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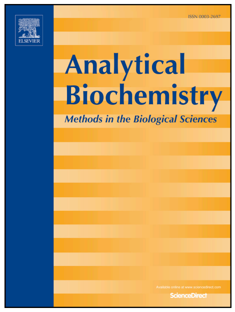

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A New Approach for Extracellular RNA Recovery from *Rhodovulum sulfidophilum*

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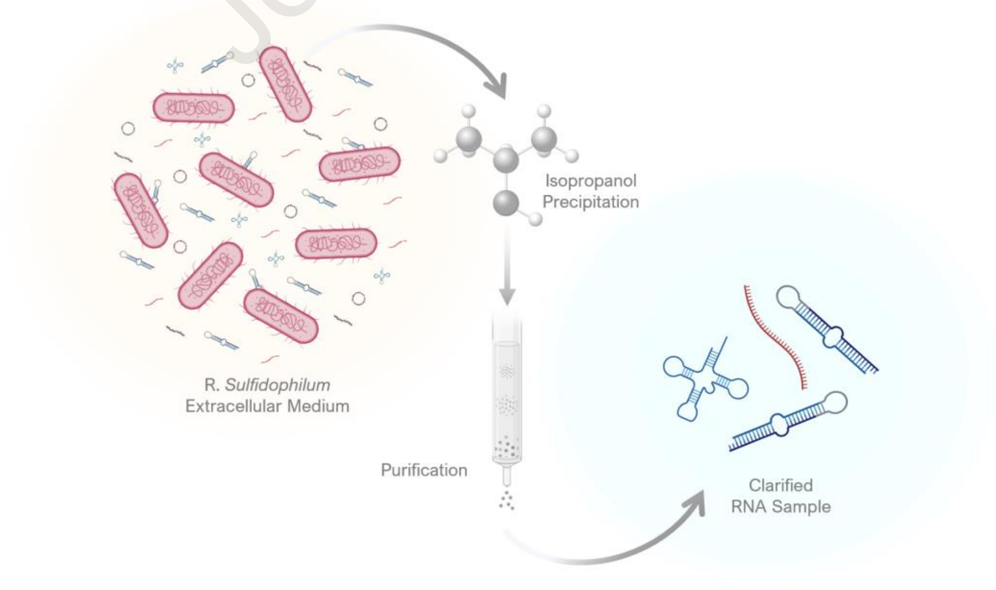
Graphical Abstract



Abstract

The development of RNA-based drugs is highly pursued due to the possibility of creating viable and effective therapies. However, their translation to clinical practice strongly depends on efficient technologies to produce substantial levels of these biomolecules, with high purity and high quality. RNAs are commonly produced by chemical or enzymatic methods, displaying these limitations. In this sense, recombinant production arises as a promising, cost-effective method, allowing large-scale production of RNA. *Rhodovulum sulfidophilum (R. sulfidophilum)*, a marine purple bacterium, offers the advantage of RNA secretion into the extracellular medium, which contains low levels of RNases and other impurities. Therefore, RNA recovery can be simplified compared to standard extraction protocols involving cell lysis, resulting in a more clarified sample and an improved downstream process. In this work, *R. sulfidophilum* was transformed with a plasmid DNA encoding pre-miR-29b-1, which is known to be involved in the Alzheimer's disease pathway. After production, the pre-miR-29b-1 was recovered through different extraction methods to verify the most advantageous one. A protocol for extracellular RNA recovery was then established, revealing to be a simpler and less time-consuming method, reducing around 16 h in execution time and the quantity of reagents needed when compared to other established methods. The new strategy brings the additional advantage of eliminating the toxic organic compounds routinely used in intracellular RNA extractions. Overall, the optimized process described here, using isopropanol as the precipitation agent, offers a greener, safer, and faster alternative for recombinant RNA recovery and concentration, with an extracellular RNA recovery of 7 µg/mL and target pre-miRNA-29b-1 recovery of 0.7 µg/L of medium.

Keywords: RNA, *Rhodovulum sulfidophilum*, Extracellular RNA recovery, Clarification, Green process.

1. Introduction

Ribonucleic acids (RNA) are gaining increased relevance as biopharmaceuticals due to significant advances in the discovery of their respective biological roles. For example, RNA methylation can play a critical role in epigenetic regulatory roles from plants to animals, controlling growth and development [1]. RNA interference technology is also a novel tool used to regulate gene expression in eukaryotic cells, showing high potential as a therapeutic approach for genetic diseases. This technology is mainly supported by the use of noncoding RNAs, such as microRNAs (miRNAs), small interfering RNAs, piwi-interacting RNAs, and long non-coding RNAs [2]. Indeed, there are plenty of opportunities to exploit the RNA potential. Still, for a better understanding of their role in cellular mechanisms and to perform biochemical and biophysical studies, an efficient RNA production process is needed [3]. The main objectives are related to producing substantial levels of these biomolecules in a cost and time-effective manner, while also ensuring a green and safe option [4, 5]. Techniques suitable for molecular biology can be used, but preparing RNAs for therapeutic purposes imposes additional challenges, and the procedures must be adapted to ensure a high-quality product.

Most RNAs are obtained through chemical, enzymatic, or recombinant production [4], but the first two present some limitations mainly related to low production yields and considerably higher costs [3, 6]. Given this, recombinant production emerges as a promising method for large-scale production, being the most cost-effective process. Moreover, recombinant techniques using *in vivo* agents are more capable of producing RNAs that can retain the structure, function, and safety properties of natural RNAs [2, 4]. Recombinant RNA expression has been mostly achieved using *Escherichia coli* (*E. coli*) as the host organism [7-9], and the RNA extraction relies on cell lysis through the acid guanidinium thiocyanate-phenol-chloroform method [10], which results in a sample with cellular components as the major impurities [4, 11]. As an alternative, *Rhodovulum sulfidophilum* (*R. sulfidophilum*), a marine gram-negative non-sulfur phototrophic bacterium, is known to naturally secrete nucleic acids in soluble form in the culture media [12-14], while not secreting RNases, contributing to increased stability of the produced RNA [7, 12]. With this host, Kikuchi and co-workers produced recombinant artificial RNAs [5], while our group produced recombinant human pre-miR-29b extracellularly [8]. After production, the extracellular RNA can be directly recovered from the culture medium by alcoholic precipitation, avoiding cell lysis and release of the main bacterial-associated impurities, resulting in an RNA product that might be non-immunogenic [5, 8, 11, 12]. Isopropanol is more effective at precipitating nucleic acids, particularly for smaller fragments or low concentrations, and requires less volume and shorter incubation time when compared to ethanol [15]. Accordingly, this work describes a low-cost, sustainable, simple, and fast method for recovering extracellular nucleic acids from *R. sulfidophilum* culture medium, using isopropanol as the precipitation agent. Moreover, the optimized method is expected to yield a high quantity of specific RNAs, contributing to a simplified downstream processing.

