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¿Biología sintética, solución para los problemas del futuro?

Synthetic biology, solution for the problems of the future?

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Contents

Abstract

Synthetic biology is a cutting edge area of science, recently emerged from the combination of biology, computing, chemistry and engineering. This work focuses on its influence on the future world issues, mainly concentrating on the upcoming power crisis.

Synthetic bacteria with the capability of producing biofuels can represent a viable solution for the depletion of fossil fuels. By their properties, butanol and hydrogen represent two plausible fuels to substitute those being used nowadays. Pathways which enable the synthesis have been described in many know bacteria, some also having been used in the past for this purpose.

Toxic products require cell adaptations to solvent saturated mediums. Strategies include membrane and capsule structural modifications, membrane composition variations, hydrophobic and hydrophilic surface shifts and efflux pumps.

Aspects of the bacteria's metabolism such as its carbon source are also reviewed. Certain organisms' catabolic routes could rend a double benefit in biofuel production, as it could also influence in bioremediation.

Most of the information and techniques needed to construct such organisms is already available. The increasing knowledge in biofuel synthesis involved pathways will most likely allow the creation of synthetic organisms for this purpose in the future.

Introduction

Synthetic Biology is a multidisciplinary field that combines biology, engineering, chemistry and computing, and aims to modify or create living organisms in order to perform specific functions. It can be focused on the production and manipulation of cell networks; orthogonal biosystems; minimal genomes; metabolic routes; regulatory systems; protocells and synthetic cells; productions platforms; and biosensors [1]. The main purpose of all of these usages is to produce, detect or decompose compounds. Genetic circuits are designed and created in similar way as electronic ones, each gene carrying out a specific function within the whole, which in turn is designed to fulfil a desired task.

The recent rise of this discipline has been favoured by the advances in science during the last decades. Large-scale studies of complex, interrelated biological processes, for example, metabolomics, have significantly increased and complement the enlarging number of available gene and protein sequences for genetic engineering. DNA synthesis, editing and sequencing techniques are becoming highly reliable and precise. Current computing data analysis and design allows to process and organize all this information selecting that of interest and generating models which with high probability can be reproduced in the laboratory.

The Synthetic Biology annual research market in Europe and the US has significantly increased in the last decade reaching 600M USD by 2010 and expecting to reach 3.5B USD in the next ten years [2]. This is mainly because of the increased interest and research on the topic due to its important applications in health, energy, environment and food. A rough calculus of the budget in research on Synthetic Biology determines that since 2005, the US has spent 450M USD, while the EU spent 160M. 4% and 2% of this money respectively has been spent on studying the implications of this research, such as safety issues.

There has been controversy on the difference between Synthetic Biology and Biotechnology [3]. Some argue that it is only an extension of Genetics Engineering, not being a discipline on its own, as both share most of their tools and techniques. Leaving aside the controversy, Synthetic Biology has been recognized as a cutting edge area of

science with high economic interests. One of the advantages of Synthetic Biology over the traditional Genetics Engineering is the possibility of starting from scratch, which increases significantly the level of control over the inserted modifications [4]. Ever since the late twentieth century, with the rise of modern Genetics Engineering, it has been argued the need for the standardization of biological parts. By biological part it is understood each DNA particle with a defined function in the whole synthetic gene circuit, such as promoters, coding sequences, enhancers. This would ease the process of assembly and would allow laboratories from all over the world to share those, facilitating research. It was for this purpose that in 2006 the BioBricks foundation was formerly established [5]. It is an online database where standardized sequences are stored and available for free, seeking to ensure the open access of biological engineering.

Synthetic biology is starting to be regarded as a branch of science that could offer solutions for future problems. The main world problems according to the UN [6] could be classified into three groups: social issues, physiological issues and environmental issues. The first group englobes overpopulation, poverty, hunger, armed terrorism, wars and refugees, human rights and racism. The second group is focused in human physiology: ageing, diseases and disabilities. The last group includes climate change, environmental destruction, ocean pollution, rise of atomic energy and agriculture efficiency. Another future problem that should also be taken into consideration is the energy crisis consequence of the depletion of fossil reserves [7].

