Programa de actividades propuestas para el período sabático

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1. Introducción

La presente propuesta se busca aplicar las tecnologías masivas de cultivos microbianos desarrolladas por el Prof. Jochen Büchs de la Universidad Técnica de Aquisgrán, Alemania (RWTH Aachen) para evaluar circuitos genéticos desarrollados en nuestro grupo de trabajo. Particularmente, se desea evaluar circuitos de autoinducción de proteína recombinante por agotamiento de glicerol. El uso de las tecnologías desarrolladas en la RWTH Aachen permitirá acelerar drásticamente la obtención de datos sobre el funcionamiento de nuestros diseños moleculares. Existen antecedentes de colaboración previa de nuestro grupo con el grupo del Prof. Büchs. Alvaro R. Lara realizó una visita de tres meses a la RWTH en 2007, de la que se generaron dos publicaciones en revistas indizadas. En 2016 realizó otra estancia de la que emanaron 3 artículos de investigación. El proyecto se enmarca en actividades de un proyecto con apoyo del CONACyT.

2. Duración del período sabático

El período sabático planeado es de 12 meses (1 de agosto de 2021 a 31 de julio de 2022). Sin embargo, existe el riesgo de que la emergencia sanitaria actual impida la realización de la visita a la RWTH Aachen, en cuyo caso se solicitaría al Consejo Académico de la DCNI la cancelación del goce de periodo sabático. Esto se haría con la mayor anticipación posible para evitar afectaciones a las necesidades docentes.

3. Comisiones del Prof. Alvaro R. Lara

Actualmente el Dr. Lara es miembro de la Comisión Dictaminadora Divisional. De aprobarse el período sabático, se presentará oportunamente la renuncia al Dr. Roberto Bernal (Presidente de la División) y al Dr. Javier Valencia (Secretario de la DCNI).

5. Situación de los estudiantes bajo la dirección del Dr. Lara

Actualmente el Dr. Lara únicamente dirige una tesis de maestría, de la alumna Elisa Alejandra Ramírez Campos, quien se encuentra redactando su ICR. Se espera que obtenga su grado en segundo trimestre del año, previo al inicio del período sabático propuesto.

3. Acerca de la RWTH Aachen.

La RWTH Aachen es consistentemente reconocida como una de las mejores universidades de tecnología a nivel mundial. En el último reporte de la *QS World University Rankings*, fue ubicada en el lugar 37 a nivel mundial en el área de Ingeniería Química, y ocupa el primer lugar en dicha área en Alemania. El Prof. Jochen Büchs es reconocido internacionalmente como pionero en el diseño de microbiorreactores y por sus novedosos desarrollos en tecnología de fermentaciones. Cuenta con cerca de 300 publicaciones y 7500 citas. Sus desarrollos tecnológicos han sido transferidos a numerosas empresas en Europa.

3. Plan de trabajo.

El plan de trabajo se detalla en un documento anexo. Se muestra en inglés, ya que se extrajo directamente del protocolo acordado con el Prof. Büchs.

4. Financiamiento

Actualmente se cuenta con un proyecto aprobado por CONACyT en el marco de la convocatoria de Investigación Científica Básica 2018 (*Esquemas de control dinámico para mejorar la producción de proteína recombinante en Escherichia coli*), en el que se enmarca la investigación propuesta. La síntesis de los circuitos genéticos y construcción de cepas será financiada por este proyecto. Se han sometido solicitudes de apoyo al Servicio Alemán de Intercambio Académico (DAAD) y a la convocatoria Kármán de la RWTH Aachen para financiar parte de la estancia.

Se anexan copias de las cartas de apoyo enviadas a la RWTH Aachen y al DAAD.

Recombinant protein production auto-induced by glycerol depletion

Abstract

Recombinant protein production requires controlled expression of the gene of interest (GOI). In the present proposal, we aim at developing a self-controlled, simple batch scheme, as an alternative to traditional fed-batch cultures with chemical or thermal induction. By integrating metabolic engineering and synthetic biology tools, high cell-densities in batch mode will be attained using a mixture of glycerol and glucose as carbon sources. This is possible due to mutations on the glucose transport system of *Escherichia coli*, which allow the simultaneous consumption of glycerol and glucose, resulting in high growth rates and very low aerobic acetate production. Once glycerol is depleted, a genetic circuit will trigger the expression of the GOI. Based on previous studies, it is expected that the engineered strains will strongly express the GOI when glucose is the only carbon source, while the biomass formation will be very slow, mimicking a fed-batch scheme. Therefore, this should be a simple, easy to scale production platform. While the biological material is being developed by our team in Mexico, we would strongly benefit by the microbioreactors systems developed at the RWTH Aachen University. The combined expertise and facilities will be key for the fast evaluation of the proposed scheme.

Background

The production of recombinant proteins at industrial scale requires inducible expression systems in order to decouple biomass formation from product synthesis. This is normally achieved in fed-batch cultures that consist of a phase of fast biomass formation with no product synthesis, followed by a phase of slow biomass formation, controlled by the addition of substrate to the bioreactor. The addition of the substrate (most commonly glucose), is carried out at in such a way that aerobic by-products, like acetate, are avoided. This is of prime importance and a common issue for attaining high-cell densities in cultures of *Escherichia coli* (*E. coli*). Once enough biomass has been accumulated, induction of the GOI is performed. This can be done by the addition of certain chemicals or increase of the temperature, among other strategies. The strength of the induction represents a compromise that should balance the mRNA formation, its efficient translation and whether the formation of soluble or insoluble (inclusion bodies) protein is wanted. Moreover, at this point it is desirable that most of the cellular resources are reallocated to product synthesis and maintenance functions, and as little as possible to biomass formation.

Although the above described technique is commonly used in industry, it is not without disadvantages. Namely, the addition of substrate requires continuous operation of a pump, which implies a risk for contamination, and technical failures, like unexpected stop. Moreover, attaining high cell-densities may take more than 24 hours, because low substrate uptake rates (and consequently low growth rates) should be maintained to avoid by-products synthesis. The addition of chemical inducers may be undesirable from technical and environmental standpoints. Furthermore, some chemical inducers like IPTG are expensive.

