºC and 35 ºC, presented 30 ºC as the optimum temperature when considering the X_{max} as the target output [52]. This achievement is also in accordance with other authors in which 30 ºC was the cultivation temperature chosen for both dark aerobic cultivation and light anaerobic cultivation of this bacterium [53, 56, 57, 65, 72, 73, 104].

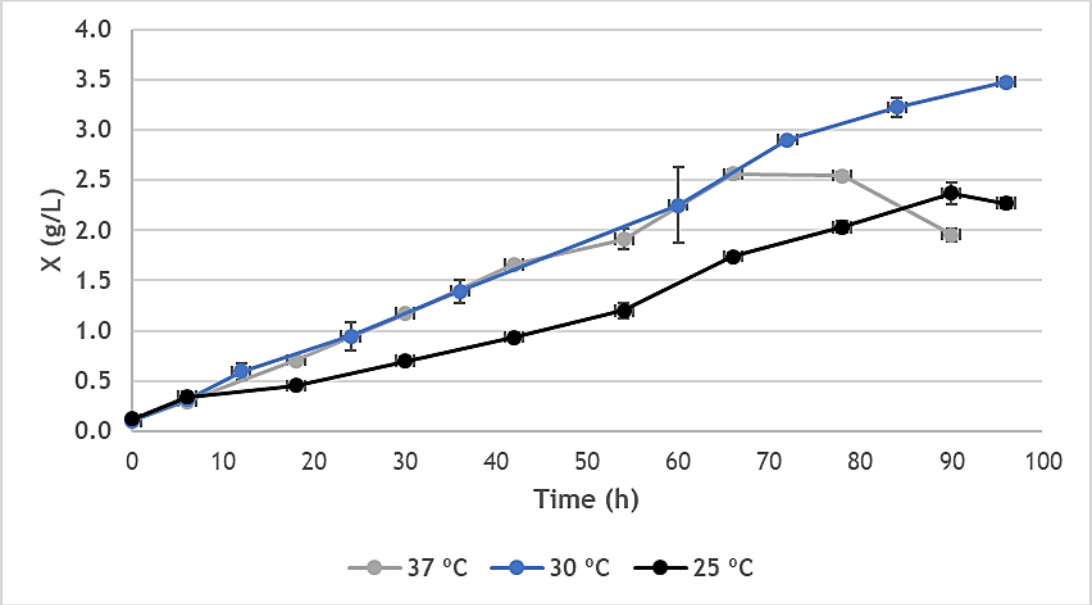


Figure 16 - Influence of temperature on the growth of R. sulfidophilum DSM 1374 in mini-bioreactors cultivation in GLU10 medium under aerobic conditions in the dark. The data presents the average of 2 independent cultivations, and the bars in figure indicate standard deviation.

Once *R. sulfidophilum* is facultative anaerobic host, it was also important to determine the effect of the constant oxygen supply in the aerobic growth of the bacteria. Thus, fermentations without oxygen supply were performed (Table 8, grey tags), basically representing the oxygen condition in the shake flask scale where the fermentation is carried out with only the oxygen existent in the flask. Figure 17 illustrates the growth under such conditions and compares it with the growth under a constant oxygen supply.

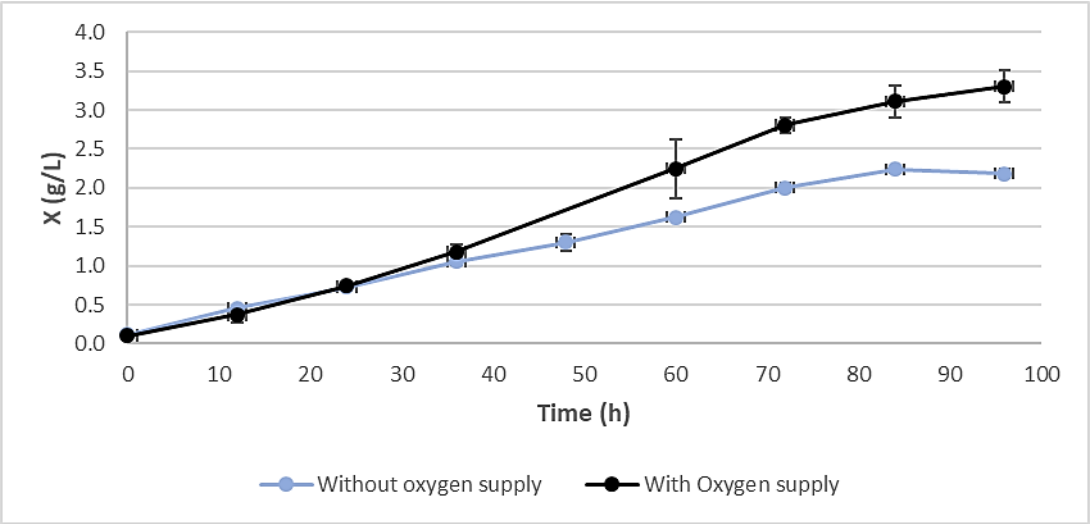


Figure 17 – Effect of oxygen availability on the growth of *R. sulfidophilum* DSM 1374 in mini-bioreactors cultivation in GLU10 medium under aerobic conditions in the dark at 30 ºC. The data presents the average of 2 independent cultivations, and the bars in figure indicate standard deviation.

This finding suggests that the oxygen supply does play an important role in this bacterium growth [58, 98] since the fermentation performed without oxygen supply lead to lower X_{max} and lower μ (Table 8, grey tag). Despite the important role of oxygen in *R. sulfidophilum* fermentations, its uptake does not occur at a rate that leads to oxygen limitations and does not lead to the activation of the oxygen cascade and hence the agitation keeps in 250 rpm through all the fermentation period. However, by the analysis of the monitor panel given by the software (Figure 18) it was possible to verify a decrease (70% to 38%) in the available oxygen in a specific period of fermentation (54 h) which corresponds to the log phase of the typical growth curve of *R. sulfidophilum* in GLU10 medium (Figure 19). Oxygen availability during the fermentation depends on many factors, being the metabolic activity one of the factors. Hence this decrease in the oxygen level could represent an increasing in the metabolic activity of the bacteria. It makes sense so, since the oxygen availability decreases in the log phase, and starts to increase at the beginning of the stationary phase (Figure 18). Variations of oxygen level due to shifts in the oxygen uptake were already described for bacterial fermentation by Pinches and Pallent [105].

Figure 18 - Monitoring panel of mini-bioreactor fermentation through Iris V5.3 software. Blue line: Oxygen percentage over the fermentation period.

One of the important characteristics of *R. sulfidophilum* was the ability to grow under both anaerobic light and aerobic dark conditions. However, in the absence of a luminous energy source, oxygen is required in the chemoorganotrophic processes and have a key role on energy and biomass formation [54]. The results observed here, are in accordance with other studies where under aerobic condition both growth rate and yield was found to be twice as high as microaerobic or anaerobic conditions [65, 98].

Taking all the results of Table 8 together, independent cultivations were performed in order to define the typical growth profile under condition 2 (18% inoculum, 30 ºC, with oxygen supply) (Figure 19).

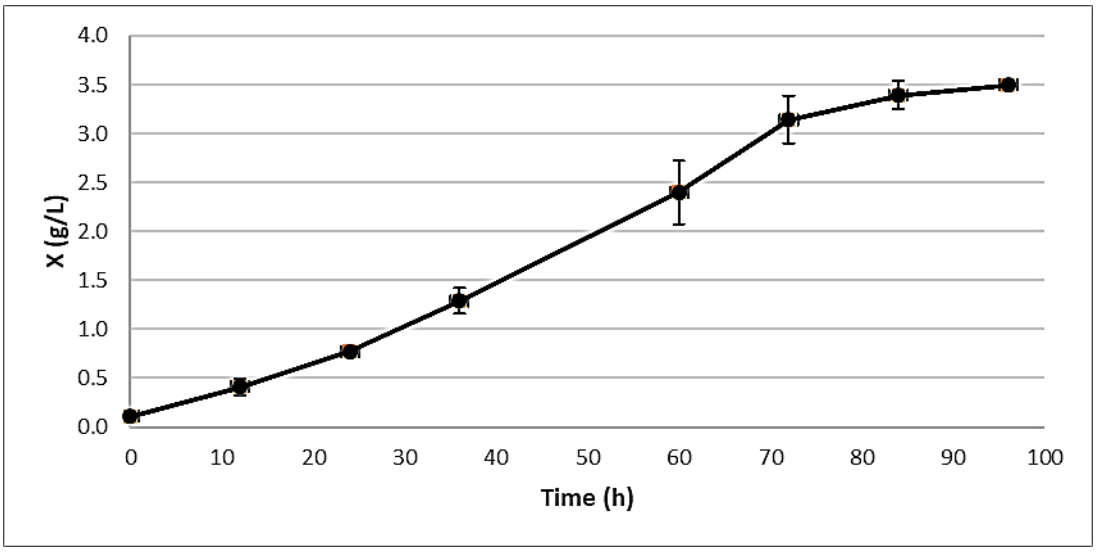


Figure 19 – Typical growth profile of *R. sulfidophilum* DSM 1374 in mini-bioreactors cultivation in GLU10 medium with 18% inoculum under aerobic-dark conditions at 30 ºC. The data presents the average of 4 independent cultivations. Bars in figure indicate standard deviation.

During these fermentations, it has been clear that the automatic acid and base adding for the purpose of pH control was minimal or even null. In some bacteria, such as *E. coli*, fermentations

having glucose as main carbon source lead to accumulation of metabolites that may cause variations on the fermentation broth, such as acetate accumulation. *R. sulfidophilum* lacks biochemistry networks models representing substrates conversions. However, the biochemistry network of glucose conversions in most bacteria identifies acetate, fumarate, lactate, and ethanol as the most common metabolites [106].

These metabolites were screened, by HPLC with a refraction index detector, in extracellular medium retrieved at each 12 h of fermentation of *R. sulfidophilum* in mini-bioreactors and the chromatograms demonstrated that there was no presence of any of these metabolites in the culture broth (data not shown). This finding is in accordance with the fact that it was not noticed any pH variation during the fermentation.

In parallel with the metabolites screening, the substrate concentration (S) over the fermentation period was also assessed (Figure 20). In the chromatograms used to glucose quantification, 3 peaks were identified (Figure 20). Peak 1 was identified as the salts present in the medium in higher concentration (NaCl, $CaCl_2.2H_2O$, $(NH_4)_2SO_4$, and $MgSO_4.7H_2O$) via separately injections of each of the salts of the medium. Peak 2 was not identified to correspond to none of the medium constituents. Peak 3 was identified as glucose. The culture medium was prepared to have an initial S (S_i) of glucose of 10 g/L, but the HPLC data indicated that the fermentations start with about 8 g/L of glucose. This shift from the theorical concentration can be a result of experimental errors.

Noteworthy, after about 40 h of fermentation only a small quantity of glucose was consumed. After that time, glucose is consumed until concentrations close to zero (Figure 21).

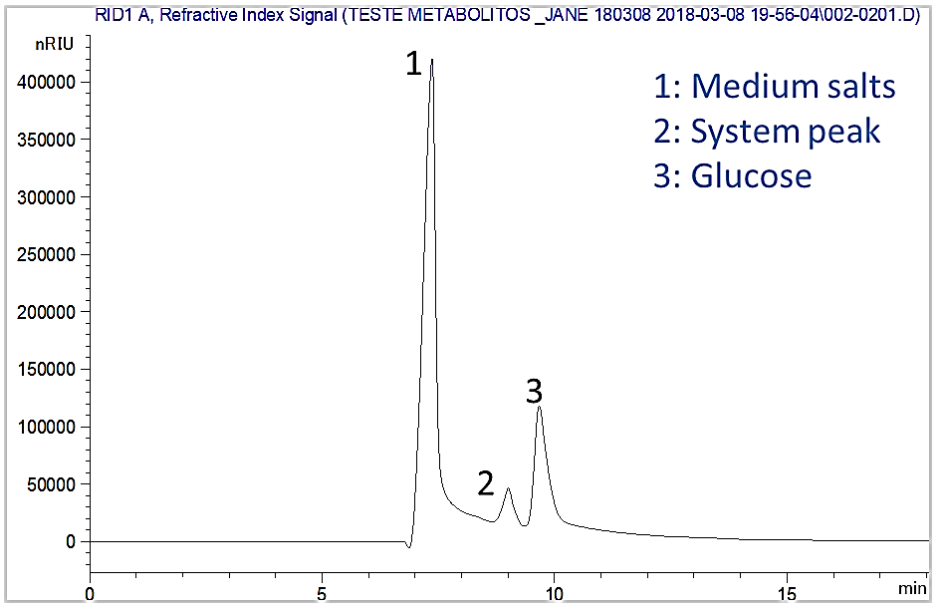


Figure 20- Typical chromatogram obtained by HPLC-RID for the quantification of glucose in fermentations at GLU10 medium. Retention time of glucose: 9.6 min.