From all of the listed problems, the impact of the future advances in Synthetic Biology could be mainly noticeable in the fields of medicine, power sources, environmental protection, agriculture and engineering [8]. With this new discipline it is intended to substitute the biotechnological paradigm and to improve the efficiency, competence and yield of life science products. However, even though synthetic biology may be used to solve the majority of these problems, it is impossible to deal with all of them in the present work. Given the fact that the modern civilization crucially depends on continuous power supply, the problem of fossil fuel depletion seems to be the most imminent (as explained later on) and my study was therefore focused on this topic.

While referring to fossil fuels, the main difference between a resource and a reserve is the possibility of entering the market. A resource is any font of fuel found in the earth's crust, while a reserve is the part of those which is economically viable to extract. Fuel reserves, contrarily to what it may appear, have increased in these last years [9]. The reserves have increased as a consequence to the improvement of the extraction and prospection methods. The consumption has also raised, almost in the same measure as the reserves. It is expected that in upcoming years the demand will overpass the reserves, consequence of the depletion of the fossil fonts.

One first calculation of the years it would take to the ending of the oil reserves stated that these would finish within 33 and 37 years. A modified version of the equation used for this first estimation established its exhaustion year in 2044 [10]. The International Energy Agency calculates a rough 25 years of demand satisfaction on liquid fuels [11]. These data are worrisome given the fact that by 2030, 84% of the energy of the planet will still be produced by fossil fuels. Finding novel energy sources is essential for maintaining the power demand when those reserves will be depleted.

Of the fuels capable of being synthetized by bacteria, the most studied are butanol, ethanol and hydrogen. Taking a close look at the pathways involved in its biosynthesis it can be seen that they are all closely related (Figure 1). Pathways which derive in butanol or ethanol synthesis originate from pyruvate. Ethanol can be also synthetized starting from acetyl-coA, which can furthermore be converted into hydrogen.

A fact that should be taken into account is that all of these pathways derive from glucose degradation. It would be very interesting to enable the bacteria to use different carbon sources. This would not only lower the global cost of the process by sparing the previous glucose synthesis, but it could also turn out to be a way of degrading substances from diverse origins.