We have previously reported an *E. coli* strain lacking the phosphoenol pyruvate: carbohydrate phosphotransferase system (PTS, the natural system of glucose uptake), in which glucose in mainly transported through a chromosomally overexpressed GalP symporter (de Anda et al., 2006). Such strain, named VH33, displays very low acetate production, and high cell-densities (*ca*. 50 g/L cell dry weight) can be attained in batch mode using unusually high initial amounts of glucose, of up to 130 g/L (Borja et al., 2012; Knabben et al., 2010). VH33 was cultured using 100 g/L glucose and 50 g/L yeast extract, reaching 52 g/L biomass and 8 g/L of green fluorescence protein (GFP) induced with IPTG. This contrasts with the production of biomass and GFP (34 and 4 g/L, respectively) attained by the wild-type strain (W3110) under the same conditions (Lara et al., 2008). We have recently evaluated the performance for constitutive GFP expression of a library of 14 transport mutants created by Fuentes and co-workers (Fuentes et al., 2014). The mutants display a wide range of glucose uptake and growth rates. Table 1 shows the data of the best mutant strains, compared to the wild type, grown in a medium that contained glucose and yeast extract (unpublished data). The strain WHIC was cultured in batch mode with 100 g/L glucose and 50 g/L yeast extract constitutively expressing the GFP. This strategy was compared to fed-batch cultures of the wild-type strain using the same amount of total glucose. While both strategies reached similar biomass concentrations (*ca*. 50 g/L), the strain WHIC produced 450 mg/L GFP, contrasting with the 220 mg/L produced by the wild-type (unpublished data).

Table 1. Maximum GFP concentration obtained in batch cultures of the wild-type and mutant strains with 20 g/L glucose and 8 g/L yeast extract. GFP was quantified by microfluidics electrophoretic chips (Agilent Technologies). Average and standard deviation of triplicate experiments are shown. Unpublished data.

Strain (deleted genes)	Maximum GFP conc. [mg/L]
W3110 (wild type)	1.52 ± 0.00
WGM (*ptsG, manX*)	1.91 ± 0.40
WHIC (*ptsHIcrr, mglABC*)	5.15 ± 0.76

So far, it has been demonstrated that the glucose transport mutant strains can be grown to high cell-densities in batch mode and produce superior amounts of recombinant proteins. A potential drawback of this strategy is that the growth rate of the mutant strains using glucose as the only carbon source in a mineral medium can be severely affected when bearing a plasmid and expressing a GOI. For instance, under the mentioned conditions, the growth rate of the wild-type strain in glucose is 0.35 h^{-1} (constitutively expressing GFP from a high copy-number plasmid), while for the strain WHIC is 0.05 h^{-1} (unpublished data). The use of complex media restores the growth rate, however, it can difficult the process and physiological control, as well as decrease the reproducibility of the cultures. Moreover, the above mentioned results show that inducible GFP expression results in higher production levels, compared to the constitutive expression. Therefore, it is desirable to develop a strategy that does not make use of complex components, allow for fast biomass formation, decouple biomass and product synthesis and integrates auto-inducible induction of the GOI.

E. coli mutants on the PTS are able to consume glucose and other carbon source simultaneously, because they lack of catabolite repression by glucose (Martínez et al., 2008). Particularly, the simultaneous consumption of glucose and glycerol results in growth rate recovery, while maintains very low acetate production (Martínez et al., 2008). For instance, the growth rate of strain WHIC bearing the plasmid for constitutive GFP expression when growing in a mixture of glycerol and glucose was 0.34 h^{-1} (unpublished data). Therefore, we propose to culture *E. coli* mutant strains in a mineral medium with a mixture of glycerol and glucose. Glucose will be present in a greater proportion (for example, 2:1 or 3:1). This will allow fast biomass formation during simultaneous consumption of the carbon sources, followed by a second phase of slow growth when only glucose is consumed, after glycerol exhaustion. This will mimic a fed-batch, as conceptually shown in Figure 1.