The results from Figure 20 suggest that glucose is a potential growth limiting factor in GLU10 fermentations since the growth experiences a depletion in low glucose concentration. However, despite the efforts to find "the" limiting factor for the host growth, multiple limiting factors (physical and nutritionals) can interact and induce the interruption of the growth [85]. Considering that temperature was kept constant over the fermentation period, that the pH does not significantly vary over the fermentation time, the salinity variation is not really a concern in batch fermentation, and formation of toxic metabolites, as acetate, was not reported, the options are narrowed down to nutritional limiting factors. In marine bacteria, carbon sources, nitrogen sources, and inorganic ion components and salinity are suggested as potential limiting factors [85, 107]. Then, with the results from figure 20 it is rational to consider glucose as one of the limiting growth factors but not as "the" only limiting factor or the more relevant.

The time taken for the glucose exhaustion together with the delay in the significative utilization of glucose encouraged us to perform fermentations experiments with a different carbon source.

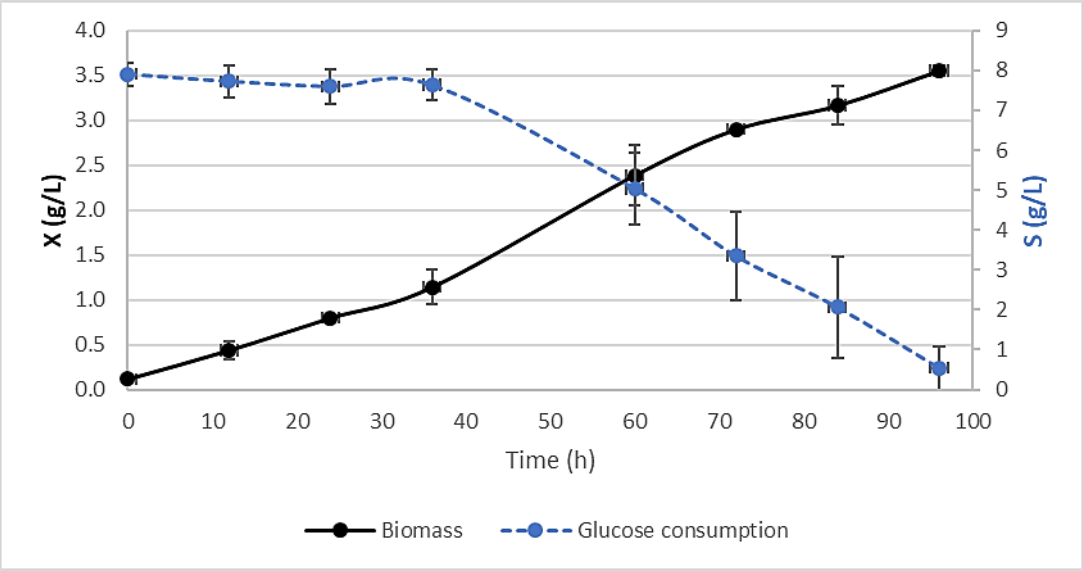


Figure 21 - Glucose consumption in mini-bioreactor fermentation of *R. sulfidophilum* DSM 1374 in GLU10 medium under aerobic conditions in the dark at 30 ºC. The data presents the average of 2 independent cultivations. Bars in figure indicate standard deviation.

4.1.2 Importance of the carbon source type and concentration in the growth profile of *R. sulfidophilum*

Complex media are easy to prepare, are cheap, and foster fast growing [76], but are also difficult to analyze and variation between batches can occur, becoming more difficult the attempt to optimize of the fermentation processes. Regardless of the medium complexity, the choice of the carbon source is crucial to the biomass formation and target biomolecule productivity [76].

The data obtained from GLU10 fermentations in terms of growth profile, and X_{max}, justified the test of a different carbon source. Then, glycerol was the choice since it is a cheap carbon source, with a higher *K*, and metabolized by several bacteria [76, 83, 84]. Therefore, fermentations with medium GLY20 and GLY10 were performed and glycerol quantity over the fermentations was assessed (Figure 21).

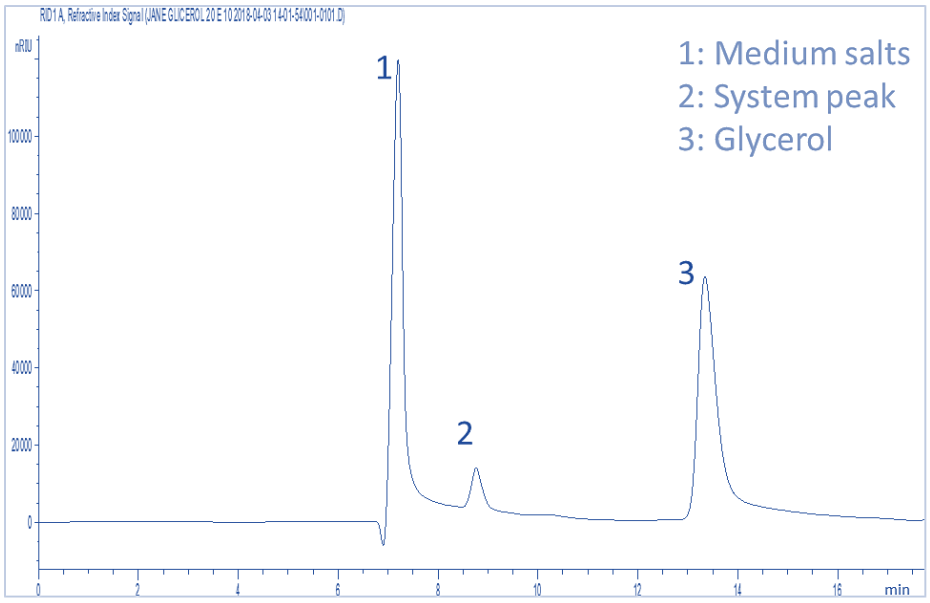


Figure 22- Typical chromatogram obtained by HPLC-RID for the quantification of glycerol in fermentation, using GLY10 and GLY20 media. Retention time for glycerol: 13.3 min.

Considering the substrate consumption, the results show that likewise what happens in GLU10, after about 40 h of fermentation in GLY10 and GLY20 only a small quantity of glycerol was consumed. Furthermore, even extending the fermentation time, there was no glycerol exhaustion (Figure 23). In GLY10 the S after 108 h was 2 g/L but the cell growth stopped earlier in concentrations around 3.5 g/L. On the other hand, in GLY20 the S after 108 h was almost 8 g/L. Even with considerably high quantities of glycerol in the medium, the growth started to decline after 96 h of fermentation. Considering biomass formation, the results obtained showed that both GLY10 and GLY20 lead to higher X_{max} when comparing with GLU10. Noteworthy, regardless of whether GLU20 has twice the S_i than GLU10, both provide similar X_{max} (Table 9).

Unlike in the GLU10 fermentations in which the results from the growth profile in comparison with glucose consumption suggest glucose as one of the limiting factors, in GLY10 and GLY20 fermentations the results bring doubts in terms of glycerol being a limiting factor. First, because in GLY10 the growth starts to decline before the S achieve residual/low values (Figure 23A). Second, because in GLY20 the growth slows down even with considerably high S in the culture medium (Figure 23B).

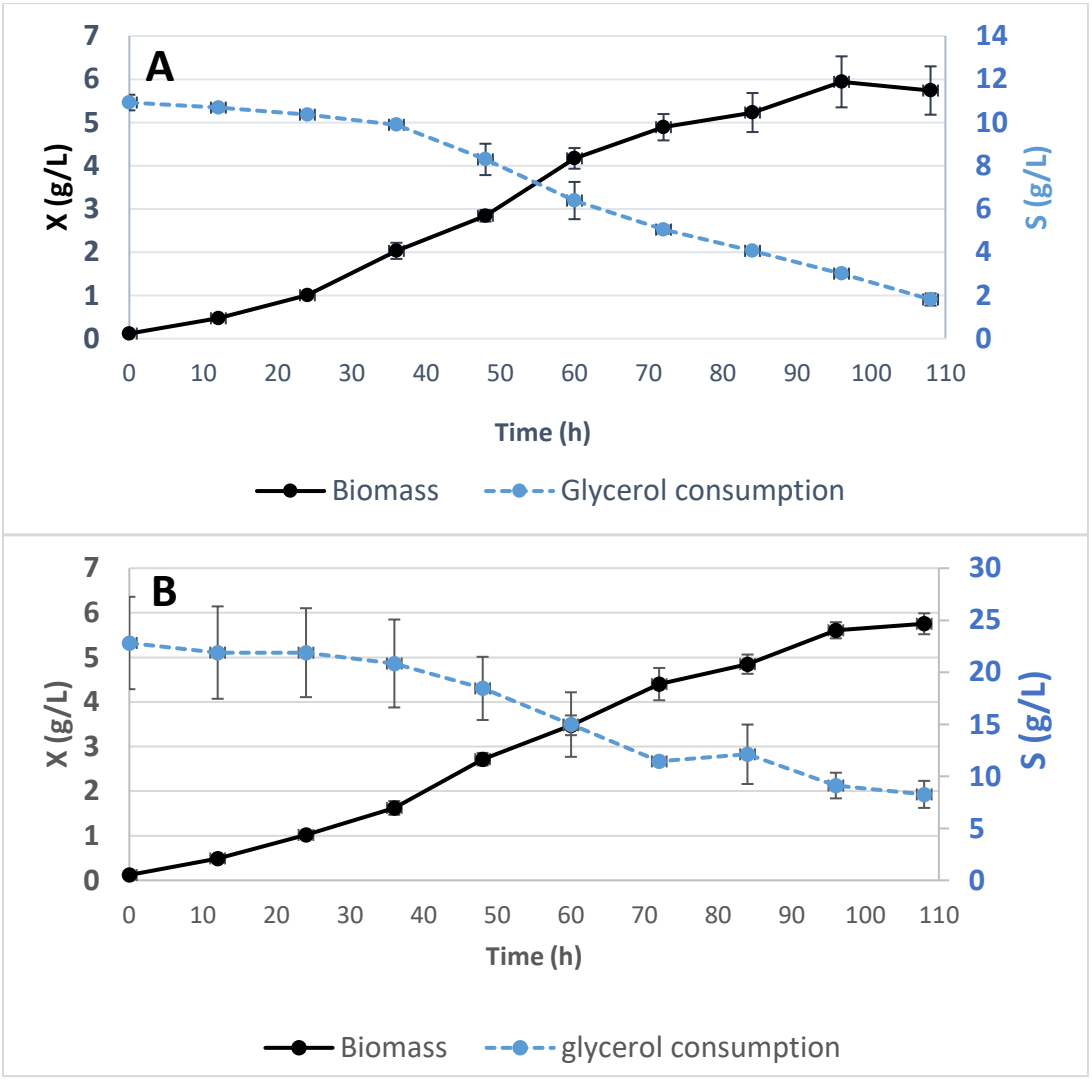


Figure 23 - Glycerol consumption in in mini-bioreactor fermentation of *R. sulfidophilum* DSM 1374 in (A) GLY10 medium and (B) GLY20 medium under aerobic conditions in the dark at 30 °C. The data presents the average of 2 independent cultivations. Bars in figure indicate standard deviation.