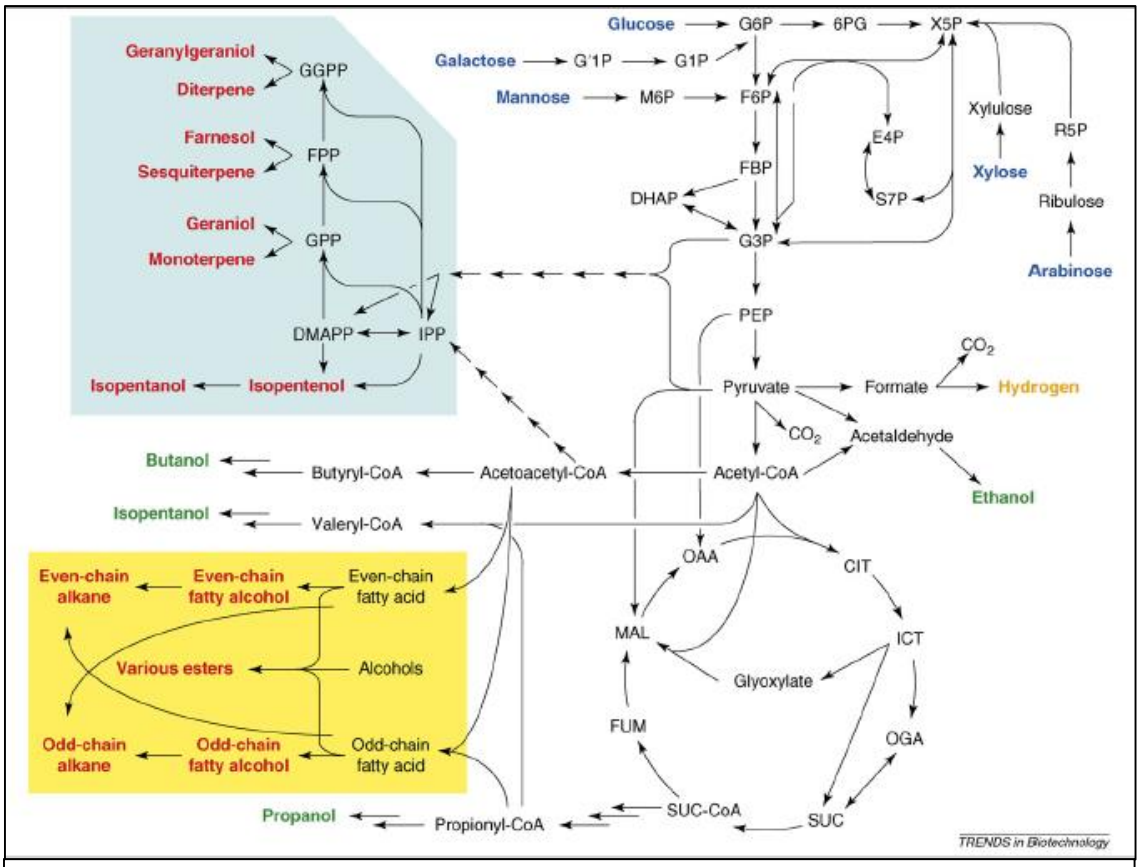

Figure 1. Bacterial metabolic pathways and the molecules produced by them [80].

Another important feature that a biosynthetic bacteria should own is the ability to survive in a medium saturated in its own products. It isn't a very serious problem while producing hydrogen in an aqueous medium, as it is a gas and would be expelled into the aerial phase on synthesis. The problem worsens with butanol and ethanol, both very toxic solvents that remain in the culture medium.

Therefore, if the goal is to create a synthetic organism which could represent a solution for the depletion of the fossil reserves it should have three main features: a metabolic pathway which generates either pyruvate or acetyl-coA; the set of genes necessary for the biofuel synthesis; and a set of resistance genes to overcome the product toxicity due to medium saturation.

Objectives

1) Review the current literature on bacterial biosynthesis of fuels that could overcome the deployment of fossil reserves, proposing as well genes that can:

2) help a bacteria to live in a fuel saturated medium and

3) allow the bacteria to degrade alternative substrates.

If the gene is not available in the literature the natural strategies used by bacteria will be reviewed instead.

Methods

The first step was to decide which databases to use for the literature research in Synthetic Biology. Web of Science (webofknowledge.com) and Google Scholar (scholar.google.es) provided the major part of the results. Institutions and journal databases were consulted for extra resources on specific topics. Institution webpages consulted include: The International Energy Agency (IEA); United Nations (UN); Woodrow Wilson International Centre for Scholars; ERASynBio; BioBricks Foundation; The International Union of Pure and Applied Chemistry (IUPAC); U.S. Energy Information Administration (EIA); US Department of Commerce; Shell Global; United Nations Office for Disarmament Affairs (UNODA); SYNBIOSAFE; and International Thermonuclear Experimental Reactor (ITER). Journal databases consulted include: Nature Biotechnology database (nature.com/nbt), Biotechnology for Biofuels database (biotechnologyforbiofuels.com). Other database: ElSevier (elsevier.es) and Massachusetts Institute of Technology (MIT) database (synbio.mit.edu).

All the search results from non-journal databases were then reviewed using ISI Web of Science Journal Citations Report® (webofknowledge.com) to determine the impact factor of each journal, using this as a marker for the reliability of each paper. Papers from journals under Q2 were mainly discarded, unless the article was found via a reference in a paper from a Q2 or above.

The results were obtained with combinations and variants of the following keywords, as specified in the following table.