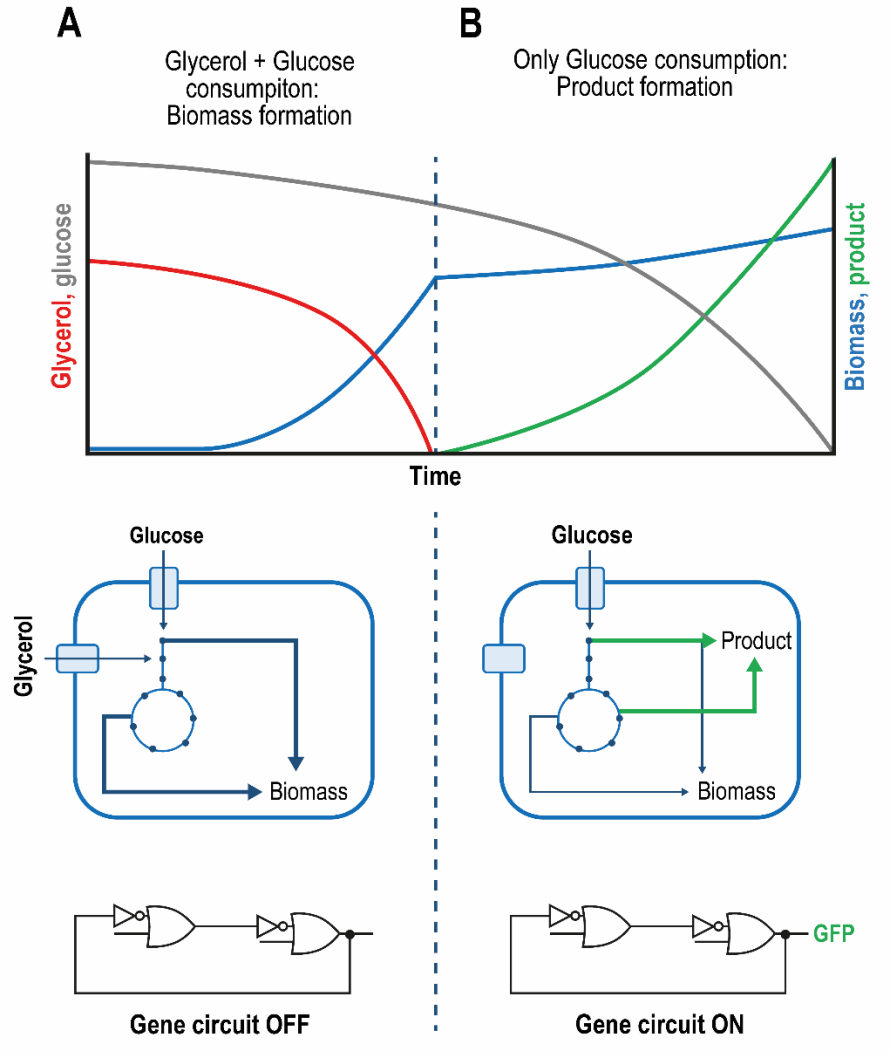


Figure 1. Overview of the proposed Project. **A)** The mutant strains can co-consume glycerol and glucose, the latter at a lower rate. Biomass is rapidly formed. Since such strains display a very low overflow metabolism, high cell densities can be attained in batch mode. When glycerol is available, the C-sources are used for biomass synthesis and the gene circuit output is OFF. **B)** Upon glycerol depletion, glucose is the only C-source and the growth rate is low, mimicking a fed-batch process. Glucose is mainly used for recombinant protein production, which is activated by the auto-induced ON circuit output.

Using glycerol as the second carbon source is particularly attractive due to its relatively high degree of reduction, carbon yield, and thermodynamic maximum biomass yield. The phase on growth on glucose as the only carbon source will be used to induce the expression of the GOI. In this way, it is expected that the resource reallocation will favor protein production over biomass synthesis, as already observed in the mentioned preliminary studies (unpublished data). This strategy will require the design of a proper expression system inducible by glycerol exhaustion, in which the GOI will not be expressed when glycerol is present, and expressed if only glucose is present. Such situation is rather unconventional and we present two approaches, based on synthetic biology tools, to address this issue. A complete description of the proposed genetic circuits is presented below.

Hypothesis

The combined use of *E. coli* strains lacking the PTS, cultured in batch mode in a mixture of glycerol and glucose, with the use of a purpose-designed genetic circuit, will allow the auto-inducible production of recombinant protein, decoupling biomass from product synthesis, thus mimicking the traditional fed-batch schemes.

Objectives

1. To evaluate the performance of the genetic circuits in the wild type (W3110) and PTS mutants (VH33, WGM and WHIC) *E. coli* strains growing in a mineral medium using glucose (5 g/L), glycerol (5 g/L) or a mixture of glycerol and glucose (5 + 15 g/L) in microbioreactors.

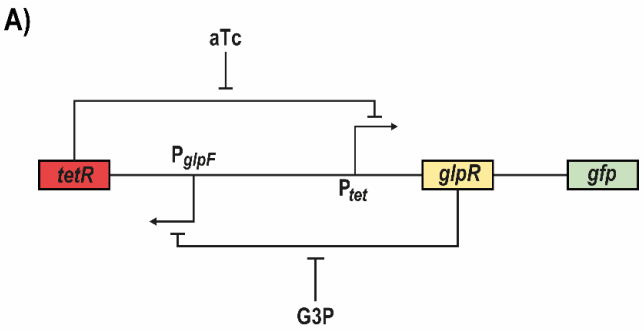
2. To select the best combination of genetic circuit and mutant strain and evaluate its performance in 2 L stirred tank bioreactor using 30 g/L glycerol + 90 g/L glucose.

Experimental approach

The *E. coli* strains are available in our groups in Mexico City and Cuernavaca. The strains will be transformed with the plasmids containing the genetic circuits described below. We propose to use two genetic circuits for recombinant protein expression auto-induced by glycerol exhaustion.

The first circuit consists on a genetic toggle switch. Such a circuit is composed of two promoters, each expressing a repressor of the next one. The toggle switch displays two stable steady states, corresponding the “ON” and “OFF” outputs (Gardner et al., 2000). In our design, the “ON” output is GFP expression and “OFF” is GFP repression.

A diagram of the proposed toggle switch, its Boolean representation and table of logic values are shown in Figure 2. GFP expression is under control of the repressible promoter P_{tet}. The use of P_{tet} in toggle switches is well documented (Gardner et al., 2000; Bothfeld et al., 2017; Lugagne et al., 2017).



C)

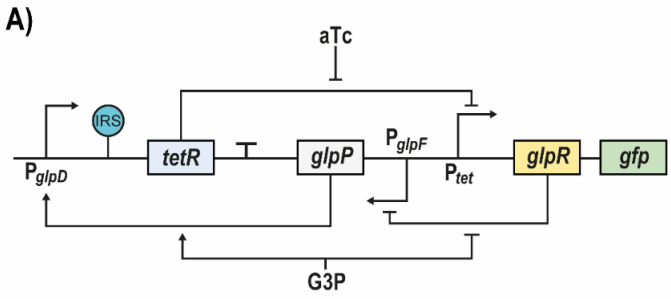
CONDITION	INPUT		GFP
	G3P	aTc	
I	0	0	1
II	0	1	1
III	1	0	0
IV	1	1	1

Figure 2. Toggle switch to express GFP by auto-induction upon glycerol exhaustion using two promoters. A) Genetic design; B) Boolean diagram of the toggle switch using "IMPLY" logic gates. The inputs are denoted in blue, and the output in green; C) Logical values of the switch. Conditions I and III correspond to the "ON" and "OFF" auto-inducible stable states. aTc can be added to test the behavior of the switch as indicated in conditions II and IV. P_{tet}: tetracycline promoter; P_{glpF}: promoter of the glycerol facilitator protein of *E. coli*; TetR: P_{tet} repressor; *glpR*: P_{glpF} repressor; *gfp*: green fluorescence protein gene; aTc: anhydrotetracycline; G3P: glycerol 3-phosphate.