Thus, taking together the results (Table 9), glycerol seems to be betther assimilated by the bacteria when compared to glucose since it provides higher cell densities and higher growth rate.

Table 9 - Summary of the effect of the carbon source (type and concentration) on the growth profile of *R. sulfidophilum* in mini-bioreactors.

	X_i (g/L)	X_{max} (g/L)	μ (h^{-1})	t_{max} (h)	S_i (g/L)	S_f (g/L)	S consumed (%)
GLU10	0.12	3.30	0.027	96	7.90	0.55	93
GLY10	0.12	5.94	0.033	96	10.92	1.81	83
GLY20	0.12	5.75	0.030	108	22.81	8.26	64

In fact, the cell growth is closely related to the consumption of the substrate. Despite in terms of consumptions kinetics, glycerol and glucose showed almost the same pattern, and despite both start being trully consumed after a period of 40 h, glicerol yields a higher X_{max} in the same t_{max} than glucose fermentation. This result indicates a more efficient "Glycerol → biomass" conversion which can be a result of the significantly higher *k* of glycerol ($C_3H_8O_3$: *k* = 4.67) than glucose ($C_6H_{12}O_6$: *k* = 4.00). Actually, when compared with glucose, glycerol leads to a higher accomulation of both reduced co-enzymes and pyruvate [106].

In aerobic fermentations it is estimated that approximately one half of the carbon source enters biomass formation. Therefore, the synthesis rate of biomass precursors, such as pyruvate may be important [81]. Actually, piruvate is an important biomass precursor [81]. So, the higher glycerol → biomass conversion can be sustained in the fact that glycerol, due to its higher *k*, can lead to higher accomulation of reduced co-enzymes and pyruvate [82].

The results suggest that glycerol is not consumed faster than glucose by the bacteria (same t_{max} than glucose), as reported for other hosts [82, 106]. But, since it leads to higher X then the μ is increasedr. Yet, the same long periods of fementation garuantee that the μ is maintained in the same magnitude of values.

Since only 63% of glycerol in consumed in GLY20 (Table 9), GLY10 medium was considered the best option for glycerol fermentation in *R. sulfidophilum* in batch strategy. On the other hand, the previous achievements of our research group relative to the biosynthesis of ncRNAs in *R. sulfidophilum* were in shake flask scale and in GLU10 medium. Thus, the next step was to qualitatively evaluate the biosynthesis of ncRNAs by this bacteria having glycerol as mais carbon source instead of glucose.

4.1.3 Biosynthesis evaluation

The great interest in getting high cell densities of this bacteria is related to its potential as a host for recombinant ncRNA biosynthesis. Then, it is of huge importance to evaluate the biosynthesis of ncRNA. Considering that interesting results in terms of X_{max} were achieved in fermentations in GLY10, and also considering that there are no results of ncRNAs biosynthesis in *R. sulfidophilum* fermentations having glycerol as the main carbon source, it was important to evaluate the biosynthesis under such conditions and compare it with the biosynthesis having glucose as main carbon source.

To elucidate the relationship between cell growth and the biosynthesis of ncRNA in GLU10 and in GLY10, the total RNA was extracted using the Trizol reagent extraction in normalized samples over the fermentation period. The integrity and quality of the RNA samples were assessed by agarose gel electrophoresis (Figure 24A and Figure 24A). In both cases, GLU10 and GLY10, revealed the presence of bands with high molecular weight, corresponding to fragments of

gDNA, bands of 23S and 16S rRNA and bands of small RNAs (sRNAs) which are clearly in higher concentration. The sRNAs bands include 6S RNA (184 bp), transfer RNAs (73 to 94 bp), and regulatory ncRNAs.

Since both RNA and DNA absorb at 260 nm and contribute to the total absorbance, an intermediate step to remove the DNA from the RNA samples was carried out attempting to achieve close estimations of TRNA concentration by spectrophotometric analysis [108]. Thus, the RNA samples were treated with DNase and the removal of the gDNA bands were confirmed by agarose gel electrophoresis (Figure 24B and Figure 25B).

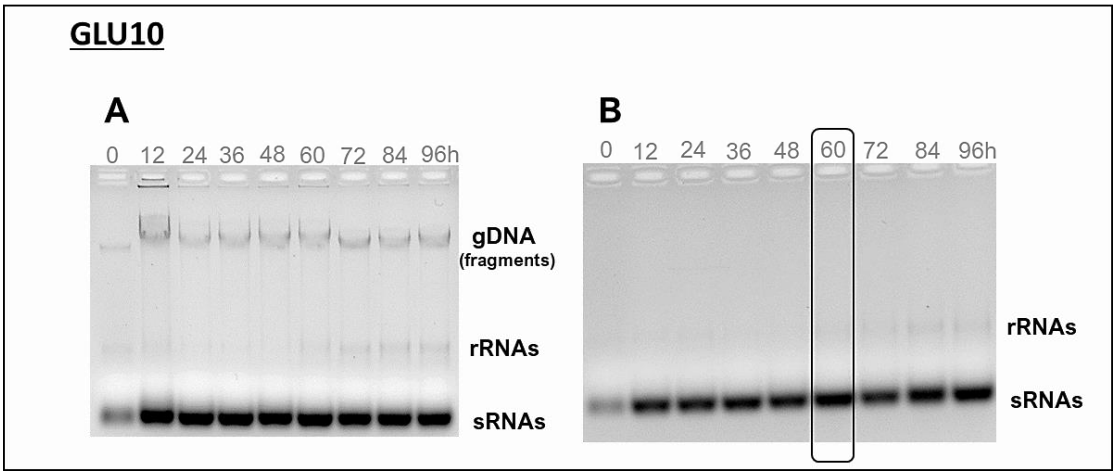


Figure 24- Electrophoretic analysis of RNA biosynthesis from GLU10 fermentation. A) Agarose gel electrophoresis analysis of intracellular RNA after RNA extraction. Samples containing gDNA, rRNAs and sRNAs. B) Agarose gel electrophoresis analysis of intracellular RNA after RNA extraction and DNase treatment. Samples containing rRNAs and sRNAs.

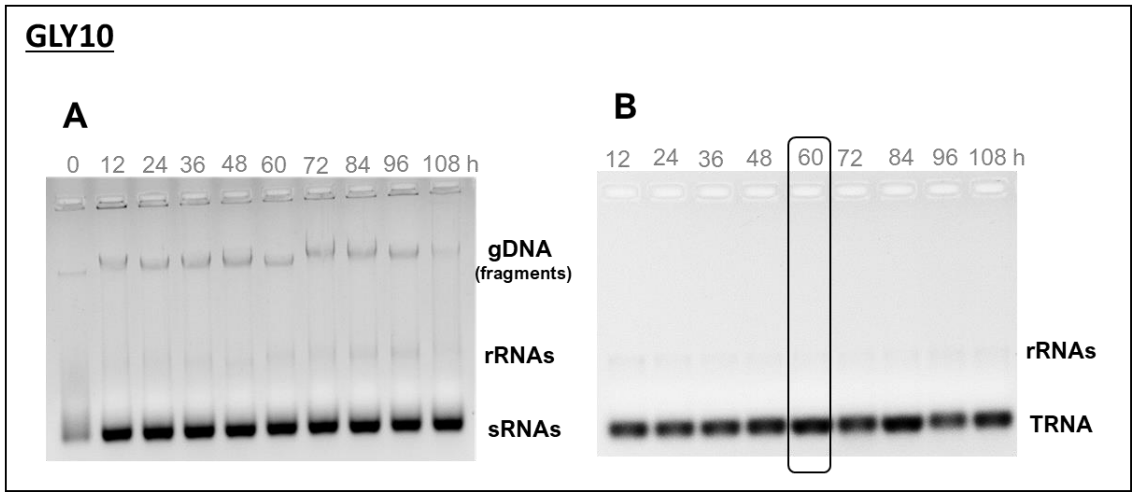


Figure 25 - Electrophoretic analysis of RNA biosynthesis from GLY10 fermentation. (A) Agarose gel electrophoresis analysis of intracellular RNA after RNA extraction. Samples containing gDNA, rRNAs and sRNAs. (B) Agarose gel electrophoresis analysis of intracellular RNA after RNA extraction and DNase treatment. Samples containing rRNAs and sRNAs.

In these gels, smaller volumes of the samples were charged in each well and so it was possible to slightly distinguish the band intensity. By analysing the band intensities both in GLU10 and GLY10 experiments, the samples from 60 h of fermentation seem to have the higher sRNAs concentration. Furthermore, only for the samples collected above 60 h of fermentation, it was possible to visualize the rRNAs bands become more intense and visually noticeable.

The estimations of TRNA concentration by spectrophotometric analysis confirms that 60h corresponde to the peak of TRNA for both GLU10 and GLY10 (Figure 26). Despite the RNA concentration in GLU10 fermentation keeps above the level of RNA concentration in GLY10 fermentation in almost all biosynthesis cycle, the diference is not significant and enforces the idea that RNA production in GLU10 and GLY10 follows almost the same patterns. In both cases, 60 h of fermentation shows a RNA biosynthesis spike (in GLU10 corresponding to 537 ± 49 µg/mL and in GLY10 corresponding to 446 ± 58 µg/mL).

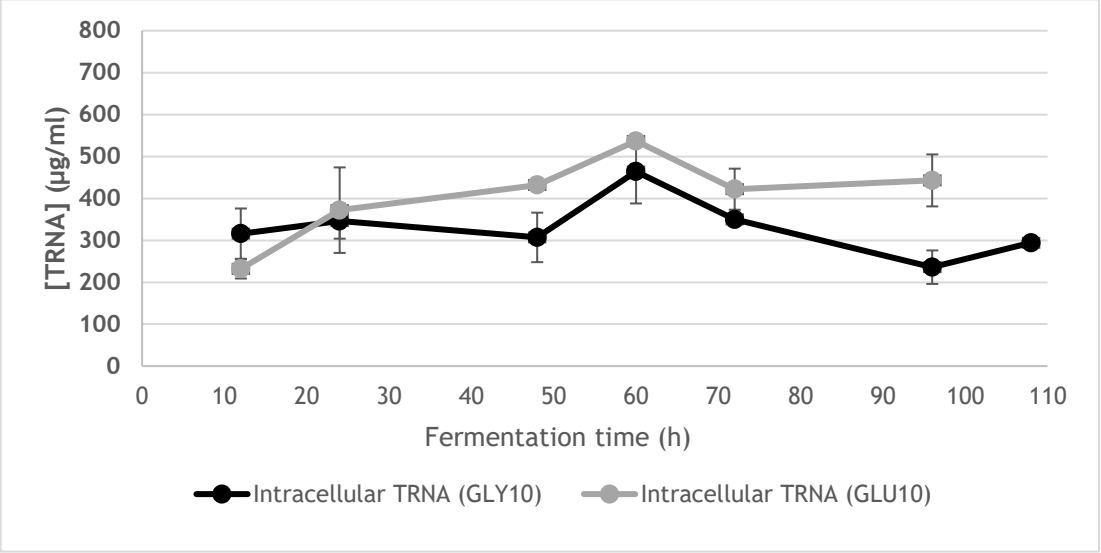


Figure 26 - Time-course analysis of intracellular Total RNA biosynthesis in *R. sulfidophilum* in GLU10 and GLY10 Total RNA production measured spectrophotometrically. Error bars indicate standard deviations calculated from 2 independent samples.

These results were in accordance with the change of oxygen level from 54h of fermentation. Considering that the bacteria increase its metabolic activity from 54h of fermentation, this will be reflected in more biosynthesis in general and, hence, more RNA levels. However, an increasing in TRNA biosynthesis cannot accurately mean more biosynthesis of recombinant small noncoding RNAs.

Whereas in agarose gel the RNAs species cannot be separated, in polyacrylamide gel electrophoresis sRNAs can be separated, which means that it allows distinguishing bands of each of its elements (Figure 27). Once again, by analysing the bands intensities and now focusing just on the ncRNA bands [52], 60 h seems to have the higher ncRNA concentration.