Subpart	Keywords
Introduction	“world issues”, “fuel reserves”, “petroleum”, “depletion”, “synthetic organism features”
Butanol	“butanol”, “synthesis”, “bacteria”, “Clostridium”, “yield”, “bioreactor”, “pathway”, “ABE pathway”, “host”
Solvent resistance	“solvent”, “resistance”, “bacteria”, “extremophile”, “thermophile”, “strategies”, “features”, “partition ratio”, “butanol”, “toxicity”, “membrane”, , “fatty acid”, “phospholipid”, “modification”, “efflux pump”
Hydrogen	“hydrogen”, “biohydrogen”, “bacteria”, “pathway”, “yield”, “enzymes”, “electrolysis”, “fuel cell”
Degradative genes	“degradation”, “petroleum”, “byproduct”, “organic solvent”, “plastic”, “polyurethane”

Results

Butanol

Butanol, also known as butyl alcohol or 1-butanol, is a four carbon primary alcohol (C_4H_9OH). It is used in modern industry as additive in fuels, solvent and reactive in industrial processes [12]. Given its similar properties to those of the petroleum derived fuels it could be directly burned in a gasoline engine.

Butanol synthesis pathway is endogenous to a group of bacteria from the *Clostridium* genre. These microorganisms are anaerobic, Gram+ and spore-forming. The organism used as a standard for the study and production of this compound is *C. acetobutylicum*.

At the beginning of the nineteenth century, butanol was synthetized using this bacteria, but the method became inefficient when the demand of this compound increased, so it was substituted by petrochemical synthesis, much cheaper and more efficient. Due to

the progressive deployment of the fossil reserves, which in turn rises oil prices, biosynthesis has re-gained its relevance as an economically suitable method for obtaining butyl alcohol.

The ABE synthesis process begins when glucose enters the Embden-Meyerhof glycolytic pathway, with which pyruvate is obtained. The pyruvate-ferredoxin oxidoreductase (pfor) then catalyses the reaction from this product to acetyl-CoA. Acetyl-CoA can follow five different branches of the ABE pathways which can result in the production of acetone, ethanol, acetate, butyrate or butanol (Figure 2). All five pathways are regulated by an ATP/NADPH equilibrium. When the bacteria is in acidogenesis, acetone, acetate and butyrate are generated. When it shifts to solventogenesis, the bacteria produces ethanol and butanol. A bacteria will always tend to acidogenic synthesis while in non-stress medium conditions, which has no industrial interest [13].