The TetR repressor is under control of the promoter P_{glpF} of *E. coli*. Genes under control of P_{glpF} (related to glycerol import) are overexpressed from 6 to 7 times more when *E. coli* is growing in glycerol, compared to growth in glucose (Martínez et al., 2012). In the switch, P_{tet} also controls the expression of the GlpR repressor, which prevents the expression of genes under P_{glpF}. The inputs of the switch are anhydrotetracycline (aTc) and glycerol 3-phosphate (G3P). aTc interacts with TetR and relieves P_{tet} repression. aTc can be added to the culture in order to induce "ON" states, but is not necessary for auto-induction. In *E. coli*, glycerol is transformed to G3P by the glycerol kinase (Weissenborn et al., 1992). Therefore, the availability of glycerol will be sensed by the presence of G3P, which interacts with GlpR and makes the latter unable to repress P_{glpF} (Weissenborn et al., 1992).

Therefore, when cells consume glycerol (and no aTc is added), the output of the circuit is "OFF" (Condition III, Figure 2C) and no GFP is expressed. When glycerol is depleted, G3P is totally consumed and P_{glpF} is inactive (partially blocked by the GlpR coded from the genome of *E. coli*). Then, P_{tet} becomes active, expressing GlpR and GFP from the switch, which corresponds to the auto-induced stable steady state (Condition 1, Figure 2C). The output is expected to behave in an analogic rather than digital way.

A second circuit is designed to include an additional transcriptional control layer, as shown in Figure 3. As in the toggle switch, GFP and the GlpR repressor are under transcriptional control of P_{tet}, which will allow direct comparison of the circuits.



C)

CONDITION	INPUT		GFP
	G3P	aTc	
I	0	0	1
II	0	1	1
III	1	0	0
IV	1	1	1

Figure 3. Gene circuit to express GFP by auto induction upon glycerol exhaustion using three promoters. A) Genetic design; B) Boolean diagram of the toggle switch using "AND", "IMPLY" and "N-IMPLY" logic gates. The inputs are denoted in blue, and the output in green; C) Logical values of the switch. Conditions I and III correspond to the "ON" and "OFF" auto inducible stable states. aTc can be added to test the behavior of the switch as indicated in conditions II and IV. P_{tet}: tetracycline promoter; P_{glpF}: promoter of the glycerol facilitator protein of *E. coli*, P_{glpF}: promoter of the glycerol uptake facilitator from *Bacillus subtilis*; IRS: inverted repeated sequence; tetR, P_{tet} repressor, *glpR*: P_{glpF} repressor, *gfp*: green fluorescence protein; aTc: anhydrotetracycline, G3P: glycerol 3-phosphate, GlpP: glycerol uptake operon antiterminator from *Bacillus subtilis*.

The inputs are aTc and G3P. In contrast with the toggle switch, the TetR repressor is under control of the P_{glpD} promoter from *Bacillus subtilis* and is positively regulated by G3P, which acts as an antiterminator and requires an inverted repeated sequence (IRS) (Lewin et al., 2008). Advantages of this promoter are that its function is based on positive control and does not involve a DNA-binding protein (Lewis et al., 2008). P_{glpD} is activated by GlpP, which will be transcribed from P_{glpF} from *E. coli* when G3P is present (Figure 3A). In this circuit, G3P has two functions: direct activation of P_{glpD}, and activation of P_{glpF} by interacting with GlpR. Similar to the toggle switch, the output is "ON" only if glycerol is depleted (provided that no aTc is added to the medium, Figure 2B and 2C).

Similar to the toggle switch, this circuit is also expected to work in an analogical way. Different to the toggle switch proposed, the second layer of control is expected to better manage the OFF state and faster turn to the ON output.

The sequences of each genetic element will be taken from public databases. Proper terminators and ribosome binding sites will be added. The whole sequence will be synthesized by a specialized company and cloned into a high copy-number plasmid like pUC57mini or pUC57Kan. The resulting plasmid will be introduced in strains W3110, VH33, WGM and WHIC. Colonies will be screened and plasmid identity tested by restriction analyses. A cell bank will be prepared and samples will be sent to the RWTH Aachen University. The experimental work at the RWTH Aachen will consist in two working packages (WP), described below.

WP1. Characterization of the genetic circuits in microscale cultures.

The different strains/circuits will be cultured in mineral medium containing glucose (5 g/L), glycerol (5 g/L) or a mixture of glycerol + glucose (5 + 15 g/L). Control experiments with the addition of aTc will be included in order to test the behavior of the circuits. Arabinose will be used as a carbon source to test the ON output using a different substrate. Cultures will be carried out in 96-wells microtiter plates using the μRAMOS device. Such system allow to perform parallelized cultures monitoring relevant parameters online, like GFP fluorescence, biomass growth, dissolved oxygen tension (DOT), and respiratory activity (Flitsch et al., 2016; Ladner et al., 2016). Therefore, they represent a unique platform to connect physiological parameters with the performance of the genetic devices.

While GFP fluorescence and cell growth can be monitored online, it is difficult to follow the exact moment of glycerol exhaustion. The μRAMOS device can follow the respiratory activity through the precise measurement of the oxygen transfer rate (OTR) in each well of the plate (Flitsch et al., 2016). The OTR is closely linked with to the substrate uptake and oxidation. Therefore, it will be useful to infer, together with the cell growth and DOT

signals, the transition from the glycerol and glucose co-utilization to the glycerol-exhausted phase. Since P_{tet} is not a very strong promoter, and GFP is known to be highly soluble, no inclusion bodies formation is expected. Therefore, the GFP fluorescence will be proportional to the GFP concentration. We have already confirmed it in previous studies using a constitutive promoter of medium strength and complex medium (unpublished data).

All the experiments will be performed in technical triplicates to allow proper statistical analysis. The best combination of genetic circuit and mutant strain will be selected based on the following criteria: low or no GFP fluorescence emission when glycerol is present, fast induction upon glycerol exhaustion (inferred from the OTR data), high induction level (based on specific GFP fluorescence per biomass unit), high final GFP emission, high specific GFP fluorescence emission rate.

WP2. Validation of the technology in cultures in 2 L stirred tank bioreactors.

The selected combination of strain and genetic circuit will be cultured in 2 L stirred tanks bioreactors to attain high cell-densities in batch mode and to collect more physiological information. The initial amount of glycerol will be 30 g/L, while glucose concentration will be 90 g/L. Off gases composition will be continuously analyzed. Samples will be taken regularly to measure cell dry weight, carbon sources concentration and by-products accumulation (acetate, formate and pyruvate will be analyzed). GFP fluorescence will be monitored off-line. Previous studies found that glycerol uptake rate for a PTS mutant strain was only slightly slower than for its wild-type when used as the only carbon source or in combination with glucose (Martínez et al., 2008). It will be interesting to analyze whether the specific mutants to be used in the present project behave similarly. The extracellular rates during growth in glucose as the only carbon source will be used to estimate the metabolic fluxes using flux balance analysis and our previously published model (Jaén et al., 2017).