2. Materials and methods

2.1 Materials

All chemicals and reagents used were of analytical grade. Isopropanol, ethanol (purity 99.99%) and acetic acid (99-100%) were purchased from Thermo Fisher Scientific Inc. (Massachusetts, USA). Sodium acetate and tris(hydroxymethyl)aminomethane (Tris) were acquired from Merck (Darmstadt, Germany). Sephacryl SF-1000 was purchased from GE Healthcare (Chicago, EUA). Ethylene-diamine tetra acetic acid (EDTA) and sodium chloride were acquired from Panreac Química S.L.U. (Barcelona, Spain). All solutions were prepared with ultra-pure grade water, purified with a Milli-Q system from Merck Millipore (Darmstadt, Germany) treated with 0.01% of diethyl pyrocarbonate (DEPC) from Sigma-Aldrich (St. Louis, Missouri, USA). Green-Safe and agarose were purchased from Grisp (Porto, Portugal). The phototrophic marine alphaproteobacterium *R. sulfidophilum* DSM 1374 (Number 35886, Taxonomy ID1188256) was purchased from ATCC (Virginia, USA). pNZY28-pre-miR-29b-1 used to transform the host bacteria was bought from NZYTech (Lisbon, Portugal). For quantitative polymerase chain reaction (qPCR), the Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) purchased from Thermo Fisher Scientific Inc. (Massachusetts, USA), Xpert cDNA Synthesis Kit from Grisp (Porto, Portugal), and specific primers for pre-miRNA-29b-1 amplification synthesized by STAB VIDA (Caparica, Portugal) were acquired. NZY DNase I was obtained from NZYtech (Lisbon, Portugal) and MEGAclear Transcription Clean-Up Kit was from Thermo Fisher Scientific Inc. (Massachusetts, USA).

2.2. Methods

2.2.1 Plasmid Design and Bacterial Growth

For the pNZY28-pre-miR-29b-1 plasmid design, the pNZY28 commercial vector purchased from NZYtech (Lisbon, Portugal) was used with some modifications. The ampicillin-resistance sequence was removed and replaced by the kanamycin-resistance gene because wild-type (WT) *R. sulfidophilum* is naturally resistant to ampicillin. Then, the T7 promoter was removed by GenScript (New Jersey, USA), and a DNA fragment containing the *rrn* promoter, the pre-miR-29b-1 sequence, flanked by ribozyme sequences, and *puf* terminator region was cloned into the plasmid, as a service purchased to NZYtech. Previously, our research group demonstrated that this DNA fragment has the key elements for its amplification and transcription in *R. sulfidophilum* [8]. The hsa-pre-miR-29b sequence was obtained through miRBase information (https://www.mirbase.org accessed in January 2023). The final plasmid, with around 5 kb, was sequenced by GenScript to confirm the identity and orientation of the DNA fragment in the recombinant plasmid.

For the heat shock transformation procedure, 100 µL of competent *R. sulfidophilum* cells were incubated with 25 ng of plasmid at 35 ºC for 1 min, as described in a previous work [8]. The culture was then plated on agar plates with the specific medium supplemented with kanamycin and incubated at 30 ºC for 3 days. To confirm the transformation of cells, several colonies were screened by PCR for the presence of the pNZY28-pre-miR-29b-1 plasmid containing the pre-miR-29b-1 sequence, using the primers previously designed [8]. From a positively transformed colony, a bacterial culture was grown, and the plasmid was isolated and purified using the NZYMiniprep Kit (NZYtech, Lisbon, Portugal) and validated again by PCR to confirm the identity of the designed vector. The *R. sulfidophilum* WT and the transformed strain (T) were cultivated in a semi-defined medium, as previously described [8].

2.2.3 Extracellular Nucleic Acids Recovery

For extracellular nucleic acids recovery [14], 10 mL of *R. sulfidophilum* DSM 1374 culture medium was centrifuged at 4304 rpm, for 10 min at 4 ºC. The supernatant was then transferred to a tube and centrifuged a second time at 6600 rpm, for 10 min at 4 ºC to remove residual cells. To precipitate the nucleic acids from the medium, 1 volume of ice-cold isopropanol was added to the supernatant and an incubation for 10 min on ice was performed. Then, the sample was centrifuged at 6600 rpm, for 10 min, at 4 ºC, and the nucleic acids pellet was air-dried for 10 min at room temperature (RT) and solubilized in 1 mL 0.01% of DEPC-treated water. The sample was stored at -80 ºC and after defrosting it was centrifuged at 6600 rpm, for 1 min at RT, to remove the salt that precipitates during storage. After nucleic acids solubilization, the samples were purified by size exclusion chromatography. For this, a column packed with 5 mL of Sephacryl SF-1000 was used to separate RNA and DNA. The column was equilibrated with 20 mL of buffer (10 mM Tris-EDTA, 150 mM NaCl pH 7), and 500 µL of sample was added. The elution was carried out by adding 20 mL of the same buffer. Fractions of 1 mL were collected for further evaluation. For each sample, this procedure was repeated two times to purify the nucleic acids sample resulting from precipitation protocols (1 mL). At the end of each assay, columns were washed with DEPC-treated water. The RNA fractions were desalted and concentrated with concentrators Vivaspin 10000 Da (Vivascience).