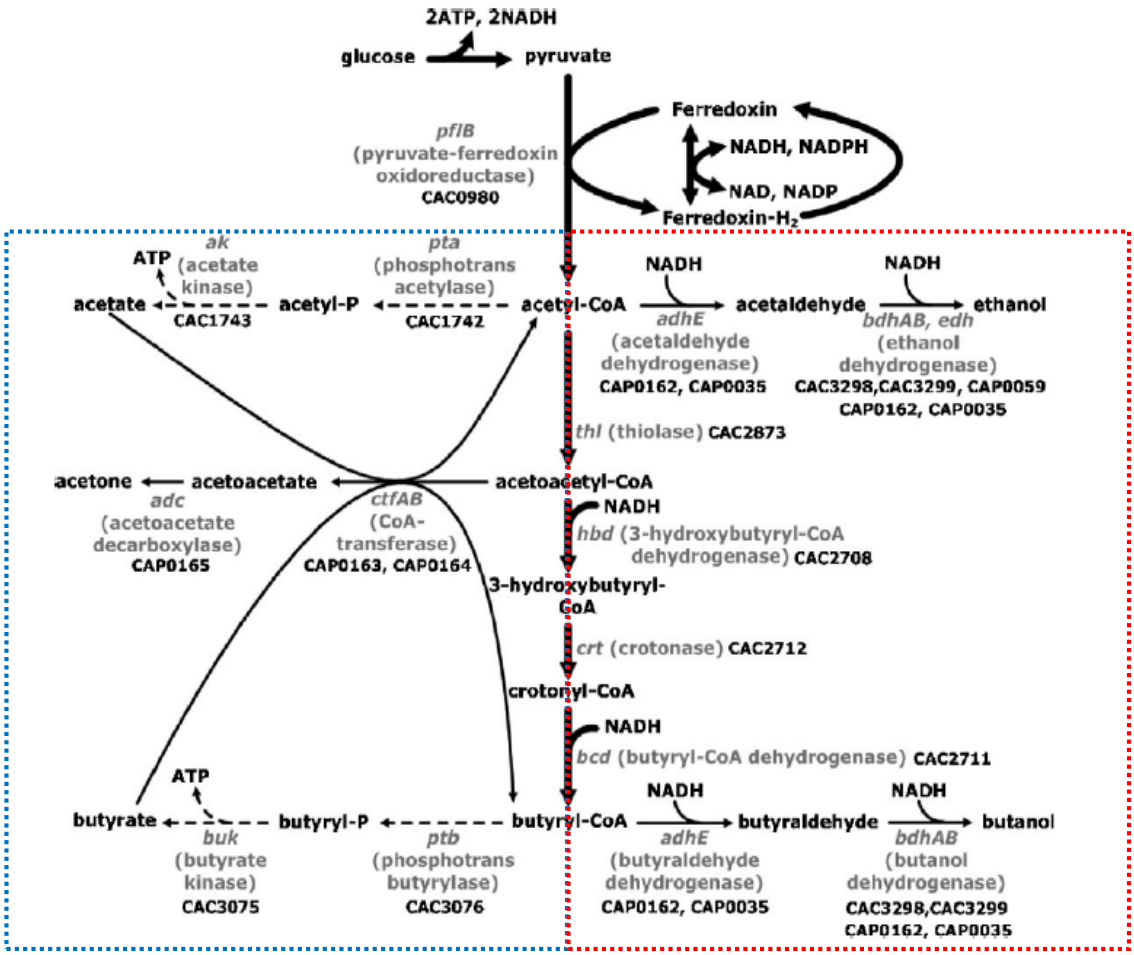

Figure 2. Clostridium acetobutylicum *glucose metabolism: ABE pathway (Acetone, Butanol, Ethanol). The blue rectangle on the left englobes the acidogenic pathways, and the red rectangle on the right englobes the solventogenic ones* [81].

When the intracellular levels of ATP increase the bacteria enters in acidogenesis, while when ATP levels decrease and those of NADH/NADPH increase solventogenesis is promoted, with butanol and ethanol synthesis. Butyl alcohol is toxic for bacteria. It lowers intracellular pH and alters the membranes [14]. If solventogenic synthesis is prolonged, the environmental stress induces the sporulation of the microorganism. Since the beginning of the production of butanol by this method in the early nineties, solventogenic sporulation was the main problem found for industrial scale synthesis. Butanol can't be highly concentrated because of its toxicity, making bioproduction less competitive than petrochemical methods [15].

Wild bacteria produce all of the ABE synthesis pathway products. It would be interesting to be able to create an organism capable of synthetizing butanol, leaving aside all the pathways which interfere with its production. Due to the negative feedback produced by this compound, the maximum butyl alcohol concentration obtained from wild strains is 13g/L. With metabolic engineering of *C. acetobutylicum* only a slightly higher concentration is obtained, 14.1g/L [16].

Other synthesis methods which are being researched include continuous culture production, metabolic pathway expression in alternative hosts and co-expression of genes with the wild ABE pathway [17]. All of those search to avoid the negative effects of solventogenesis in *Clostridium* by retiring the product from the medium as soon as it is generated or by allowing the bacteria to live in solvent saturated medium.

Alternative hosts of interest known to this moment are: *E. coli*, *Methylobacterium extorquens* and *Zymomonas mobilis*. They all share a common trait, they synthetize an intermediate product of the ABE pathway that can be derived into butanol by adding the required genes.

The reference model for exogenous pathway addition is *E. coli.* It is so because of its easy culture and the high genomic, physiologic and metabolic knowledge that it's had on this bacteria.