The wild-type strain bearing the gene circuit selected above will be cultured in fed-batch mode. The batch phase will be carried out with 30 g/L glycerol. It is known that acetate production is low when glycerol is used as carbon source (Martínez et al., 2008). Therefore, we expect that this amount of glycerol will not result in inhibitory levels of acetate during the batch phase. After glycerol depletion, 90 g/L of glucose will be added at an exponentially increasing rate matching that of the mutant strain growing in glucose as the only carbon source. The same group of data will be collected and flux balance analysis during growth in glucose will be performed.

As we have consistently observed that the product yield of the mutant strains is higher than that of the wild-type, we expect that the estimated metabolic fluxes will provide useful information to better understand the physiological adaptations in the mutant strains that result in greater production. Metabolic fluxes will be also

useful to understand the resources reallocation upon auto-induction in the two different scenarios. After careful comparison of the fed-batch and the batch culture using a mutant strain, conclusions on the feasibility of the latter will be drawn.

Expected Results

The proposed study is a combination of metabolic engineering and synthetic biology approaches to deal with bioprocessing issues. Such a combination is not commonly found in literature. We consider that the proposal is highly original and that the results will be of interest for industrial and academic researchers. Therefore, we expect that the results could be published in a reputed journal like *Biotechnology and Bioengineering* or *ACS Synthetic Biology*.

Relevance of the collaboration with the RWTH Aachen University

The Chair of Biochemical Engineering headed by Prof. Jochen Büchs is a world leader in the development and application of microscale culture systems with high monitoring capabilities. Such systems are unique, and while some of them are now commercially available, others can be found only at the RWTH Aachen University. Synthetic biology applications require the precise and dynamic measurement of signals, coupled to physiological information in high-throughput systems. The devices developed by Prof. Büchs are ideal for such purposes. Furthermore, the vast experience on bioprocess development at the Chair of Biochemical Engineering will be key for the successful development of this proposal.

Time schedule and itinerary

The experimental work at the RWTH Aachen University will be carried out in 12 months. A detailed time schedule is provided in a separate sheet. It includes the time needed for bench-scale cultures (at least 6) and metabolite analyses. Flux balance analysis and the preparation of the potential manuscript for submission will be performed back in Mexico.

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Time Schedule and itinerary

The planned activities are described in the following chronogram, organized in months (M) of the research stay:

Activity	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
Reception of strains, culture medium and cell bank preparation, inocula characterization												
Microscale cultures with single and combined carbon sources at different concentrations												
Data analysis												
Cultures in 2 L stirred tank bioreactor												
Quantification of extracelular metabolites												
Flux balance Analysis												
Project closing. Manuscript preparation.												

All the activities will be performed at the Chair of Biochemical Engineering, RWTH Aachen University, Forckenbeckstraße 51, 52074, Aachen.

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Chair of Biochemical Engineering
Prof. Dr.-Ing. Jochen Büchs
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Letter of support

Dear ladies and gentlemen,

I have known Prof. Alvaro Lara since November 2005, when I first visited Mexico. I had been invited by Prof. Enrique Galindo to give an oral presentation for the "Instituto de Biotecnologia Universidad Nacional Autonoma de Mexico (UNAM)" in Cuernavaca and, in addition, several classes for his students. During my stay, I got into closer contact with Prof. Alvaro Lara, who was at that time a Ph.D. student at the lab of Professor Octavio Tonatiuh Ramirez. We discussed and developed plans for joined research and started arranging common experiments. In parallel, we applied for funding from the "Deutsche Forschungsgemeinschaft (DFG)" and "Consejo Nacional de Ciencia y Tecnología (CONACYT)" for a high level workshop of Mexican and German scientists in the field of Biotechnology, to be held at Cuernavaca. Prof. Alvaro Lara was the main person on Mexican side to coordinate the application and to organize the workshop. Finally, the funding was granted and the workshop was held on September 21.-23., 2008 with 25 senior participants. From October to December 2007, Prof. Alvaro Lara visited our chair at the RWTH Aachen University. From that visit two papers evolved. On January 2008, Dr. Alvaro Lara was assigned the position as a full professor at the "Departamento de Procesos y Tecnología, Universidad Autónoma Metropolitana Unidad Cuajimalpa". We also saw each other at the XIII Mexican National Congress on Biotechnology and Bioengineering, Acapulco, 24. 6. 2009. In May 2011, I hired a German student, who had worked at the laboratory of Prof. Lara, for a Ph.D. work. This also strengthened the ties between our chairs. Another visit of Prof. Lara at the RWTH Aachen University followed in 2016 (April to November). From this visit, three common papers resulted. As evident from the scientific history described above, the collaboration of Prof. Lara and our chair was always very fruitful. We are very confident that also our future work will result in interesting findings and publications.

Prof. Lara has designed an ambitious project plan for his visit, which is scheduled for autumn

of this year. He intends to develop a completely new type of expression system based on *E. coli*. Under batch conditions, glucose is present in excessive amounts for most of the fermentation time. Under these conditions, *E. coli* produces a lot of inhibiting anaerobic side products, mainly acetate. Therefore, globally fed-batch operation is the state of the art for technical scale *E. coli* fermentations. However, the necessity of fed-batch operation complicates the whole process. Suitable times for initiating the feeding of the limiting carbon source and the feed profile have to be investigated. Another essential point, additionally complicating the development of fermentation processes with *E. coli*, is the induction of the cells. This is usually conducted by a temperature shift or by addition of inducers, like e.g. lactose or IPTG. In the latter case, the amount and time of addition of the inducer is crucial. Laborious experiments are required to optimize the induction protocol. According to the plan of Prof. Lara, special *E. coli* strains will be used, which are defective in the glucose uptake system (phosphotransferase system; PTS). Our past common work has demonstrated that these strains can be cultured in high-glucose-media under batch conditions without excessive acetate formation. Additionally, the expression strains will be genetically modified such that when cultured on a mixture of glucose and glycerol, the recombinant protein production strains will be auto-induced by glycerol depletion. That means a fermentation can be conducted in batch mode without any requirement of intervention during the process. This is a highly innovative and promising concept, drastically simplifying reactor operation for screening and technical scale production. The unique small scale bioreactors with a wide variety of online measuring capabilities developed in our chair will assist in efficiently characterizing the microbial systems, which Prof. Lara will develop.