The nucleic acids precipitation with Ethanol Overnight Protocol (EOP) was based on previous work from our research group with some adjustments [8]. In this case, 10 mL of *R. sulfidophilum* DSM 1374 culture medium was centrifuged at 4304 rpm, for 10 min at 4 ºC. The supernatant was then transferred to a new tube and centrifuged a second time at 6600 rpm, for 10 min at 4 ºC to remove residual cells. After incubation overnight at -20 ºC, the supernatant was transferred to a clean tube to separate the salt pellet precipitated overnight. The sample was stored at -80 ºC and after defrosting, it was centrifuged at 6600 rpm, for 1 min at RT, to remove the additional salt that had precipitated. As described above, after nucleic acids solubilization the samples were purified by size exclusion chromatography. The RNA fractions were desalted and concentrated with concentrators Vivaspin 10000 Da (Vivascience). Figure 1 shows a schematic representation of the described methods.

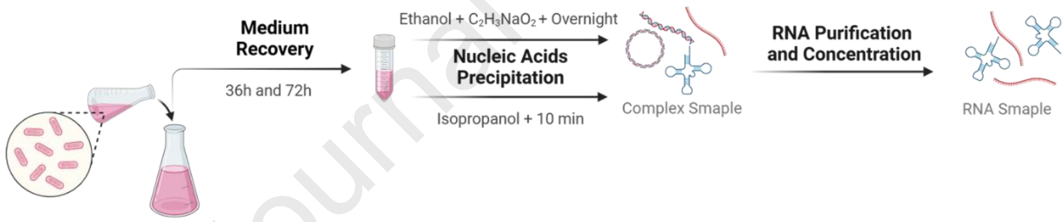


Figure 1: Schematic representation of extracellular nucleic acid recovery by isopropanol (IP) or ethanol overnight (EOP) precipitation. *10 mL of R. sulfidophilum DSM 1374 culture was recovered* at 36 h and 72 h and after *centrifug*ation to separate the medium from cells, the nucleic acids were precipitated from the medium by ethanol or isopropanol protocol. The resulting sample was then *purified by size exclusion chromatography* and concentrated *to obtain RNA.*

2.2.4 Intracellular Nucleic Acids Extraction

RNA extraction was performed by the method of acid guanidinium thiocyanate-phenol-chloroform, as described in the literature [16, 17]. After cell lysis and separation of cell debris from nucleic acids by biphasic extraction, the upper phase enriched in RNA was carefully transferred to new tubes, avoiding DNA contamination from the bottom phase. The resultant pellets were washed several times with isopropanol and ethanol and air-dried. RNA pellets were dissolved in DEPC-treated water and stored at -80 °C.

For extraction of all nucleic acids from *R. sulfidophilum* cells, the protocol was initiated with chemical lysis as previously described [18]. The recovered nucleic acid pellets were resuspended in DEPC-treated water and incubated in a 60 °C water bath for 10 min, and finally centrifuged, recovering the supernatant. The samples were stored at - 80 °C. A schematic representation of these procedures is shown in Figure 2.

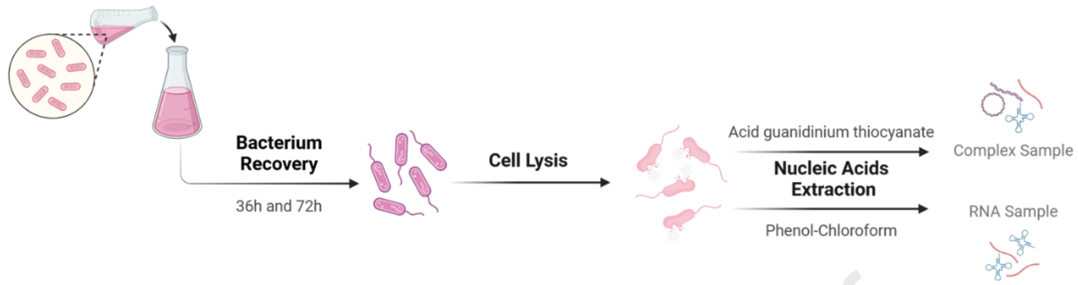


Figure 2: Schematic representation of the phenol-chloroform extraction protocol and total nucleic acids extraction. *10 mL of R. sulfidophilum DSM 1374 culture was recovered at 36 h and 72 h. Then the cells were lysed, and the nucleic acids were extracted by acid guanidinium thiocyanate or by phenol-chloroform protocol, resulting in a total nucleic acid sample or RNA sample, respectively.*

2.2.5 Analysis of the Recovered Nucleic Acids

Recovered nucleic acids by each protocol were analyzed in terms of integrity and relative purity by agarose gel electrophoresis in 1 % agarose gel. Nucleic acids were stained in the gel with Green Safe (0.012 µL/mL), and electrophoresis was run at 120 V for 30 min in TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA, pH 8.0). The gels were visualized under ultraviolet (UV) light exposure with a UV chamber (UVITEC Cambridge, Cambridge, United Kingdom). Sample concentration was measured in the Nano Photometer (IMPLEN, United Kingdom) at 260 nm.

2.2.6 pre-miR-29b-1 Analysis by Quantitative Polymerase Chain Reaction

pre-miR-29b-1 production was assessed by qPCR as described in a previous work [19]. cDNA synthesis was performed using Xpert cDNA Synthesis Kit (Grisp, Portugal), according to the manufacturer's instructions. About 26 ng of RNA sample was used to initiate cDNA synthesis, with a 20 pmol gene-specific primer described in [19]. Then, PCR reactions were carried out, in a 20 µL reaction containing 10 µL of Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) and 0.3 µM of each specific primer. Specific primers for cDNA and qPCR were reconstituted in nuclease-free water to a final concentration of 100 µM and designed based on previous work [19]. DNase I (2U/µL) was used to treat extracellular RNA samples, to remove any remaining DNA, and MEGAclear Transcription Clean-Up Kit was used to clean the samples from DNase I.

3. Results and Discussion

3.1 pNZY28-pre-miR-29b-1 vector construction

Several modifications were made to the pNZY28 commercial vector to construct the pNZY28-pre-miR-29b-1 plasmid. The kanamycin-resistance sequence and the DNA fragment containing the *rrn* promoter, the pre-miR-29b-1 sequence, flanked by ribozyme sequences, and *puf* terminator region were cloned in the vector backbone. To confirm the identity and orientation of DNA sequences, the plasmid was tested experimentally and sequenced (Supporting Information). Figure S1 shows the electrophoresis corresponding to the plasmid digestion by *NcoI,* an enzyme that recognizes the sequence 5'- CCATGG – 3' present in the plasmid. The expected molecular weight of the plasmid is around 5 kb after linearization, which was confirmed in the agarose gel (Figure S1). The analysis of sequencing data revealed that the plasmid presents all the intended sequences, kanamycin-resistance gene, *rrn* promoter, pre-miR-29b-1 gene, ribozymes and *puf* terminator (data not shown). The reference sequences for the ribozymes can be accessed in the RNAcentral database (https://rnacentral.org/). Part of the sequence of the pNZY28-pre-miR-29b-1, demonstrates the presence of the intended pre-miR-29b-1 sequence, flanked by ribozyme sequences (Figure S2).