This result suggests that the biosynthesis of recombinant small ncRNA in *R. sulfidophilum* follows the same pattern of RNA biosynthesis regardless of the carbon source, glucose or glycerol. In both cases, the peak of biosynthesis of both TRNA and small ncRNAs happens at 60h of fermentation.

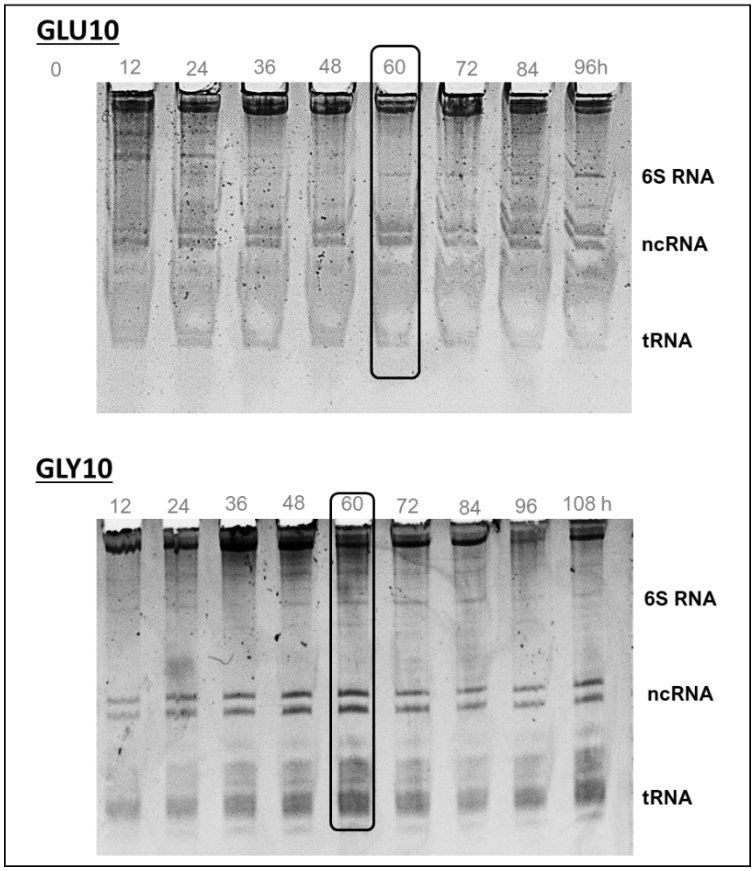


Figure 27 - Polyacrylamide gel electrophoresis of RNA samples from GLU10 and GLY10 medium.

Taking advantage of the fact that the samples from RNA extraction have gDNA fragments and regarding the potential applicability of this method to obtain recombinant ncRNA, it was also important to evaluate the presence of contaminants over the fermentation. gDNA was determined by quantitative real-time PCR and Figure 28 shows the results from both the fermentation in GLU10 and GLY10. The results are in accordance with the agarose gel electrophoresis in which the bands of sRNAs are much more intense than the bands of gDNA fragments. Despite the variations on gDNA concentration in RNA samples over the fermentation period, in both cases rapid or significant increase was not noticed and the higher concentrations in both cases kept under 100 ng/µL.

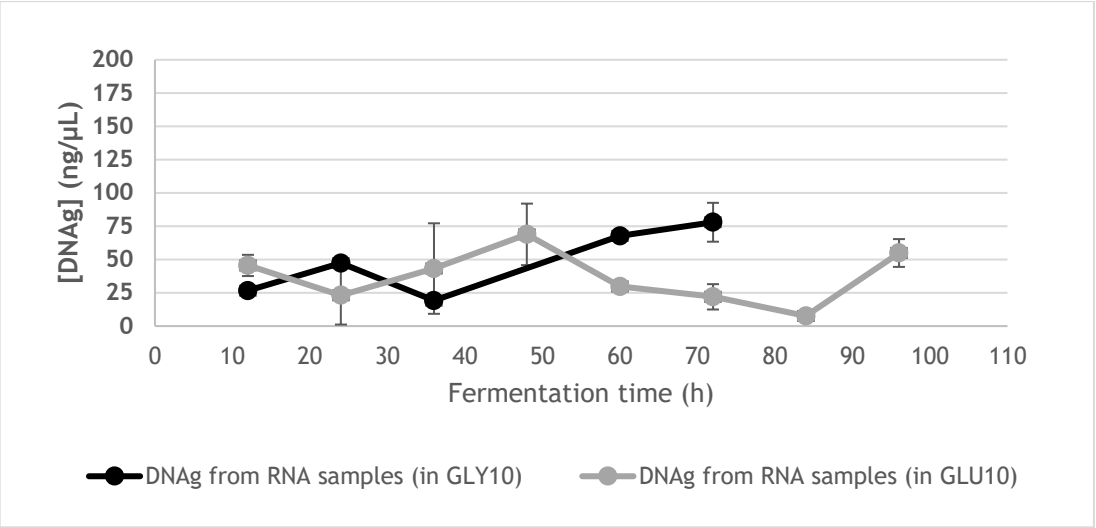


Figure 28 - Time-course analysis of gDNA contamination in RNA samples from *R. sulfidophilum* fermentation in GLU10 and GLY10. gDNA measured by RT-PCR. Error bars indicate standard deviations calculated from 2 independent samples.

Considering that it was the first-time that glycerol was used as carbon source in *R. sulfidophilum* fermentation for small ncRNA production it is also important to mention that glycerol do not demonstrate a negative effect on the RNA biosynthesis of this bacteria since it seems very similar to the pattern of RNA biosynthesis in glucose. Despite the TRNA quantification in glycerol fermentation resulted in lower concentration when compared to Glucose fermentation, it is important to highlight the much higher biomass obtained from glycerol fermentation. At 60 h of GLU10 fermentation, it is obtained 2.40 g of *R.sulfidofilum* cells per liter of fermentation. On the other hand, at 60 h of GLY10 fermentation, it is obtained *4.20 g of R.sulfidofilum* cells per L of fermentation which is almost twice from glucose fermentation.

In higher growth rates, both the RNA/protein and RNA/DNA ratios increase, as well as the RNA synthesis capacity of the cell. In bacterial systems, total RNA amount may varies from 14% to 24% of DCW depending on the growth kinetics [109]. Noteworthy, RNA expression depends also in the synthesis of proteins, more precisely enzymes involved in the RNA biosynthesis, which are also expressed in a growth rate dependence manner [29, 109].

So, the great growth conditions provided by glycerol represents an advantage to the upstream bioprocess of recombinant biosynthesis of RNAs in *R. sulfidophilum*.

4.1.4 Bioreactor scale versus shake flask scale

Bioreactor fermentations intend to provide a controlled environment, in terms of physical and nutritional factors, to achieve optimal growth and/or optimal product formation in a particular bioprocess [79].

In previous optimizations of shake flask fermentations of this strain, it was only tested a maximum work volume of 100 mL [52], and in bioreactor fermentations it was already possible to improve the conditions, establishing a higher culture volume of 250 mL, being verified that the growth was achievable, highlighting the importance of well-controlled conditions in the adaptation and growth of *R. sulfidophilum*.

After the optimization of the growth conditions of *R. sulfidophilum* in batch fermentations in bioreactor it is possible to compare the obtained X_{max}, t_{max}, and total RNA concentration with the obtained in shake flask scale. Table 10 summarize these outcomes.

Table 10 - Outcomes from *R. sulfidophilum* DSM 1374 fermentation from shake flask and bioreactor scale.

Fermentation Scale	Fermentation Medium	X_{max} (g/L)	t_{max} (h)	Max [TRNA] (µg/mL)	Time to achieve Max [TRNA] (h)
Shake Flask	GLU10	2.6	72	395	60
Bioreactor	GLY10	5.9	96	446	60

Bioreactor fermentations provided higher biomass of *R. sulfidophilum,* and Figure 29 illustrate the increase in biomass in comparison with shake flask fermentations. Despite in bioreactor the X_{max} is achieved later (higher t_{max}), it increases the biomass 2.3 times.

In terms of total RNA biosynthesis, the optimized bioreactor fermentations provided higher concentration (Table 10) and have an evident peak at 60 h (Figure 30). In shake flask fermentations, total RNA production remains constant and do not present an evident production peak (Figure 30).

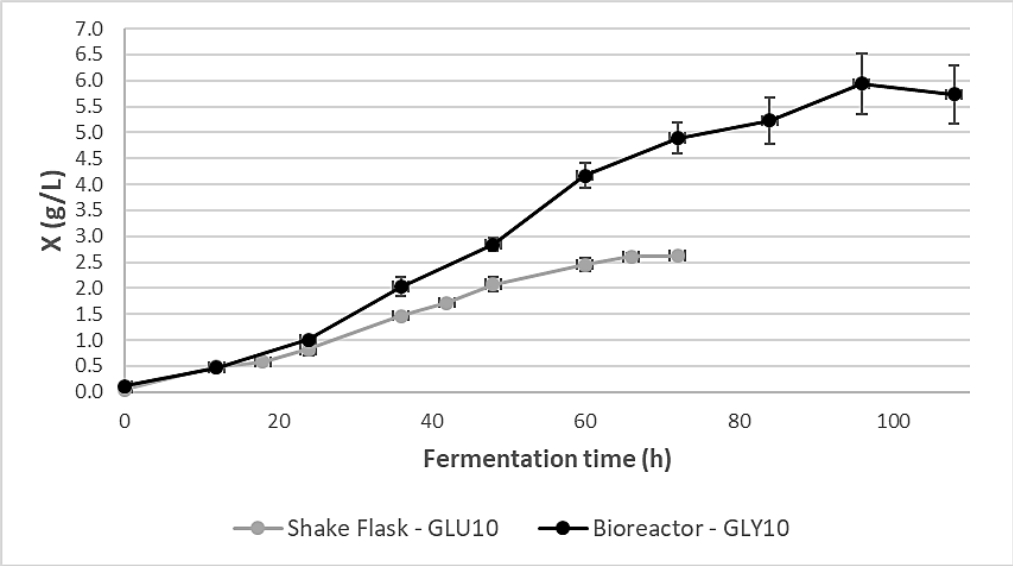


Figure 29 – Growth profile of *R. sulfidophilum* DSM 1374 in mini-bioreactors cultivation in GLY10 medium with 18% inoculum in comparison with shake flask cultivation in GLU10 medium with 13% inoculum, both under aerobic-dark conditions at 30 ºC. The data presents the average of 2 independent cultivations. Bars in figure indicate standard deviation.

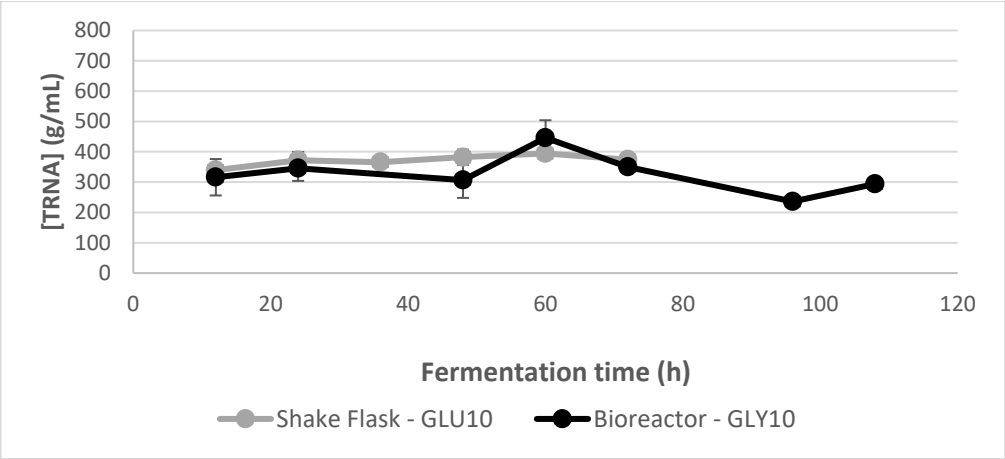


Figure 30 – Comparison of the time-course analysis of intracellular RNA biosynthesis in *R. sulfidophilum* in Bioreactor scale (medium GLY10) and Shake flask scale. Total RNA production measured spectrophotometrically. Error bars indicate standard deviations calculated from 2 independent samples.