I am very happy that Prof. Lara is interested in again joining forces with us in Aachen. He is sincerely invited and welcomed to stay at our chair and he will get all the support he requires. I am looking forward to collaborating with him again.

Aachen, January 5th, 2021

Jochen Büchs

(Prof. Dr. Jochen Büchs)

AVT·BioVT | RWTH Aachen | Forckenbeckstraße 51 | 52074 Aachen | GERMANY

Chair of Biochemical Engineering
Prof. Dr.-Ing. Jochen Büchs
RWTH Aachen University
Jochen.Buechs@avt.rwth-aachen.de

Antrag im Rahmen des 11th Call for proposals: *Theodore von Kármán Fellowship* – visiting scholars program for incoming international scientists

1. **Namen der Antragsteller:**

Prof. Dr. Jochen Büchs, AVT - Lehrstuhl für Bioverfahrenstechnik

Prof. Dr. Lars Blank, Lehrstuhl für angewandte Mikrobiologie

2a. **Motivation zur Einladung von Herrn Professor Alvaro Lara aus Mexico**

Sehr geehrte Mitglieder des ERS International Board,

ich stelle, in Absprache mit Herrn Kollegen Lars Blank (iAMB), hiermit den Antrag für ein „Theodore von Kármán Fellowship – for incoming international scientists" für Herrn Prof. Alvaro Lara von der „Universidad Autónoma Metropolitana Unidad Cuajimalpa" in Mexico.

Ich kenne Herrn Prof. Alvaro Lara seit meinem ersten Besuch 2005 in Mexico am "Instituto de Biotecnologia, Universidad Nacional Autonoma de Mexico (UNAM)" in Cuernavaca. Dieses ist in Mexico mit seinem stark zentralisierten Bildungs- und Forschungssystem das Spitzeninstitut für Biotechnologie und Bioverfahrenstechnik. Herr Prof. Lara hat bei Prof. Octavio Ramirez promoviert, einem der zwei führenden Bioverfahrenstechniker Mexicos. Wir haben gemeinsam 2008 einen Workshop für deutsche und mexikanische Biotechnologen in Cuernavaca organisiert, bei dem Herr Prof. Lara die Koordinierung und Organisation übernommen hat. Finanziell wurde dieser Workshop, an dem insgesamt 25 Lehrstuhlleiter aus beiden Ländern teilnahmen, durch die "Deutsche Forschungsgemeinschaft (DFG)" and das "Consejo Nacional de Ciencia y Tecnología (CONACYT)" unterstützt.

Herr Prof. Lara war von Oktober bis Dezember 2007 zu seinem ersten Forschungsaufenthalt an der RWTH Aachen. Aus den Arbeiten in diesem kurzen Zeitabschnitt sind 2 begutachtete Publikationen hervorgegangen. Seit Januar 2008 ist Herr Lara selbständiger Professor an der Universidad Autónoma Metropolitana Unidad Cuajimalpa. Zwischen April und November 2016 führte Herr Prof. Lara nochmal einen Forschungsaufenthalt bei uns an der RWTH durch. Aus den gemeinsamen experimentellen Arbeiten während dieser kurzen Zeit gingen drei hochwertige begutachtete Publikationen hervor. Es wird aus dieser Historie, denke ich, deutlich, dass die Kooperationen mit Herr Prof. Lara immer ausgesprochen fruchtbar waren. Wir gehen unbedingt davon aus, dass aus den Arbeiten, für die wir hiermit die Lebenshaltungskosten beantragen, wieder neue Veröffentlichungen in hochwertigen Journalen hervorgehen. Herr Prof. Lara hat sich in den letzten Jahren zu einem „aufsteigenden Stern" in der Bioverfahrenstechnik in Mexico entwickelt. Er hat beste Kontakte zu führenden mexikanischen Molekularbiologen am UNAM (Prof. Bolivar, einer der weltweiten Pioniere der Molekularbiologie, und seinem Schüler, Prof. Gosset). Über diese Kontakte hat er Zugang zu hoch interessanten Stämmen und genetischen Konstrukten, die auch für uns interessant sind. Er ist selber aber auch sehr in der Molekularbiologie bewandert und baut sich mit seiner Forschungsgruppe interessante Stämme mit vollkommen neuen Eigenschaften zusammen. Herr Prof. Lara ist auch mit anderen führenden Arbeitsgruppen (z.B. Delft, Berlin, Bielefeld) in Europa gut vernetzt.

Für seinen geplanten Aufenthalt hat Herr Prof. Lara einen ambitionierten Plan (s. Anlage) aufgestellt. Er fokussiert mit dem Bakterium *E. coli* **das** Arbeitspferd der modernen Biotechnologie. Er will mit molekularbiologischen Methoden zwei derzeit bestehende immanente Probleme von *E. coli* Fermentationen lösen. Im einfach durchzuführenden Batch-Betrieb liegen die längste Zeit in einer Fermentation hohe Konzentrationen an Kohlenstoffquelle (meist Glucose) vor. Unter diesen Bedingungen bildet *E. coli* in großen Mengen Essigsäure und verdirbt sich damit selber seine

Lebensbedingungen. Daher ist es international Stand der Technik, dass *E. coli* im technischen Maßstab in Fed-batch Betrieb fermentiert wird. Das ist aber sehr aufwändig. Man muss in aufwändigen Versuchen den Startzeitpunkt der Zufütterung und deren Kinetik (Feedprofil) ermitteln. Herr Prof. Lara plant daher einen (im Anhang erläuterten) speziellen Stamm zu verwenden, der in Mexico entwickelt wurde, und der unter Batch-Bedingungen kaum Essigsäure ausscheidet. Ein weiteres immanentes Problem bei *E. coli* Fermentationen ist die Entwicklung des Induktionsprotokolls. Auch hier sind gewöhnlich sehr aufwändige Experimente notwendig, um z.B. den Induktionszeitpunkt und die Induktormenge zu optimieren. Herr Prof. Lara plant seine Stämme auf einer Mischung von Glucose und Glycerin zu kultivieren und so zu modifizieren, dass bei Verbrauch von Glycerin die Produktbildung auf Glucose automatisch einsetzt (Autoinduktionsmedium). Würde dieses Vorhaben gelingen, hätte das einen riesigen praktischen Vorteil bei *E. coli* Fermentationen und die Screening- und Produktionsverfahren würden drastisch vereinfacht.