3.2 Bacterial Growth and Nucleic Acids Production

After the design and construction of the vector and transformation of the bacteria, a comparison of the growth profile of both transformed (T) and non-transformed (WT) *R. sulfidophilum* was performed. The optical density (OD) was measured every 12 h along 72 h of culture, with the resulting growth curves being shown in Figure 3. The growth curve corresponding to the WT *R. sulfidophilum* strain reveals that the OD reaches approximately 5 during the culture time, in accordance with previous studies [5, 8]. The growth curve of the strain transformed with the plasmid pNZY28-pre-miR-29b-1, designed for the recombinant production of the pre-miR-29b-1, is also shown in Figure 3. The growth of the transformed strain is significantly lower than that of the WT strain (OD of 1 versus 4.5 at 72 h of growth). This suggests that the transformation induces alterations in cell metabolism, leading to a significant decrease in the growth rate, which can directly affect the nucleic acids produced and secreted to the extracellular medium. However, not only the incorporation of the plasmid can explain the decreased bacterial growth. The incorporation of the kanamycin antibiotic in the culture medium can also restrain the growth of non-transformed bacteria, thus resulting in a lower cell density, corresponding to the selective growth of the transformed bacteria. Despite this, the growth of *T R. sulfidophilum* can be improved by developing perfusion cultures to increase viable cell density [20].

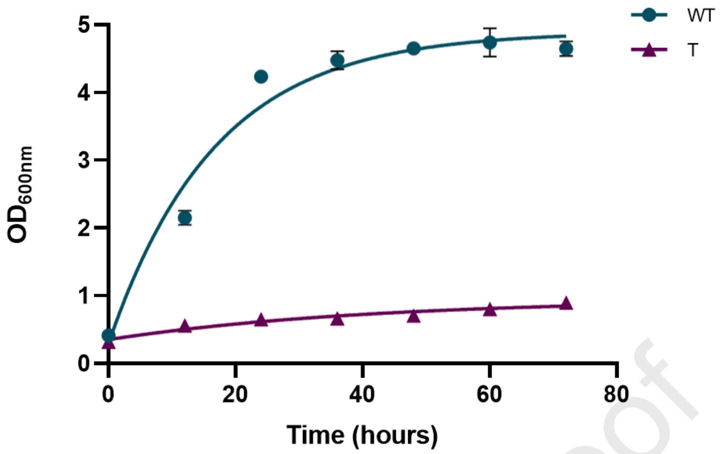


Figure 3: Growth curves profile of R. sulfidophilum DSM 1374 cultivated in a semi-defined medium, at 30 °C, 250 rpm, under dark aerobic conditions. *WT- Wild type R. sulfidophilum DSM 1374; T - R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1.*

Based on the obtained results, it is important to confirm if the decrease in the bacteria growth had any impact on nucleic acids production. Different protocols described in Materials and Methods were used for the extraction and recovery of nucleic acids from the WT and T *R. sulfidophilum.* The concentration of total nucleic acids obtained with these methods using 10 mL of culture is displayed in Figure 4A. For the IP (Isopropanol Protocol) and LP (Lysate Protocol), the quantity of extracted nucleic acids was approximately the same in both WT and T strains. On the contrary, this is not observed when the EOP (Ethanol Overnight Protocol) is performed. In this case, the content of nucleic acids recovered from the extracellular medium was considerably higher in the WT than in the T bacteria. However, considering the difference in the growth of both strains, a normalization with the OD was performed. The assessment of the recovery considering the number of cells would give more information regarding the nucleic acids yield in both WT and T strains, similar to a comparison between the specific *versus* the volumetric productivities. The results from the normalization with the OD_{600nm} corresponding to each time point and strain are shown in Figure 4B. The levels of nucleic acids are higher for the T strain compared to the WT, except for the EOP protocol. These results suggest that the optimized protocol based on the IP can be more advantageous for the recovery of total nucleic acids from the extracellular medium than the method previously reported by Sousa and co-workers [8]. It should be noted that all the quantifications from here on are represented with the OD_{600nm} normalization.

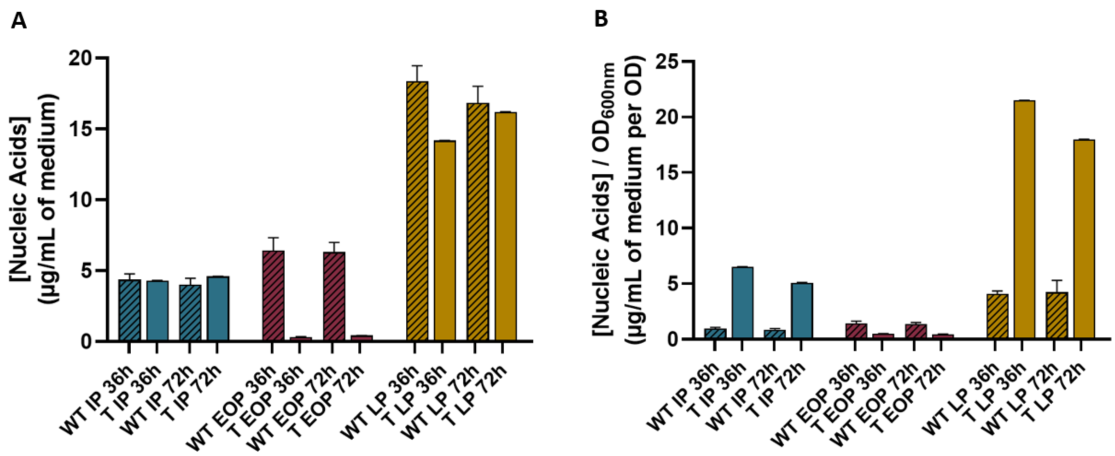


Figure 4: Extra- and intracellular nucleic acid levels, recovered at 36 and 72 hour of bacteria cultivation, for IP, EOP, and LP. *WT- Wild type R. sulfidophilum DSM 1374; T - R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1. A: Nucleic acids concentration; B: Nucleic acids concentration after normalization with the* OD_{600nm}*; Data shown are mean ± SD from three repeats of triplicate cultivations.*