In a study by the Universitiy of California [18], a synthetic pathway constituted by six genes from *C. acetobutylicum* was introduced in *E. coli* (figure 3). This bacteria produces

acetyl-CoA, which can be turned into butanol with the activity of these six enzymes: thl (thiolase), hbd (3-hydroxybutyryl-CoA dehydrogenase), Crt (crotonase), Bcd (butyryl-CoA dehydrogenase); Etf (electron transfer flavoprotein), AdhE2 (aldehyde/alcohol dehydrogenase). Appart from adding new genes, others which interfered with these new enzymes were removed, as well as those interfering with the use of NADH. The overexpression of certain genes increased the production of this cofactor, essential for butanol synthesis. The maximum yield while producing butanol in this experiment was 552mg/L, a mere 4% of what *C. Acetobutylicum* is able to produce.

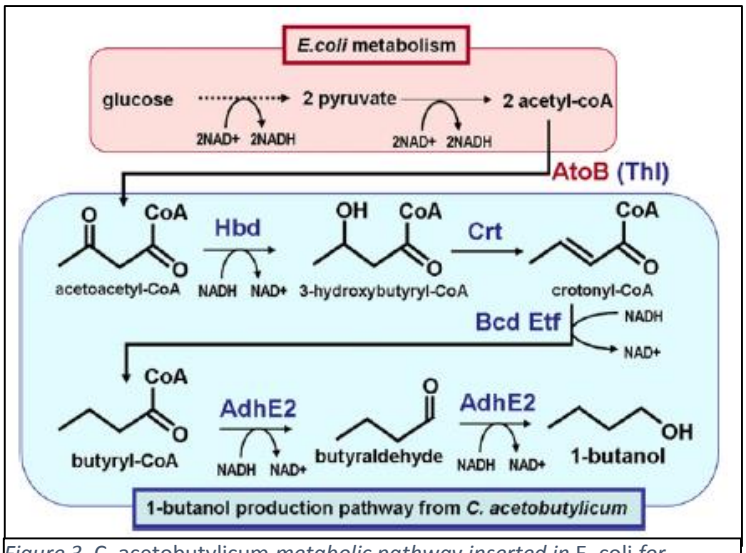

Figure 3. C. acetobutylicum *metabolic pathway inserted in* E. coli *for butanol synthesis.* [18]

Another bacteria used as a host for butanol synthesis is *Methylobacterium extorquens* [19]. It has the ethylmalonyl-CoA pathway which produces ABE synthesis intermediate products. Adding the enzymes Ter (trans-2-enoyl-CoA reductase) from *Treponema denticola* and *C. acetobutylicum* AdhE2, a maximum yield of 13.6mg/L is obtained.

Solvent resistance

The main problem while synthetizing an organic compound has been shown to be its toxicity due to the medium saturation, killing the bacteria and not allowing to highly concentrate it.

Solvents such as butanol are highly toxic due to their low partition ratio (logP). This ratio determines whether a substance is more soluble in a hydrophobic compound, usually an octane, or a hydrophilic one, in a biphasic solution constituted by both [20].

It is calculated by the formula: $(K_D)_A = \frac{[A]_{org}}{[A]_{aq}}$

K_D: distribution constant; $[A]_{org}$: concentration in organic phase; $[A]_{aq}$: concentration in aqueous phase.

Lower partition ratios indicate hydrophilic compounds; higher ratios are a trait of hydrophobicity. Solvents with partition ratios bellow 4 are considered more toxic due to their hydrophilicity. This characteristic allows the solvent to directly interact with the membranes, intracellular structures and enzymes. Butanol has one of the lowest logP, 1.8, and therefore highly impairs the structure and function of cell membranes.

Solvent resistance strategies aren't mutually exclusive, as many bacteria combine a group of stress response genes in well-defined operons. Here the most relevant strategies have been reviewed, compatible with either gram positive or gram negative bacteria. Two or more of these should be combined in order to improve the global efficiency of the solvent resistance.