Dieses Vorhaben von Herrn Prof. Lara steht in synergistischer Beziehung zu unseren eigenen Forschungsaktivitäten. Unsere eigene international anerkannte Stärke liegt in der Entwicklung von neuartigen Werkzeugen zur effizienten Charakterisierung und Optimierung von unterschiedlichsten Stammkonstruktionen im Kleinkulturmaßstab. Mit unseren hoch parallelen Techniken können unterschiedlichste Signale aus dem Kulturen online erfasst werden, aus denen sich die Eigenschaften der kultivierten Stämme herauslesen lassen. Daher hat Herr Prof. Lara großes Interesse seine Stammkonstruktionen mit unseren fortschrittlichen Techniken zu untersuchen. Herr Kollege Blank ist u.a. ein Fachmann der Flussanalyse zellinterner Stoffwechselwege. Er hat für derartige Untersuchungen entsprechende experimentelle Methoden entwickelt und sich, wie wir, mit dem Phänomen des „metabolic burden“ auseinandergesetzt. Ein zu geringer „metabolic burden“ zeigt an, dass die Stämme vornehmlich nur wachsen und zu wenig Energie für die Produktbildung aufwenden. Ein zu hoher „metabolic burden“ hingegen überfordert die Stämme. Es gilt die optimale Balance zu finden. Dieses Phänomen des „metabolic burden“ wird auch bei den Stämmen von Herrn Prof. Lara auftreten. Wir planen daher dieses Phänomen gemeinsam mit den drei beteiligten Forschungsgruppen zu untersuchen und vor allem zu quantifizieren. Kollege Blank und wir selbst sind für unsere weiteren Forschungsarbeiten sehr an den Stämmen interessiert, die Herr Prof. Lara während seines Aufenthalts hier an der RWTH Aachen erzeugen möchte. Evtl. können gemeinsame Patente eingereicht werden. Prof. Lara ist auch für unsere Studenten und wissenschaftlichen Mitarbeiter interessant. Man kann sicher davon ausgehen, dass die Vorträge und Diskussionsangebote von Herrn Prof. Lara lebhaft genutzt werden. Ich bin froh, dass sich Herr Prof. Lara für einen weiteren Aufenthalt bei uns an der RWTH Aachen interessiert und hoffe, dass er die finanzielle Unterstützung erhält, um gemeinsam mit Herrn Kollegen Blank und meiner Gruppe die Wissenschaft ein weiteres Stück voranzubringen. Ich halte Herrn Lara für einen sehr geeigneten und würdigen Kandidaten für das Theodore von Kármán Fellowship und bin sicher, dass er in Zukunft die Anknüpfungsstelle für Verbindungen der RWTH nach Mexico sein wird.

(Prof. Dr. Jochen Büchs)

Aachen, January 8th, 2021

2b. **Motivation of Prof. Alvaro Lara to come to Aachen**

Mexico City, January 5th, 2021

Dear Members of the ERS International Board,

I am addressing you to express my interest in the 11th Call for proposals: Theodore von Kármán Fellowship – visiting scholars program for incoming international scientists, as I have sought to collaborate with Profs. Jochen Büchs and Lars Blank for several years.

I have established research lines dealing with the design of bacterial and mammalian cell factories that can better cope with environmental stresses linked to large-scale conditions. Such research has enabled the development of highly innovative culture strategies for the production of plasmid DNA (to be used as DNA vaccine vector) and recombinant proteins. Currently, I am interested in designing genetic devices in combination with engineered cells to simplify the production or recombinant proteins. The evaluation of synthetic gene circuits requires the use of highly dependable cultivation devices. The group of Prof. Jochen Büchs has developed some of the finest high-throughput cultivation devices that allow the online-measurement of relevant parameters. The experience of microbial physiology and metabolism of Prof. Lars Blank can be combined with our biological designs to better interpret the online data and gain knowledge. Such interdisciplinary approach will undoubtedly be key for the successful development of the planned research.

During previous visits to the RWTH Aachen University, I have discovered the diversity of ideas, cutting-edge research and outstanding experimental facilities that characterize this world-class University. I am sure that during my stay, I will strongly benefit from the highly stimulating intellectual atmosphere. I particularly enjoy discussing and interacting with young RWTH researchers, who are always very energetic and passionate about science and technology. My experimental results of my previous collaborations with the RWTH Aachen University have been published in a number of scientific articles in reputed journals. I am totally committed to continue such path of productivity and very confident that our collaboration will enhance the links between our institutions and countries.

Thanks you for considering my application.

With all best regards,

Alvaro R. Lara

3. **CV of the visiting scientist and the** five most important **publications**

Dr. Alvaro R. Lara

Professor of Biological Engineering
Department of Processes and Technology
Universidad Autónoma Metropolitana-Cuajimalpa
Vasco de Quiroga 4871, Santa Fe, Cuajimalpa, C.P. 05348, Mexico City
Tel: +52 55 5814 6500 Email: alara@cua.uam.mx
Scopus ID: 12801772900 Orcid ID: orcid.org/0000-0003-3535-7619 Publons ID: publons.com/a/1299580/

RESEARCH INTERESTS

Biochemical Engineering, Biological Engineering, Synthetic Biology, Biomanufacturing

EDUCATION

Universidad Nacional Autónoma de México (UNAM), Mexico 2003-2007
Institute of Biotechnology, Degree: Ph.D. (with Honours Mention)
Advisor: Octavio T. Ramírez
PhD Thesis: *"Bioreactor scale-down and cell engineering for improving recombinant protein production in Escherichia coli"*