4.2 Fed-Batch fermentation of *R. sulfidophilum*

Batch fermentations are simple and robust and are importante in the defenition of the variables that can affect the productivity or the quality of the target product. On the other hand, fed-batch stretagies are powerful biostrategies that regulate the nutrient concentration properly and have been widely used to produce many recombinant products since it allows to achieve high cell density and productivity [110–112]. The main feature of this fermentation method is the feed of a concentrated growth limiting substrate. The batch fermentation of this bacteria identified the carbon source as the growth limiting substrate. The fed-batch strategies start by a bacth fermentation phase and, ideally, the feed should start when the initial carbon source provided to the system is almost consumed.

Considering the results of glycerol consumption in batch fermentation GLY10 (Figure 23A), if GLY10 was the starting point of a fed-batch of this strain in bioreactor it would take more than 100 h for the feed to start, which would make this strategy a method incompatible with the laboratory scale as well as the industrial scale.

With the aim of lowering the batch fermentation period of this strain in minibioreactors, fermentations were performed in media with lower S_i of glicerol: GLY5 and GLY2.5. The results of such fermentations (Figure 31) once again reinforce the hypothesis of the principal carbon source, in this case, glycerol, to be the growth limiting factor. In GLY5, the complete glycerol exhaustion accours after about 70 h of fermentation and it reaches X_{max} of about 4 g/L. In GLY2.5 the complete glycerol exhaustion accours earlier, after about 50 h of fermentation and reaches X_{max} of about 3 g/L.

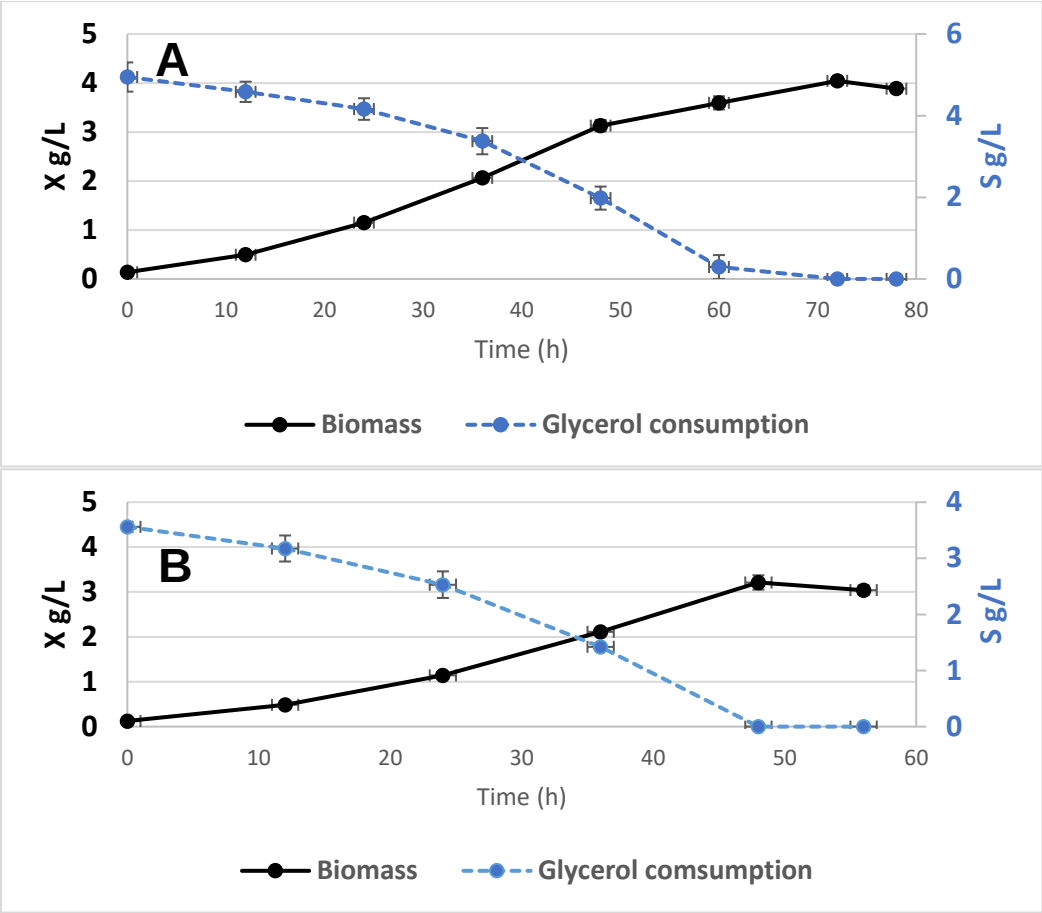


Figure 31 - Batch fermentation of *R. sulfidophilum* DSM 1374 in mini-bioreactors under aerobic conditions in the dark at 30 °C in (A) GLY5 and in (B) GLY2.5. The data presents the average of 2 independent cultivations. Bars in figure indicate standard deviation.

Based on these results and considering both the X_{max} and the t_{max}, GLY5 was chosen to be the medium of the batch phase of fed-batch fermentations of this strain in mini-bioreactors.

Fed-batch fermentations were operated to investigate whether a higher density of cells was attainable in the mini-bioreactor and which would be the effects in the productivity of the target product. By analysing the consume of glycerol in GLY5, the batch period was set to 50 h, period at which the glycerol concentration is very low (Figure 31A). The feed rate was set as 1g/L/h considering the slow rate of carbon source uptake demonstrated by the previous experiments.

Despite the results from Fed-batch fermentation were not reproducible (Figure 32A and Figure 32B), the growth profile was exactly the same of the growth in batch fermentation (Figure 31A), suggesting that a feed only based in the main carbon source is not suitable for *R. sulfidophilum*. In fact, even keeping glycerol to a non-limiting level, the growth of the bacteria stops after 70 h of fermentation likewise in the batch fermentation suggesting that other medium substrates are limiting the growth of the bacteria.

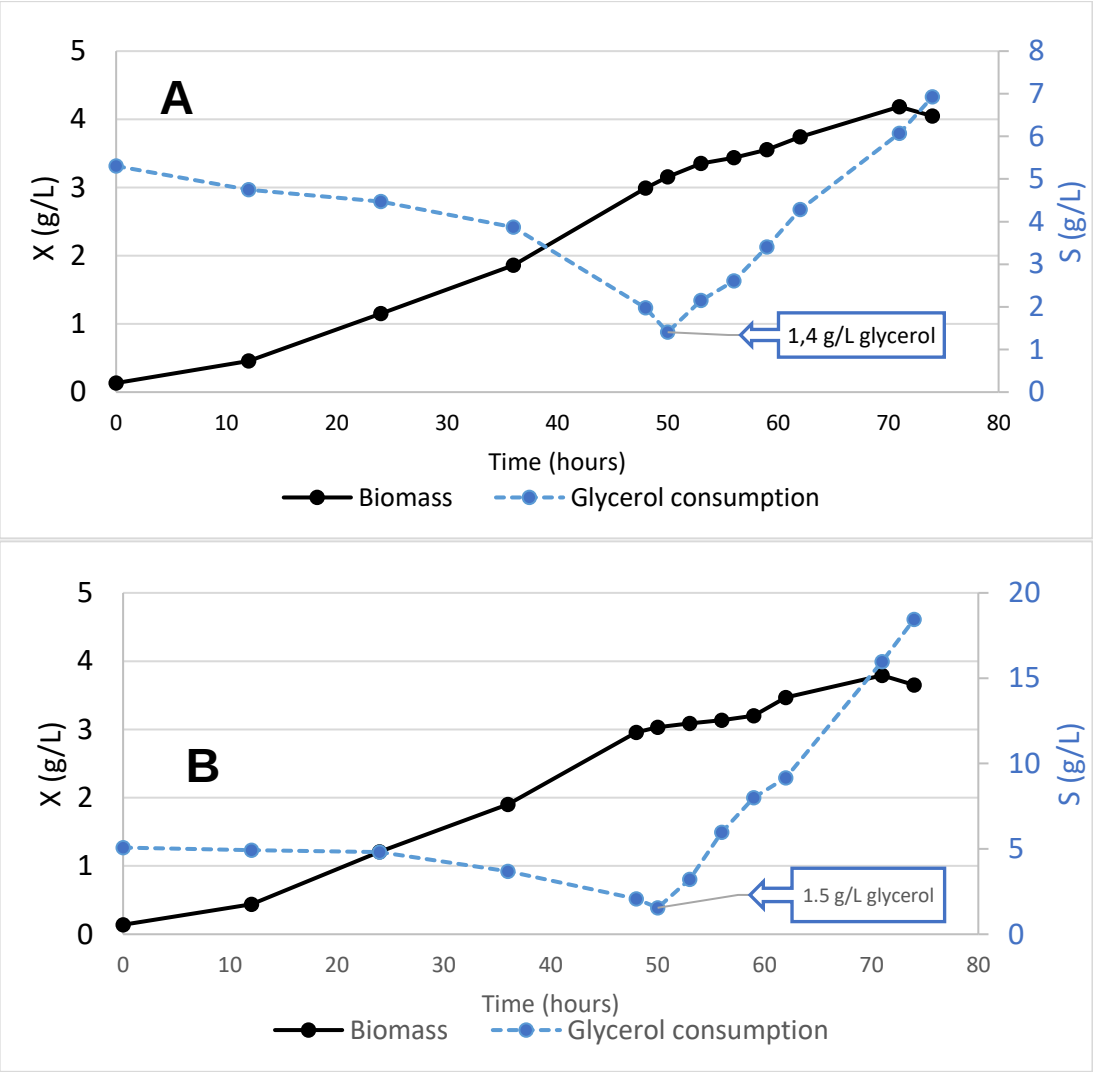


Figure 32- Constant fed-batch fermentation of *R. sulfidophilum* DSM 1374 in mini-bioreactors under aerobic conditions in the dark at 30 ºC having GLY5 media as batch media.

Salinity and the work volume could be a concern in a fed-batch strategy in which a feed solution is added to the system. However, the concentration of the feed solution was set in a way that the addition of volume did not significantly vary the work volume nor the medium salinity. However, other essential requirements, such as nitrogen sources and inorganic ions, could be limiting the growth of the bacteria and should be considered in the formulation of a possible feed solution for *R. sulfidophilum* fed-batch fermentation.

Considering the carbon source as one of the limiting factors of *R. sulfidophilum* fermentations, a good strategy to unravel other limiting factors would be keep glycerol in a non-limiting concentration while concentrate solutions of nitrogen sources and inorganic ions are added separately.

Chapter 5 - Conclusion and Future Prespectives

The efficiency of recombinant production by bacteria depends on both the species and the nutritional and physical conditions provided for growth. Recombinant RNA produced by bacteria have become a solution to cost-effectively produce large quantities of RNA that better reflect the biologic RNAs in terms of structure and biologic activity. The prospect on the utilization of ncRNAs as RNA drugs together with the fact that the RNase-free *R. sulfidophilum* favor the integrity and quality of RNAs, highlight the relevance to increase the production of ncRNA in these bacteria.

Herein, and for the first time, the fermentation of *R. sulfidophilum* DSM 1374 in bioreactors was described. By controlling several physical and nutritionals parameters this study allowed to optimize the growth of *R. sulfidophilum* in bioreactors which refletcs in an increase of the biomass and consequently of volumetric productivity.

The growth conditions for the fermentations in bioreactor had as reference the growth conditions in shake flask fermentations, previously described by our research group. However, some conditions must be adapted such is the case of the inoculum size. The need for the adaptation of the inoculum size from shake flask to bioreactor fermentation highlights how shifts in the scale, even that small, can alter the ideal growth conditions. The inoculum size that adequate the most to the growth in bioreactor scale was 18% leading to the higher biomass in a smaller period. In what concerns to temperature for the cultivation of *R. sulfidophilum* in bioreactor, likewise in shake flask fermentations, 30 ºC is the choice due to the higher biomass provided in the scale-up. Moreover, the aeration conditions supplied in aerobic fermentations in bioreactor allow to identify the oxygen availability as an important factor in the growth of this strain even being a facultative anaerobic bacterium.