Besides measuring sample concentration, it is also important to identify the different nucleic acid species that are present. The agarose gels presented in Figures 5A and 5B show the results from the extracted samples from WT and T strains, respectively. Analyzing each extracellular extraction protocol allows to show that the IP sample from the T strain contains a higher quantity of RNA compared to the WT one which contains ribosomal RNA. Regarding EOP samples, a faint DNA band is observed, and the RNA quantity is almost negligible when compared to the IP strategy, which once again validates that the IP extraction protocol is more efficient for RNA recovery. Concerning the intracellular extraction (LP), the concentration of nucleic acids is considerably higher than that of the extracellular samples, which is expected since the nucleic acids present in the medium always have a high dilution factor. Despite this, a precipitation protocol for the extracellular medium is much simpler and faster than an intracellular extraction, resulting in a more clarified sample as it avoids cell lysis. Given the aim of this work, which is to produce RNA for pharmaceutical purposes, the IP resulting sample seems to be the most beneficial, given the considerable amount of RNA recovered, with low DNA contamination.

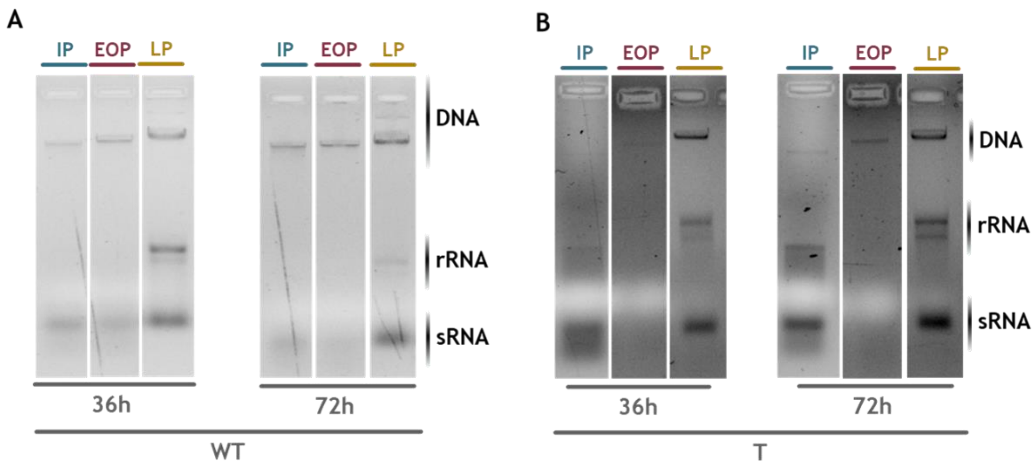


Figure 5: Characterization of extra- and intracellular nucleic acids at 36 and 72 hour cultivation for IP, EOP, and LP strategies. *rRNA – ribosomal RNA; sRNA – small weight RNA; WT- Wild type R. sulfidophilum DSM 1374; T - R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1. A - 1% agarose gel electrophoresis of recovered extra and intracellular nucleic acids from wild-type R. sulfidophilum DSM 1374 by IP, EOP, and LP at 36- and 72-hour cultivation times. B – 1% agarose gel electrophoresis of extra and intracellular nucleic acids recovered from R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1 by IP, EOP, and LP at 36- and 72-hour cultivation times.*

3.3 Extracellular RNA Recovery Optimization

The comparison of extracellular recovery processes (IP and EOP) is analyzed in Figures 6A and 6C. As observed, both DNA and RNA are recovered from the extracellular medium, which is expected since it is proven that *R. sulfidophilum* can secrete both RNA and DNA into the culture medium due to some cells that may suffer disruption, leading to host DNA release [7, 13]. Nevertheless, from the results shown in Figures 6B and 6D, there is an increased amount of RNA, with apparently no DNA contamination upon the RNA purification/concentration step. In general, the transformed *R. sulfidophilum* secreted higher levels of RNAs than WT when the nucleic acids were recovered by IP. By contrast, only a faint RNA band was detected when the EOP protocol was used. Overall, the IP protocol allows a better RNA recovery from T *R. sulfidophilum* medium than EOP, revealing an increased relevance when the extraction of recombinant species is aimed. This is in agreement with previous reports [15] revealing that precipitating nucleic acids with ethanol requires a larger volume (2 to 3 volumes of sample), while it can be achieved with isopropanol using a lower volume (0.7 to 1 volume of sample). Moreover, isopropanol is recommended when nucleic acids concentration is low, which is the case in this study due to the high dilution factor of the culture medium [15, 21]. The yield in nucleic acids recovery is highly dependent on the composition and environment of the solvent in which they are present; therefore, in this particular case with *R.*

sulfidophilum, our data converge to prove that isopropanol is more efficient in the recovery of the target biomolecule.