Instituto Politécnico Nacional (IPN), Mexico 1996-2001
Department of Biochemical Engineering. Degrees: B.S. (with Honours Mention)

APPOINTMENTS

Associate Professor of Biological Engineering 2011-present
Department of Processes and Technology, Universidad Autónoma Metropolitana (UAM)

Associate Professor (Tenure track) 2008-2011
Department of Processes and Technology, Universidad Autónoma Metropolitana (UAM)

AWARDS AND HONORS

Awards and Honors (not exhaustive)

Bill and Melinda Gates Foundation fellowship to attend the Microbial Engineering Conference (ECI)	2018
Carlos Casas Campillo Award, Mexican Society for Biotechnology and Bioengineering.	2014
European Science Foundation Fellowship to attend the Conference on Microbes and Industrial Biotechnology and the School on Advanced Techniques in Bacterial Genome Research in Bielefeld, DE	2010
Fellowship of the German Academic Service to perform research at RWTH-Aachen	2007
Fellowship of the Huygens Programme of NUFFIC to perform a research stay at TU-Delft	2005

RESEARCH VISITS

Visiting Researcher, Institute of Biochemical Engineering, RWTH-Aachen, Germany	2016
Visiting Researcher, Centre for Biotechnology, Bielefeld University, Germany	2012
Visiting Researcher, Department of Biotechnology, Delft University of Technology	2005

EDITORIAL BOARDS

Microbial Cell Factories (Impact Factor: 4.187),	2017-present
Bioprocess and Biosystems Engineering (*Impact Factor: 2.419*),	2018-present
Microorganisms (*Impact Factor: 4.152)*	2019-present
Engineering in Life Sciences (*Impact Factor: 1.934*)	2019-present

PUBLICATIONS

Independent citations (IC) as of 5/01/2021: 817 *h* index (Scopus): 17

A) Research Articles: 47. Ratio of articles as first or corresponding author: 31/46 = 0.67.

B) Book chapters by invitation: 5. Ratio as first or corresponding author: 3/5 = 0.60

C) Editorial Notes: 2

D) Edited Special Issues and Books: 3

E) Patentes: Granted: 3, Submited: 2. Ratio as first or main inventor: 5/5 = 100 %

Key 5 Publications * = Corresponding author IF: Journal´s Impact Factor.

1) Jaén KE, Velázquez D, Sigala JC, Lara AR*. 2019. Design of a microaerobically inducible replicon for high-yield plasmid DNA production. Biotechnology and Bioengineering. 116(10): 2514-2525. *IF: 4.260*.

2) Lara AR*, Jaén KE, Mühlmann M, Sigala JC, Regestein L, Büchs J. 2017. Characterization of endogenous and reduced promoters for oxygen-limited processes using *Escherichia coli*. ACS Synthetic Biology. 6: 344-356. *IF: 5.382.*

3) Pablos TE, Sigala JC, Le Borgne S, Lara AR*. 2014. Aerobic expression of *Vitreoscilla* hemoglobin efficiently reduces overflow metabolism in *Escherichia coli*. Biotechnology Journal. 9(6): 791-799. *IF: 3.490.*

4) Borja MG, Meza E, Gosset G, Ramírez OT, Lara AR*. 2012. Engineering *E. coli* to increase plasmid DNA production in high cell-density cultivations in batch mode. Microbial Cell Factories. 11: 132. *IF: 3.310.*

5) Lara AR*, Taymaz-Nikerel H, van Gulik W, Heijnen JJ, Ramírez OT, van Winden W. 2009. Fast dynamic response of *Escherichia coli* fermentation metabolism to aerobic and anaerobic glucose pulses. Biotechnology and Bioengineering. 104: 1153-1161. *IF: 3.377.*

FUNDING

> 480,000 Euros in different projects sponsored by CONACyT, DAAD, BMBF, NFRS (Belgium)
Note: Project budgets in Mexico do not include the payment of salaries for PhD researchers.

SUPERVISED THESIS AND RESEARCHERS

1 Postdoc, 2 PhD, 8 MSc, 11 BSc

4. Research topics and planned activities for the stay in Aachen

Recombinant protein production requires controlled expression of the gene of interest. In the present proposal, we aim at developing a self-controlled, simple batch scheme, as an alternative to traditional fed-batch cultures with chemical or thermal induction. By integrating metabolic engineering, synthetic biology and bioprocess engineering tools, high cell-densities in batch mode will be attained using a mixture of glycerol and glucose as carbon sources. This is possible due to mutations on the glucose transport system of *Escherichia coli*, which allow the simultaneous consumption of glycerol and glucose, resulting in high growth rates and very low aerobic acetate production. Once glycerol is depleted, a genetic circuit will trigger the expression of the gene of interest. Based on previous studies, it is expected that the engineered strains will strongly express the gene of interest when glucose is the only carbon source, while the biomass formation will be very slow, mimicking a fed-batch scheme. Therefore, this should be a simple, easy to scale production platform.

While the biological material is being developed by our team in Mexico, we would strongly benefit by the microbioreactors systems developed by the group of Prof. Jochen Büchs (AVT – Biochemical Engineering, RWTH Aachen University) and the knowledge on microbial metabolism of Prof. Lars Blank (Institute for Applied Microbiology - RWTH Aachen University). The combined expertise and facilities will be key for the fast evaluation of the proposed scheme. The Mexican partner will provide the synthetic gene circuits and modified strains. However, no funding is available for covering the living expenses of the Mexican participant in Aachen. The experiments will be run from August to November, 2021.

The planned activities include a Lecture to introduce the Autonomous Metropolitan University in the second week of August. It will be focused on the advances on the development of cell factories that can better cope with large-scale conditions, emphasizing the approaches followed by the Mexican group. The Mexican researcher will remain available for meetings to interchange ideas and knowledge with students and researchers from the RWTH Aachen University. At the end of the stay, the result of the collaborative research will be shown in a talk for the RWTH community. Significance of the project and opportunities for further collaboration and researchers exchange will be highlighted.

5. Budget plan

The requested budget for this proposal is intended to pay the accommodation at the RWTH Guest House and partially other living expenses during the four-month stay (August to November 2021). We request 1750 EUR per month, yielding a total of 7000 EUR for the whole stay.