The main carbon source contributes significantly to the biomass formation. Here the substitution of glucose by glycerol had resulted in a significant increase of the biomass. Also, the initial concentration of the carbon source influenced the growth and its rate. Glycerol in an initial concentration of 10 g/L does not lead to a significant increase in the specific growth rate when compared to glucose with the same initial concentration. However, it led to higher biomass, even reaching twice the biomass in the exponential phase.

Summarizing, the maximum biomass under optimized conditions in a 0.75 L bioreactor was 5.9 g of dry *R. sulfidophilum* cells per liter of culture medium, which was 2.3 times higher than

that at a flask scale under the previously optimized conditions and 1.8 times higher than at bioreactor scale under no optimized conditions.

The biosynthesis of TRNA by the bacteria have a peak at 60 hours of fermentation, in both glucose and glycerol, suggesting that the pathways for RNA biosynthesis were not affected by the shift on the carbon source. The recombinant ncRNA biosynthesis presents a peak also at 60 h which in turn suggests that the biosynthesis of recombinant ncRNAs in *R. sulfidophilum* follows the same pathway and features of the homologous RNA biosynthesis.

Fed-batch fermentations are characterized by a significative increase in the biomass and productivity when compared to batch fermentations. However, the results achieved from the fed-batch fermentations of *R. sulfidophilum* were not conclusive due to the lack of reproducibility. Indeed, it was clear that the formulation of the feed solution was not appropriate to the bacteria since the final biomass and growth profile remained similar to the batch fermentations. Fed-batch experiments highlighted the fact that the main carbon source (in this case glycerol) is not the only growth limiting factor in *R. sulfidophilum* fermentation. Therefore, ongoing work includes the study of new feed strategies (exponential, stepwise) as well as new feed formulations.

Overall, this study represents the first step toward the optimization of the fermentation process of *R. sulfidophilum* in bioreactor. To extend and improve all this work, many other experiments and studies must be carried out.

First, it would be important to consider the fermentation parameters as overlapping variables by mean of experimental design, which allows varying several factors at the same time and seeking interactions using statistical analysis. The outcomes from experimental design would define the combination of variables that favor the growth and the productivity of ncRNAs. Then, it would also be important to explore new methods for the cost-effective quantification of ncRNAs in order to precisely asses the productivity at each combination of growth condition.

This study highlights how the principal carbon source has an important role in the biomass formation. Therefore, the analysis of the effect of other carbon sources, such as lactate and fumarate, should be considered since it could lead to surprising outcomes in terms of biomass formation, and help to elucidate biochemical pathways underlying the metabolization of each carbon source utilization by *R. sulfidophilum*.

Last but not least, considering the possibility of nucleic acids secretion by this specie under specific growth conditions it would be important to explore the growth conditions that favor the extracellular production of RNA as a strategy to simplify the ncRNA recovery and isolation steps.

Chapter 6 - Bibliography

1. Sana, J., Faltejskova, P., Svoboda, M., Slaby, O.: Novel classes of non-coding RNAs and cancer. *Journal of Translational Medicine*. 10, 1–21 (2012).

2. Inamura, K., Inamura, Kentaro: Major Tumor Suppressor and Oncogenic Non-Coding RNAs: Clinical Relevance in Lung Cancer. *Cells*. 6, 1–15 (2017).

3. Bhartiya, D., Scaria, V.: Genomic variations in non-coding RNAs: Structure, function and regulation. *Genomics*. 107, 59–68 (2016).

4. Wahid, F., Shehzad, A., Khan, T., Kim, Y.Y.: MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. *Biochimica et Biophysica Acta - Molecular Cell Research*. 1803, 1231–1243 (2010).

5. Almeida, M.I., Reis, R.M., Calin, G.A.: MicroRNA history: Discovery, recent applications, and next frontiers. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*. 717, 1–8 (2011).

6. Waldman, S.A., Terzic, A.: MicroRNA signatures as diagnostic and therapeutic targets. *Clinical Chemistry*. 54, 943–944 (2008).

7. Mairinger, F.D., Ting, S., Werner, R., Walter, R.F.H., Hager, T., Vollbrecht, C., Christoph, D., Worm, K., Mairinger, T., Sheu-Grabellus, S.Y., Theegarten, D., Schmid, K.W., Wohlschlaeger, J.: Different micro-RNA expression profiles distinguish subtypes of neuroendocrine tumors of the lung: Results of a profiling study. *Modern Pathology*. 27, 1632–1640 (2014).

8. Chou, J., Lin, J.H., Brenot, A., Kim, J., Provot, S., Werb, Z.: GATA3 suppresses metastasis and modulates the tumour microenvironment by regulating microRNA-29b expression. *Nature Cell Biology*. 15, 201–213 (2013).

9. Cortez, M.A., Nicoloso, M.S., Shimizu, M., Rossi, S., Gopisetty, G., Molina, J.R., Carlotti, C., Tirapelli, D., Neder, L., Brassesco, M.S., Scrideli, C.A., Tone, L.G., Georgescu, M.-M., Zhang, W., Puduvalli, V., Calin, G.A., Calin, G.A.: miR-29b and miR-125a regulate podoplanin and suppress invasion in glioblastoma. *Genes, chromosomes & cancer*. 49, 981–90 (2010).

10. Hou, M., Zuo, X., Li, C., Zhang, Y., Teng, Y.: Mir-29b Regulates Oxidative Stress by Targeting SIRT1 in Ovarian Cancer Cells. *Cellular Physiology Biochemistry*. 43, 1767–

1776 (2017).

11. Roshan, R., Shridhar, S., Sarangdhar, M.A., Banik, A., Chawla, M., Garg, M., Singh, V.P., Pillai, B.: Brain-specific knockdown of miR-29 results in neuronal cell death and ataxia in mice. *RNA*. 20, 1287–1297 (2014).

12. Yu, J.Y., Chung, K.H., Deo, M., Thompson, R.C., Turner, D.L.: MicroRNA miR-124 regulates neurite outgrowth during neuronal differentiation. *Experimental Cell Research*. 314, 2618–2633 (2008).

13. Fenn, A.M., Smith, K.M., Lovett-Racke, A.E., Guerau-de-Arellano, M., Whitacre, C.C., Godbout, J.P.: Increased micro-RNA 29b in the aged brain correlates with the reduction of insulin-like growth factor-1 and fractalkine ligand. *Neurobiology of Aging*. 34, 2748–2758 (2013).

14. Pereira, P., Queiroz, J.A., Figueiras, A., Sousa, F.: Current progress on microRNAs-based therapeutics in neurodegenerative diseases. *Wiley Interdisciplinary Reviews: RNA*. 8, (2017).

15. Kriegel, A.J., Liu, Y., Fang, Y., Ding, X., Liang, M.: The miR-29 family: genomics, cell biology, and relevance to renal and cardiovascular injury. *Physiological genomics*. 44, 237–44 (2012).

16. Jiang, W., Zhang, Z., Yang, H., Lin, Q., Han, C., Qin, X.: The Involvement of MIR-29b-3p in Arterial Calcification by Targeting Matrix Metalloproteinase-2. *BioMed Research International*. 11, 1–9 (2017).

17. Li, J., Chan, M.C., Yu, Y., Bei, Y., Chen, P., Zhou, Q., Cheng, L., Chen, L., Ziegler, O., Rowe, G.C., Das, S., Xiao, J.: MiR-29b contributes to multiple types of muscle atrophy. *Nature Communications*. 8, 1–15 (2017).

18. Pandey, A.K., Verma, G., Vig, S., Srivastava, S., Srivastava, A.K., Datta, M.: miR-29a levels are elevated in the db/db mice liver and its overexpression leads to attenuation of insulin action on PEPCK gene expression in HepG2 cells. *Molecular and Cellular Endocrinology*. 332, 125–133 (2011).

19. Soifer, H.S., Rossi, J.J., Sætrom, P.: MicroRNAs in Disease and Potential Therapeutic Applications. *Molecular Therapy*. 15, 2070–2079 (2007).

20. Van Rooij, E., Kauppinen, S.: Development of microRNA therapeutics is coming of age. *EMBO Mol Med*. 6, 851–864 (2014).

21. Ho, P.Y., Yu, A.M.: Bioengineering of noncoding RNAs for research agents and therapeutics. *Wiley Interdisciplinary Reviews: RNA*. 7, 186–197 (2016).

22. Duan, Z., Yu, A.M.: Bioengineered non-coding RNA agent (BERA) in action. *Bioengineered*. 7, 411–417 (2016).

23. Edelmann, F.T., Niedner, A., Niessing, D.: Production of pure and functional RNA for *in vitro* reconstitution experiments. *Methods*. 65, 333–341 (2014).

24. Beckert, B., Masquida, B.: RNA. In: Methods in molecular biology (2011).

25. Marshall, W.S., Kaiser, R.J.: Recent advances in the high-speed solid phase synthesis of RNA. *Current Opinion in Chemical Biology*. 8, 222–229 (2004).

26. Hu, S., Li, M., Zhong, L., Lu, S., Liu, Z., Pu, J., Wen, J., Huang, X.: Development of reverse-transcription loop-mediated isothermal amplification assay for rapid detection and differentiation of dengue virus serotypes 1–4. *BMC Microbiology*. 15, 265 (2015).

27. Boccaletto, P., MacHnicka, M.A., Purta, E., Pitkowski, P., Baginski, B., Wirecki, T.K., De Crécy-Lagard, V., Ross, R., Limbach, P.A., Kotter, A., Helm, M., Bujnicki, J.M.: MODOMICS: A database of RNA modification pathways. 2013 update. *Nucleic Acids Research*. 41, D303–D307 (2013).

28. Jozala, A.F., Geraldes, D.C., Tundisi, L.L., Feitosa, V. de A., Breyer, C.A., Cardoso, S.L., Mazzola, P.G., de Oliveira-Nascimento, L., Rangel-Yagui, C. de O., Magalhães, P. de O., de Oliveira, M.A., Pessoa, A.: Biopharmaceuticals from microorganisms: from production to purification. *Brazilian Journal of Microbiology*. 47S, 51–63 (2016).

29. Ponchon, L., Dardel, F.: Large scale expression and purification of recombinant RNA in *Escherichia coli*. *Methods*. 54, 267–273 (2011).

30. Pereira, P., Sousa, Â., Queiroz, J.A., Figueiras, A., Sousa, F.: Pharmaceutical-grade pre-miR-29 purification using an agmatine monolithic support. *Journal of Chromatography A*. 1368, 173–182 (2014).

31. Pereira, P., Sousa, Â., Queiroz, J., Correia, I., Figueiras, A., Sousa, F.: Purification of pre-miR-29 by arginine-affinity chromatography. *Journal of Chromatography B*. 951–952, 16–23 (2014).

32. Singh Sekhon, B.: Biopharmaceuticals: an overview. *Thai Journal pharmaceutical sciences*. 34, 1–19 (2010).

33. Dunham, C.M., Conn, G.L.: Recombinant RNA expression. *Nature Methods*. 4, 547–548 (2007).

34. Pereira, P.A., Tomás, J.F., Queiroz, J.A., Figueiras, A.R., Sousa, F.: Recombinant pre-miR-29b for Alzheimer's disease therapeutics. *Scientific Reports*. 6, (2016).

35. Ponchon, L., Dardel, F.: Recombinant RNA technology: the tRNA scaffold. *Nature methods*. 4, 571–6 (2007).

36. Pereira, P., Pedro, A.Q., Queiroz, J.A., Figueiras, A.R., Sousa, F.: New insights for therapeutic recombinant human miRNAs heterologous production: *Rhodovolum sulfidophilum vs Escherichia coli*. *Bioengineered*. 8, 670–677 (2017).

37. Ferrer-Miralles, N., Domingo-Espín, J., Corchero, J., Vázquez, E., Villaverde, A.: Microbial factories for recombinant pharmaceuticals. *Microbial Cell Factories*. 8, (2009).

38. Rosano, G.L., Ceccarelli, E.A.: Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Frontiers in Microbiology*. 5, 1–17 (2014).