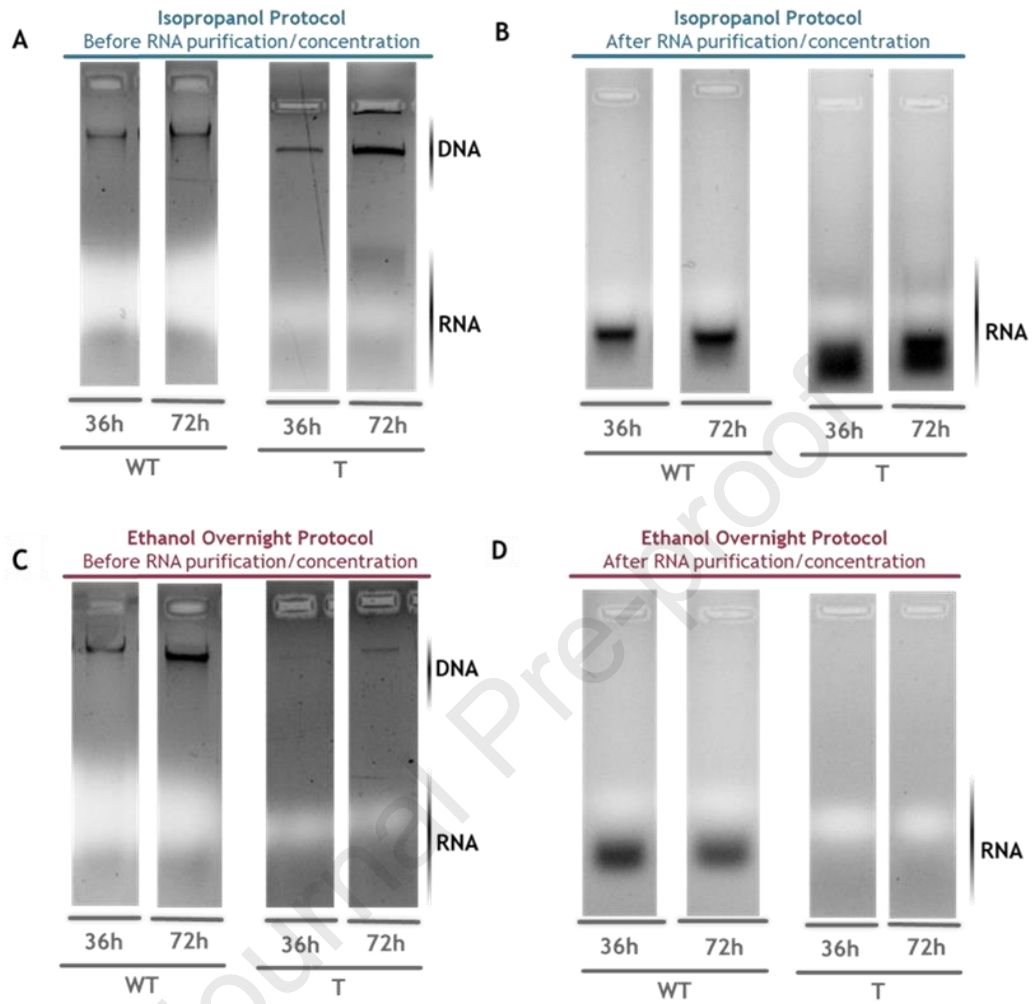


Figure 6: Extracellular nucleic acids recovered by IP and EOP, after 36- and 72-hour cultivation times, before and after purification. *WT- Wild type R. sulfidophilum DSM 1374; T - R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1. A - 1% agarose gel electrophoresis of recovered nucleic acids from WT and T R. sulfidophilum extracellular medium by IP, before purification. B – 1% agarose gel electrophoresis of recovered nucleic acids from WT and T R. sulfidophilum extracellular medium by IP, after purification. C - 1% agarose gel electrophoresis of recovered nucleic acids from WT and T R. sulfidophilum extracellular medium by EOP, before purification. D – 1% agarose gel electrophoresis of recovered nucleic acids from WT and T R. sulfidophilum extracellular medium by EOP, after purification.*

The RNA quantification (Figure 7), before and after purification, reveals similar values, meaning that the purification step did not interfere with the extracellular RNA recovery. Regarding the RNA levels, it is verified that from the WT *R. sulfidophilum,* the recovered RNA is similar for 36 and 72 h of culture, reaching a level of about 1 µg/mL of medium with both recovery methods. From the transformed strain, it is possible to recover a slightly higher RNA quantity at 36 h of growth (approximately 7 µg/mL) than at 72 h (approximately 5 µg/mL), when the RNA is recovered with IP. The RNA recovery by EOP results in RNA levels close to 0.5 µg/mL for both time points, showing a worse performance on RNA recovery when compared to the IP.

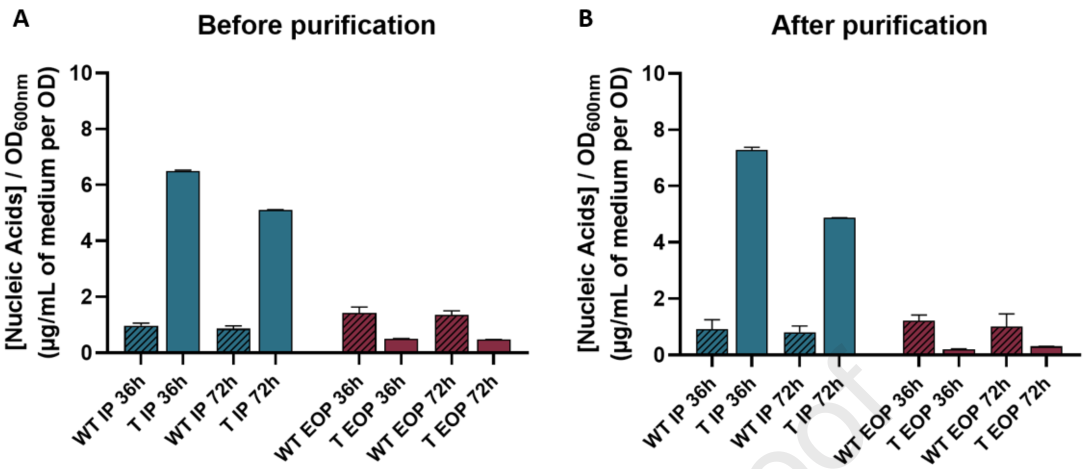


Figure 7: Extracellular nucleic acids levels, at 36- and 72-hour cultivation times, for IP and EOP recovery, before and after purification. *WT- Wild type R. sulfidophilum DSM 1374; T - R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1. A - Before purification. B- After purification. Data shown are mean ± SD from three repeats of triplicate cultivations.*

Once again, IP has shown to be more reliable for RNA recovery (Table1), being a more straightforward protocol and less time-consuming. Actually, the IP protocol reduced the global execution time by nearly 16 h compared to the overnight incubation required for the EOP method, thus allowing its completion in one day. The IP protocol also has a lower reagent requirement, only using 1 volume of isopropanol versus 3 volumes of ethanol plus two more reagents used in the EOP protocol. In the end, a clearer and more concentrated sample was obtained, with a recovery of 7 µg/mL of medium, compared to 0.5 µg/mL obtained with the EOP protocol, in the best production conditions. For these reasons, in this work, the IP was chosen as the extracellular recovery method to analyze and compare pre-miRNA-29b-1 production.