39. Umekage, S., Kikuchi, Y.: In vitro and in vivo production and purification of circular RNA aptamer. *Journal of Biotechnology*. 139, 265–272 (2009).

40. Ponchon, L., Beauvais, G., Nonin-Lecomte, S., Dardel, F.: A generic protocol for the expression and purification of recombinant proteins in *Escherichia coli* using a combinatorial His6-maltose binding protein fusion tag. *Nature Protocols*. 4, 947–959 (2009).

41. Nelissen, F.H.T., Leunissen, E.H.P., Van De Laar, L., Tessari, M., Heus, H.A., Wijmenga, S.S.: Fast production of homogeneous recombinant RNA-towards large-scale production of RNA. *Nucleic Acids Research*. 40, e102 (2012).

42. Li, M.-M., Addepalli, B., Tu, M.-J., Chen, Q.-X., Wang, W.-P., Limbach, P.A., Lasalle, J.M., Zeng, S., Huang, M., Yu, A.-M.: Chimeric MicroRNA-1291 Biosynthesized Efficiently in Escherichia coli Is Effective to Reduce Target Gene Expression in Human Carcinoma Cells and Improve Chemosensitivity. *Drug Metabolism And Disposition*. 43, 1129–1136 (2015).

43. Li, M.-M., Wang, W.-P., Wu, W.-J., Huang, M., Yu, A.-M.: Rapid Production of Novel Pre-MicroRNA Agent hsa-mir-27b in *Escherichia coli* Using Recombinant RNA Technology for Functional Studies in Mammalian Cells. *DRUG METABOLISM AND DISPOSITION*. 42, 1791–1795 (2014).

44. Umekage, S., Kikuchi, Y.: *In vivo* circular RNA production using a constitutive promoter for high-level expression. *Journal of Bioscience and Bioengineering*. 108, 354–356 (2009).

45. Ponchon, L., Catala, M., Seijo, B., Khouri, M. El, Dé, F., Dardel, R., Nonin-Lecomte, S., Tisné, C.: Co-expression of RNA–protein complexes in *Escherichia coli* and applications to RNA biology. *nucleic acid*. 41, e150 (2013).

46. Chen, Q.-X., Wang, W.-P., Zeng, S., Urayama, S., Yu, A.-M.: A general approach to high-yield biosynthesis of chimeric RNAs bearing various types of functional small RNAs for broad applications. *Nucleic Acids Research*. 43, 3857–3869 (2015).

47. Liu, Y., Stepanov, V.G., Strych, U., Willson, R.C., Jackson, G.W., Fox, G.E.: DNAzyme-mediated recovery of small recombinant RNAs from a 5S rRNA-derived chimera expressed in *Escherichia coli*. *BMC Biotechnology*. 10, 1–9 (2010).

48. Suzuki, H., Ando, T., Umekage, S., Tanaka, T., Kikuchi, Y.: Extracellular Production of an RNA Aptamer by Ribonuclease-Free Marine Bacteria Harboring Engineered Plasmids: a Proposal for Industrial RNA Drug Production. *Applied and Environment Microbiology*. 76, 786–793 (2010).

49. Hammann, C., Luptak, A., Perreault, J., de la Pena, M.: The ubiquitous hammerhead ribozyme. *RNA*. 18, 871–885 (2012).

50. Suzuki, H., Umekage, S., Tanaka, T., Kikuchi, Y.: Artificial RNA aptamer production by the marine bacterium *Rhodovulum sulfidophilum*: Improvement of the aptamer yield using a mutated transcriptional promoter. *Journal of Bioscience and Bioengineering*. 112, 458–461 (2011).

51. Nagao, N., Suzuki, H., Numano, R., Umekage, S., Kikuchi, Y.: Short hairpin RNAs of designed sequences can be extracellularly produced by the marine bacterium *Rhodovulum sulfidophilum*. *The Journal of General and Applied Microbiology*. 60, 222–226 (2014).

52. Pereira, P., Pedro, A.Q., Tomás, J., Maia, C.J., Queiroz, J.A., Figueiras, A., Sousa, F.: Advances in time course extracellular production of human pre-miR-29b from *Rhodovulum sulfidophilum*. *Applied Microbiology and Biotechnology*. 100, 3723–3734 (2016).

53. Hiraishi, a., Ueda, Y.: Intrageneric structure of the genus *Rhodobacter*: Transfer of *Rhodobacter sulfidophilus* and related marine species to the genus *Rhodovulum* gen. nov. *International Journal of Systematic Bacteriology*. 44, 15–23 (1994).

54. Hansen, T.A., Veldkamp, H.: *Rhodopseudomonas sulfidophila*, nov. spec., a new species of the purple nonsulfur bacteria. *ArchivMikrobiologie*. 92, 45–58 (1973).

55. Hiraishi, A., Uedat, Y.: Isolation and characterization of *Rhodovulum strctum* sp. nov. and Some Other Purple Nonsulfur Bacteria from Colored Blooms in Tidal and Seawater Pools. *International Journal of Systematic Bacteriology*. 45, 319–326 (1995).

56. Hagemann, G.E., Katsiou, E., Forkl, H., Steindorf, A.C.J., Tadros, M.H.: Gene cloning and regulation of gene expression of the puc operon from *Rhodovulum sulfidophilum*. *Biochimica et Biophysica Acta*. 1351, 341–358 (1997).

57. Masuda, S., Nagashima, K.V.P., Shimada, K., Matsuura, K.: Transcriptional Control of Expression of Genes for Photosynthetic Reaction Center and Light-Harvesting Proteins in the Purple Bacterium <i>Rhodovulum sulfidophilum<i>. *Journal of Bacteriology*. 182, 2778–2786 (2000).

58. Watanabe, M., Saasaki, K., Nishio, N., Kakizono, T.: Growth and focculation of a marine photosynthetic bacterium. *Applied Microbiology and Biotechnology*. 50, 682–691 (1998).

59. Watanabe, M., Sasaki, K., Nakashimada, Y., Kakizono, T., Noparatnaraporn, N., Nishio, N.: Promotion of growth and flocculation of a marine photosynthetic bacterium *Rhodovulum* sp. by metal cations. *Biotechnology Letters*. 20, 1109–1112 (1998).

60. Cai, J., Wang, G.: Hydrogen production by a marine photosynthetic bacterium, *Rhodovulum sulfidophilum* P5, isolated from a shrimp pond. *International Journal of Hydrogen Energy*. 37, 15070–15080 (2012).

61. Loo, P.L., Vikineswary, S., Chong, V.C.: Nutritional value and production of three species of purple non-sulphur bacteria grown in palm oil mill effluent and their application in rotifer culture. *Aquaculture Nutrition*. 19, 895–907 (2013).

62. Loo, P.L., Chong, V.C., Vikineswary, S.: *Rhodovulum sulfidophilum*, a phototrophic bacterium, grown in palm oil mill effluent improves the larval survival of marble goby Oxyeleotris marmorata (Bleeker). *Aquaculture Research*. 44, 495–507 (2013).

63. Matsunaga, T., Hatano, T., Yamada, a, Matsumoto, M.: Microaerobic hydrogen production by photosynthetic bacteria in a double-phase photobioreactor. *Biotechnology and bioengineering*. 68, 647–51 (2000).

64. Maeda, I., Miyasaka, H., Umeda, F., Kawase, M., Yagi, K.: Maximization of hydrogen production ability in high-density suspension of *Rhodovulum sulfidophilum* cells using intracellular poly(3-hydroxybutyrate) as sole substrate. *Biotechnology and*

Bioengineering. 81, 474–481 (2003).

65. Azad, A.S., Vikineswary, S., Ramachandran, K.B., Chong, V..: Growth and production of biomass of *Rhodovulum sulfidophilum* in sardine processing wastewater. *Letters in Applied Microbiology*. 33, 264–268 (2001).

66. Idi, A., Md Nor, M.H., Abdul Wahab, M.F., Ibrahim, Z.: Photosynthetic bacteria: an eco-friendly and cheap tool for bioremediation. *Reviews in Environmental Science and Biotechnology*. 14, 271–285 (2015).

67. Friedrich, C.G., Bardischewsky, F., Rother, D., Quentmeier, A., Fischer, J.: Prokaryotic sulfur oxidation. *Current Opinion in Microbiology*. 8, 253–259 (2005).

68. Ando, T., Suzuki, H., Nishimura, S., Tanaka, T., Hiraishi, A., Kikuchi, Y.: Characterization of extracellular RNAs produced by the marine photosynthetic bacterium *Rhodovulum sulfidophilum*. *Journal of Biochemistry*. 139, 805–811 (2006).

69. Suzuki, H., Daimon, M., Awano, T., Umekage, S., Tanaka, T., Kikuchi, Y.: Characterization of extracellular DNA production and flocculation of the marine photosynthetic bacterium *Rhodovulum sulfidophilum*. *Applied Microbiology and Biotechnology*. 84, 349–356 (2009).

70. Winzer, K., Hardie, K.R., Williams, P.: Bacterial cell-to-cell communication: Sorry, can't talk now - Gone to lunch! *Current Opinion in Microbiology*. 5, 216–222 (2002).

71. Nagao, N., Yamamoto, J., Komatsu, H., Suzuki, H., Hirose, Y., Umekage, S., Ohyama, T., Kikuchi, Y.: The gene transfer agent-like particle of the marine phototrophic bacterium *Rhodovulum sulfidophilum*. *Biochemistry and Biophysics Reports*. 4, 369–374 (2015).

72. Watanabe, M., Suzuki, Y., Sasakl2, K., Nakashimada, Y., Nishio ', N.: Flocculating Property of Extracellular Polymeric Substance Derived from a Marine Photosynthetic Bacterium, *Rhodovulum* sp. *Journal of Bioscience and Bioengineering*. 87, 625–629 (1999).

73. Noparatnaraporn, N., Watanabe, M., Choorit, W., Sasaki, K.: Production of RNA by a marine photosynthetic bacterium, *Rhodovulum* sp. *Biotechnology Letters*. 22, 1867–1870 (2000).

74. Suzuki, H., Umekage, S., Tanaka, T., Kikuchi, Y.: Extracellular tRNAs of the marine photosynthetic bacterium *Rhodovulum sulfidophilum* are not aminoacylated. *Bioscience, biotechnology, and biochemistry*. 73, 425–427 (2009).

75. Kleman, G.L., Strohl, W.R.: Developments in high cell density and high productivity microbial fermentation. *Current Opinion in Biotechnology*. 5, 180–186 (1994).

76. Thiry, M., Cingolani, D.: Optimizing scale-up fermentation processes. *Trends in Biotechnology*. 20, 103–105 (2002).

77. Obom, K.M., Magno, A., Cummings, P.J.: Operation of a Benchtop Bioreactor. *Journal of Visualized Experiments*. 79, e505882 (2013).

78. Stanbury, P.F., Whitaker, A., Hall, S.J.: Media for industrial fermentations. In: Principles of Fermentation Technology. pp. 93–122 (1995).

79. Garcia-Ochoa, F., Gomez, E., Santos, V.E., Merchuk, J.C.: Oxygen uptake rate in microbial processes: An overview. *Biochemical Engineering Journal*. 49, 289–307 (2010).

80. Harry Holms: Flux analysis and control of the central metabolic pathways in *Escherichia coli*. *FEMS Microbiology Reviews*. 19, 85–116 (1996).

81. Werner, S., Diekert, G., Schuster, S.: Revisiting the Thermodynamic Theory of Optimal ATP Stoichiometries by Analysis of Various ATP-Producing Metabolic Pathways. *Journal of Molecular Evolution*. 71, 346–355 (2010).

82. Pranata, W., Suryadarma, P., MAngunwidjaja, D.: Glycerol Utilization as Substrate for Ethanol Production in Escherichia coli Recombinant under an Aerobic Condition. *Journal of Tropical Life Science*. 8, 81–86 (2018).

83. da Silva, G.P., Mack, M., Contiero, J.: Glycerol: A promising and abundant carbon source for industrial microbiology. *Biotechnology Advances*. 27, 30–39 (2009).

84. Pastor, J.M., Bernal, V., Salvador, M., Argandoña, M., Vargas, C., Csonka, L., Sevilla, Á., Iborra, J.L., Nieto, J.J., Cánovas, M.: Role of central metabolism in the osmoadaptation of the halophilic bacterium chromohalobacter salexigens. *Journal of Biological Chemistry*. 288, 17769–17781 (2013).

85. Pomeroy, L.R., Wiebe, W.J.: Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. *Aquatic Microbial Ecology*. 23, 187–204 (2001).

86. Sarkar, S., Pramanik, A., Mitra, A., Mukherjee, J.: Bioprocessing data for the production of marine enzymes. *Marine Drugs*. 8, 1323–1372 (2010).

87. Schmidt, F.R.: Optimization and scale up of industrial fermentation processes. *Appl Microbiol Biotechnol*. 68, 425–435 (2005).

88. Li, B., Chen, X., Ren, H., Li, L., Xiong, J., Bai, J., Chen, Y., Wu, J., Ying, H.: Kinetic models of ribonucleic acid fermentation and continuous culture by *Candida tropicalis*. *Bioprocess and Biosystems Engineering*. 35, 415–422 (2012).

89. Echiegu, E.A.: Kinetic Models for Anaerobic Fermentation Processes-A Review. *American Journal of Biochemistry and Biotechnology*. 11, 132–148 (2014).

90. Song, H., Jang, S.H., Park, J.M., Lee, S.Y.: Modeling of batch fermentation kinetics for succinic acid production by *Mannheimia succiniciproducens*. *Biochemical Engineering Journal*. 40, 107–115 (2008).

91. Silva, F., Passarinha, L., Sousa, F., Queiroz, J.A., Domingues, F.C.: Influence of growth conditions on plasmid DNA production. *Journal of Microbiology and Biotechnology*. 19, 1408–1414 (2009).

92. Hall, B.G., Acar, H., Nandipati, A., Barlow, M.: Growth rates made easy. *Molecular Biology and Evolution*. 31, 232–238 (2014).

93. Passarinha, L.A., Bonifácio, M.J., Queiroz, J.A.: The effect of temperature on the analysis of metanephrine for catechol-O-methyltransferase activity assay by HPLC with electrochemical detection. *Biomedical Chromatography*. 20, 937–944 (2006).

94. Gallardo E, P. LA: An Improved HPLC Method for Quantification of Metanephrine with Coulometric Detection. *Journal of Chromatography & Separation Techniques*. 5, 1–7 (2014).

95. Rio, D.C., Ares, M., Hannon, G.J., Nilsen, T.W.: Purification of RNA using TRIzol (TRI Reagent). *Cold Spring Harbor Protocols*. 2010, 1–4 (2010).

96. Silva, R., Ferreira, S., Bonifácio, M.J., Dias, J.M.L., Queiroz, J.A., Passarinha, L.A.: Optimization of fermentation conditions for the production of human soluble catechol-O-methyltransferase by *Escherichia coli* using artificial neural network. *Journal of Biotechnology*. 160, 161–168 (2012).

97. Espírito Santo, G.M., Pedro, A.Q., Oppolzer, D., Bonifácio, M.J., Queiroz, J.A., Silva, F., Passarinha, L.A.: Development of fed-batch profiles for efficient biosynthesis of catechol-O-methyltransferase. *Biotechnology Reports*. 3, 34–41 (2014).

98. Watanabe, M., Sasaki, K., Nakashimada, Y., Nishio, N.: High density cell culture of a marine photosynthetic bacterium *Rhodovulum* sp. with self-flocculated cells. *Biotechnology Letters*. 20, 1113–1117 (1998).

99. Jaapar syed Syaripah, Kalil Sahaid Mohd, Ali Ehsan, A.N.: Effect of age of inoculum, size of inoculum and headspace on hydrogen production using *Rhodobacter sphaeroides*. *Bacteriology Journal*. 1, 16–23 (2011).

100. Bidlas, E., Du, T., Lambert, R.J.W.: An explanation for the effect of inoculum size on MIC and the growth/no growth interface. *International Journal of Food Microbiology*. 126, 140–152 (2008).

101. Pin, C., Baranyi, J.: Kinetics of Single Cells : Observation and Modeling of a Stochastic Process. *Applied and Environment Microbiology*. 72, 2163–2169 (2006).

102. Surette, M.G., Miller, M.B., Bassler, B.L.: Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proceedings of the National Academy of Sciences of the United States of America*. 96, 1639–44 (1999).

103. Terada, T., Kikuchi, Y., Umekage, S.: Improved bioassay for detecting autoinducer of *Rhodovulum sulfidophilum*. In: AIP Conference Proceedings. pp. 67–70 (2015).

104. Ando, T., Suzuki, H., Komura, K., Tanaka, T., Hiraishi, A.: Extracellular RNAs produced by a marine photosynthetic bacterium *Rhodovulum sulfidophilum*. 48, 165–166 (2004).

105. Pinches, A., Pallent, L.J.: Rate and yield relationships in the production of xanthan gum by batch fermentations using complex and chemically defined growth media. *Biotechnology and Bioengineering*. 28, 1484–1496 (1986).

106. Temudo, M.F., Poldermans, R., Kleerebezem, R., Van Loosdrecht, M.C.M.: Glycerol fermentation by (open) mixed cultures: A chemostat study. *Biotechnology and Bioengineering*. 100, 1088–1098 (2008).

107. Lang, S., Hüners, M., Lurtz, V.: Bioprocess Engineering Data on the Cultivation of Marine Prokaryotes and Fungi. In: Marine Biotechnology II. Advances in Biochemical Engineering/Biotechnology. pp. 29–62. Springer (2005).

108. Desjardins, P.R., Conklin, D.S.: Microvolume quantitation of nucleic acids. *Current Protocols in Molecular Biology*. 45, e2565 (2011).

109. Dennis, P.P., Bremer, H.: Modulation of Chemical Composition and Other Parameters of the Cell at Different Exponential Growth Rates. *EcoSal Plus: Cellular and Molecular Biology of E.coli, Salmonela, and Enterobacteriaceae*. 3, 1–49 (2008).

110. Shin, H.S., Lim, H.C.: Optimal fed-batch operation of recombinant cells subject to

plasmid instability and death. *Bioprocess and Biosystems Engineering*. 31, 655–665 (2008).

111. Hewitt, C.J., Nienow, A.W.: The Scale-Up of microbial batch and fed-batch fermentation processes. In: Marin Berovic, M. and Enfors, S. (eds.) Comprehensive Bioprocess Engineering. pp. 295–320. University of Ljubljana (2010).

112. Mohseni, S.S., Babaeipour, V., Vali, A.R.: Design of sliding mode controller for the optimal control of fed-batch cultivation of recombinant *E. coli*. *Chemical Engineering Science*. 64, 4433–4441 (2009).

Appendix

Poster presentation at the XIII CICS-UBI Symposium, Covilhã (July 2018): Jane Dias, Diana Duarte, Fani Sousa, Luís Passarinha: Optimization of fermentation conditions of *Rhodovulum sulfidophilum*in mini-bioreactor for the production of noncoding RNAs

CICS-UBI
Centro de Investigação em Ciências da Saúde
Health Sciences Research Centre

FACULDADE CIÊNCIAS DA SAÚDE

Optimization of fermentation conditions of *Rhodovulum sulfidophilum* in mini-bioreactor for the production of noncoding RNAs

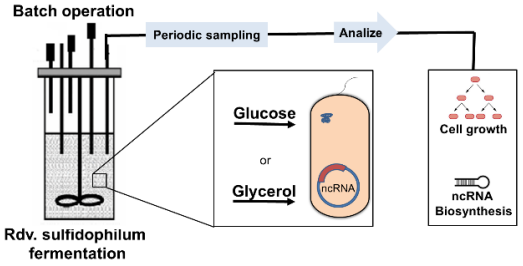
Jane Dias[1], Diana Duarte[1], Fani Sousa[1], Luís Passarinha[1, 2, 3]

[1] CICS-UBI - Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, 6200-506, Covilhã, Portugal
[2] UCIBIO@REQUINTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
[3] Laboratório de Fármaco-Toxicologia-UBIMedical, Universidade da Beira Interior, Covilhã, Portugal

Introduction

- The discoveries of novel roles of noncoding RNAs (ncRNA), such as microRNAs and small interfering RNAs, have motivated the rise of RNA-centered research. Parallelly, there is increase efforts in developing and/or improving RNA synthesis methods, highlight recombinant RNA methods.
- *Rhodovulum sulfidophilum (Rdv. sulfidophilum),* a peculiar bacteria, has been studied as a potential host for ncRNAs biosynthesis based on recombinant technology [1].
- This work aims to optimize the fermentation of *Rdv. sulfidophilum DSM 1374* taking into consideration, mainly, the bacterial growth and the biosynthesis of recombinant ncRNAs.

Materials and Methods



Results

Figure 1

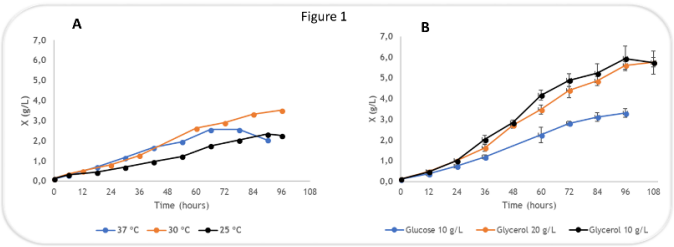


Fig 1. Growth profile of *Rdv. sulfidophilum* cultivated (A) under different temperatures and (B) having glucose or glycerol as main carbon source. Error bars indicate standard deviations calculated from 2 independent samples.

Table 1. – Summary of batch fermentation parameters under different conditions. Si: initial carbon source concentration; Sf: final carbon source concentration; Xf: final biomass; μ: specific growth rate; Tf: fermentation time.

	Glucose 10 g/L			Glycerol (20 g/L)	Glycerol (10 g/L)
	25 °C	37 °C	30 °C	30 °C	30 °C
S_i (g/L)			7.90	22.81	10.92
Sf (g/L)			0.55	8.26	3.00
μ (h^{-1})	0.020	0.022	0.027	0.030	0.033
X_f (g/L)	2.34	2.57	3.30	5.75	5.94
T_f	90	78	96	108	96

Figure 2

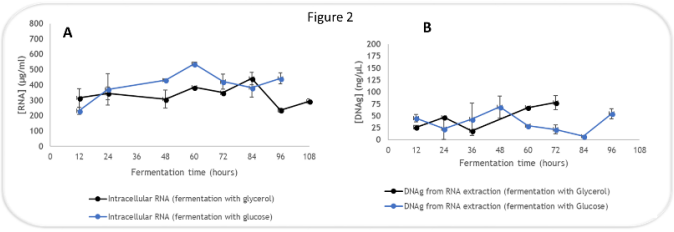


Fig 2. Time-course analysis of intracellular RNA biosynthesis in *Rdv. Sulfidophilum* having glucose (blue lines) and glycerol (black lines) as main carbon source. (A) Total RNA (tRNA) production measured spectrophotometrically. Error bars indicate standard deviations calculated from 2 independent samples. (B) DNAg obtained from RNA extraction samples and measured by RT-PCR. Error bars indicate standard deviations calculated from 2 independent samples. .

Figure 3

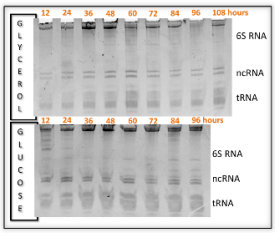


Fig 3. – Time-course electrophoretic analysis of intracellular tRNA fractions of *Rdv. sulfidophilum having glucose and glycerol as main carbon source.*

Conclusion

- The results suggest that the best conditions for batch cultivation of *Rdv. sulfidophilum* on mini-bioreactors are cultivation at 30 ºC having glycerol as main carbon source, preferentially with a initial concentration of 10 g/L since it allows higher growth rates and higher cell densities without substrate excess at the end of fermentation period and without sacrifice the production of the target molecule.

REFERENCES
[1] Peralta et al., Appl. Microbiol. Biotechnol., 100 (8), pp. 3723-3734, 2016.

FCT COMPETE 2020 PORTUGAL 2020