Table 1: Summary of isopropanol (IP) and ethanol overnight (EOP) protocols. *Comparison of execution time, reagents used, and yield obtained for both extracellular RNA recovery protocols under study.*

Method	Execution Time (h)	Precipitation Reagents	Yield (µg/mL of medium)			
			WT		T	
			36 h	72 h	36 h	72 h
IP	≈ 4	1 volume isopropanol	≈ 1	≈ 1	≈ 7	≈ 5
EOP	≈ 20	3 volumes ethanol; 1:20 volumes sodium acetate; 1.5 mL of ethanol-DEPC	≈ 1	≈ 1	≈ 0.5	≈ 0.5

3.4 Extracellular RNA Recovery *vs* Intracellular RNA Extraction

Since this work aims at developing and optimizing the RNA recovery from the extracellular medium, it is imperative to compare this process with the Phenol-Chloroform Protocol (PCP), the standard RNA intracellular extraction method Figure 8 presents the concentration of nucleic acids obtained through extracellular IP and EOP and intracellular PCP. A clear difference can be observed between the IP and PCP samples from the T *R. sulfidophilum*, with a higher RNA recovery from the extracellular environment (approximately 7 µg/mL) compared with the intracellular extract (2 µg/mL).

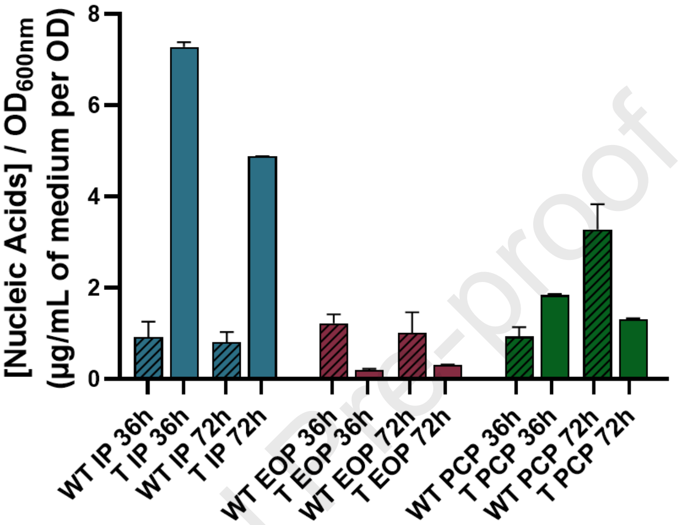


Figure 8: Extra- and intracellular nucleic acids levels, at 36- and 72-hour cultivation times, for IP, EOP, and PCP. *WT- Wild type R. sulfidophilum DSM 1374; T - R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1. Data shown are mean ± SD from three repeats of triplicate cultivations.*

Despite the high dilution factor of nucleic acids in the extracellular medium, the developed protocol is more efficient and allows a higher recovery of RNA from 10 mL of the extracellular medium (around 7 µg/mL of medium), than from the lysis of the corresponding cells (about 2 µg/mL), as summarized in Table 2. In addition, with the IP method, there is no requirement for toxic and hazardous reagents, resulting in a relevant contribution to enhance sustainability when using the developed process.

Table 2: Summary of isopropanol (IP) and phenol-chloroform (PCP) protocols. *Comparison of execution time, reagents used, and yield obtained for both RNA recovery protocols under study.*

Method	Execution Time (h)	Reagents	Yield (µg/mL of medium)			
			WT		T	
			36 h	72 h	36 h	72 h
IP	≈ 4	Isopropanol	≈ 1	≈ 1	≈ 7	≈ 5
PCP	≈ 4.2	Solution D ([16, 17]); Sodium acetate; Phenol; Chloroform/isoamyl alcohol; Isopropanol; Ethanol-DEPC	≈ 1	≈ 3	≈ 2	≈ 1

A comparison between the IP and PCP samples was examined by agarose gel electrophoresis, confirming that RNA maintains its integrity in both processes (Figure 9). This result also validates the IP as a feasible and suitable method to replace the standard PCP when using this bacterium that secretes nucleic acids to the extracellular medium.

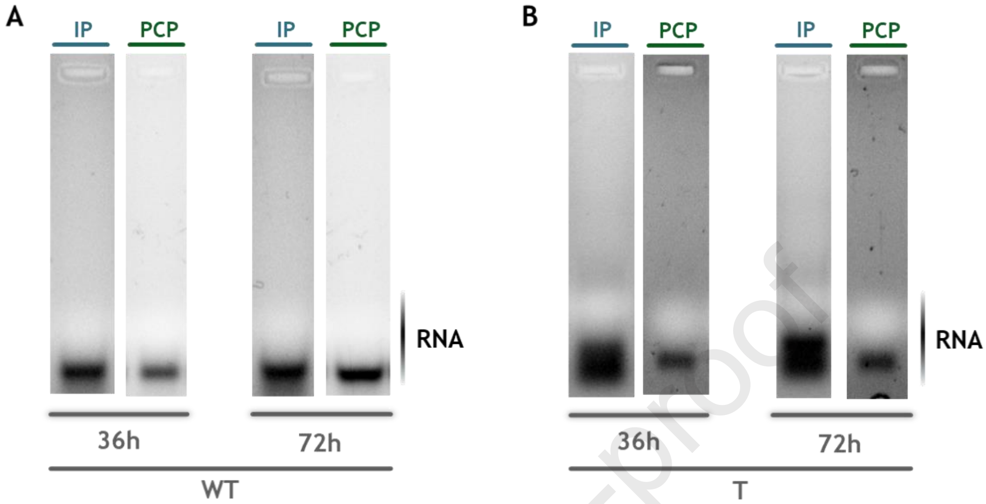


Figure 9: Intracellular nucleic acids, at 36- and 72-hour cultivation times, for IP after purification and PCP*. WT- Wild type R. sulfidophilum DSM 1374; T - R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1. A - 1% agarose gel electrophoresis of recovered extracellular nucleic acids from wild-type R. sulfidophilum DSM 1374 by IP and PCP at 36- and 72-hour cultivation times. B – 1% agarose gel electrophoresis of extracellular nucleic acids recovered from R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1 by IP and PCP at 36- and 72-hour cultivation times.*

3.5 Recombinant pre-miR-29b-1 quantification

miRNA-29 family has been shown to have an important role in several pathological disorders, being considered a potential therapeutic agent [2]. In this work, the recombinant hsa-pre-miR-29b-1 was produced instead of the mature miRNA since the processing in the cell is more efficient, and its structural characteristics, specifically the long hairpin structure, will improve stability and sample manipulation [22]. After RNA recovery and intracellular RNA extraction, the samples were analyzed by qPCR for pre-miR-29b-1 production evaluation. In Figure 10 it can be seen that WT *R. sulfidophilum* shows some non-specific amplification in the qPCR. Since WT *R. sulfidophilum* is known not to produce hsa-pre-miR-29b-1, this result suggests that a similar sequence was amplified in that sample. To guarantee that the pre-miRNA-29b-1 production was evaluated with certainty, the values obtained were statistically compared between WT and T *R. sulfidophilum.* According to the results (Figure 10), pre-miR-29b-1 was not accumulated intracellularly by the T *R. sulfidophilum,* suggesting that most of it could be secreted in the culture medium. When the extracellular medium samples of T *R. sulfidophilum* treated by IP were analyzed, a pre-miR-29b-1 concentration of 0.7 µg/L was detected for 36 h of growth. At 72 h, no pre-miR-29b-1 was detected in the samples from T *R. sulfidophilum,* which was confirmed by the lack of statistical significance compared to WT samples*.* Those data suggest that pre-miR-29b-1 production by T *R. sulfidophilum* had a peak of production at 36 h and then reduced at 72 h. This can be due to the unprotected pre-miR-29b-1 degradation after a long period in culture, as described in the literature [23]. Here, it is demonstrated that a growth period of 36 h may be enough for extracellular pre-miR-29b-1 production. The levels described in this work are comparable to or higher than other published works for other types of extracellular recombinant RNA production in *R. sulfidophilum,* which showed productions between 0.045-0.2 µg/L [5, 24, 25]. Overall, our data demonstrate the suitability and higher efficiency of the IP method for the global recovery of nucleic acids from the extracellular medium, including the target pre-miRNA with a maximum recovery of 0.7 µg/L of medium.

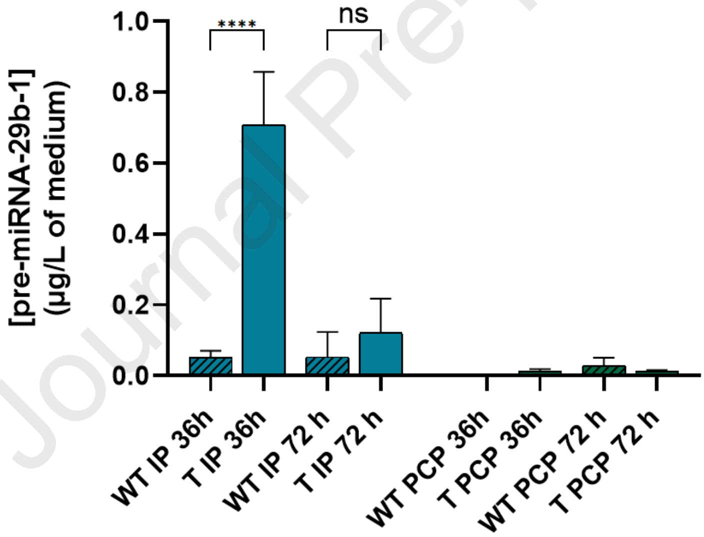


Figure 10: Extra-and intracellular pre-miR-29b-1 levels, at 36- and 72-hour cultivation times, for IP, and PCP. *WT- Wild type R. sulfidophilum DSM 1374; T - R. sulfidophilum DSM 1374 transformed with plasmid pNZY28-MR-pre-miR-29b-1. Statistics were analyzed in GraphPad Prims 9 by ordinary one-way ANOVA with Tukey test to compare different groups, where a value of p < 0.0001 was considered statistically significant. Data are shown as the mean ± SD of 3 separate experiments.*

4. Conclusion

In this work, different protocols of extracellular RNA recovery were developed and characterized. Isopropanol-based protocol had a high potential for rapid and easy RNA recovery, with better and more consistent results, enabling the recovery of 7 µg/mL of RNA from the extracellular medium, which is higher that the recovery achieved with the use of ethanol precipitation overnight and the phenol-chloroform protocol. Furthermore, extracellular samples were proven to have less DNA than intracellular samples, and a higher ratio of target RNA/total RNA. When comparing intracellular pre-miRNA-29b-1 levels with the extracellular samples, it was demonstrated that extracellular recombinant pre-miRNA-29b-1 recovery is more advantageous, resulting in 0.7 μg/L of medium. Additionally, it was shown that the designed vector can be used to produce pre-miR-29b-1 in the *R. sulfidophilum*, and it can be accumulated and recovered from the extracellular environment with only 36 h of bacterial growth. The isopropanol protocol established in this work can be more prone to recovering extracellular recombinant RNA from *R. sulfidophilum*, with the advantages of being free from toxic organic compounds, simpler, and less time-consuming compared to the routinely used intracellular RNA extraction procedures. From a future perspective, it might be of interest to scale up the production of pre-miR-29b-1, which can be achieved by implementing perfusion bioreactors to improve cell growth.

Conflict of interest

The authors declare they have no financial interests or personal relationships that could have influenced this work.

Author contributions

Micaela Riscado: Conceptualization, Methodology, Investigation, Validation, Writing - original draft; **Rita Carapito**: Conceptualization, Methodology, Investigation, Validation, Writing - original draft; **Cláudio J. Maia**: Conceptualization, Methodology, Validation, Writing - review & editing; **Chantal Pichon**: Conceptualization, Methodology, Writing - review & editing, Supervision; **Mara G. Freire**: Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition; **Mattia Sponchioni**: Conceptualization, Methodology, Writing - review & editing, Supervision; **Fani Sousa**: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Highlights

- *R. sulfidophilum* secretes nucleic acids to the extracellular medium.
- Different methods for nucleic acids recovery are evaluated.
- Recombinant pre-miR-29b-1 is successfully recovered from the extracellular medium.
- A greener, faster, and easier process for RNA recovery is established.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Micaela Riscado reports financial support was provided by Foundation for Science and Technology. Rita Carapito reports financial support was provided by Foundation for Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.