There are several characterized genes and cell features which could allow a microorganism to live in a solvent saturated medium. The attributes that should be conferred to the bacteria as to overcome this problem vary depending on what specie is being used as a template.

Both gram positive and negative bacteria share many strategies for solvent resistance. These include: efflux pumps, cis-to-trans isomeration of membrane phospholipids, retention vesicles and changes in the membrane repair rate [21].

Gram positive bacteria have six main strategies for solvent tolerance controlled by stress operons: sporulation, degradation or deactivation of organic solvents, changes in cell

morphology, hydrophobic shift of cell surface, cell membrane adaptations and efflux pumps [22]. From these, the most interesting are the last four ones.

Gram negative bacteria have three main strategies for solvent resistance: membrane composition changes and surface modifications, isolation vesicles and efflux pumps [23].

Stress operons are constituted in *Bacillus subtilis* by an average of 100 different genes [24]. This is counterproductive in a synthetic bacteria, as it is intended to have a genome as simpler as possible, but these operons englobe many genes involved in different resistance strategies. It would be interesting to isolate each particular one, characterizing those of interest. Sporulation isn't an appropriate strategy for biosynthetic bacteria because of understandable reasons: while in resistance state bacteria can't accomplish their purpose. Neither is the deactivation of the solvent, as the main objective of the process is to produce this substance.

Cell morphology changes in gram positive bacteria include increased size, filamentous growth and capsule formation. Both increased size and filamentous growth, consequence of the expression of heat shock proteins, may have an influence in the membrane/cytoplasm ratio, having less surface for absorption and thus increasing the effectiveness of other defence mechanisms such as efflux pumps [25,26]. These adaptations strategies are controlled by regulons constituted by a high number of genes. The specific function of each one should be studied in order to be used in a synthetic organism.

Hydrophilic capsule formation is used by certain strains of *Staphylococcus* [27]. The capsule is composed of carbohydrates which aren't expressed in a non-stress situation. It has an impact in the adaptation to solvents as it repels them from the cell membrane. This strategy has been reported also in gram negative bacteria. This strategy has only been described at physiological level, in order to be used in a synthetic organism of the specific genes involved in the capsule synthesis should be characterized.

In addition to the generation of a hydrophilic cell capsule, certain strains of bacteria have the ability to modify the hydrophobicity of their membrane, rendering it less affine to solvents [28]. This strategy has been reported without being combined to the capsule formation, but the resistance could be higher when both are expressed, as the capsule

prevents the solvent to reach the membrane, and the membrane is less affine to the solvent when it is reached. The strategy has been characterized both in gram positive and gram negative bacteria [29]. It is particularly interesting because it allows the bacteria to survive in mediums containing solvents with lower logP, which is the case of butanol.

When it comes to solvent adaptation at membrane level, the first feature which is modified is its fluidity [30]. Its alteration is a direct consequence of the interaction of the organic solvent with the lipid bilayer. The bacteria's initial response is to modify its basal membrane fluidity to overcome the modifications induced by the solvent. This can be achieved by a cis-trans isomeration or a change in the saturated/unsaturated fatty acid ratio, whether it's a short term or a long term response respectively.

Bacteria synthetize fatty acids in cis- conformation, but in response to medium stress caused by toxic substances it can modify those into trans- conformation [31,32]. The enzyme which catalyses this reaction is the cis-to-trans isomerase (Cti), found in the periplasm of certain gram negative bacteria, such as *Pseudomonas putida* and *P. Oleovorans* [33,34]. The enzyme appears to have a constitutive expression, but the mechanisms involving its activation to toxic substances in vivo remain unknown.

The effects of toxic compounds in the saturated/unsaturated fatty acid ratio has been studied deeply in many strains of bacteria [28,35–37]. Unsaturated fatty acids (UFA) in trans- conformation act in a similar way to saturated fatty acids (SFA) within the membrane structure. The strategy is closely related to the cis-trans isomeration, as both seek to increase the membrane rigidity. The UFA/SFA ratio is a long term defence against solvent membrane aggression and seems to be constitutive of many bacteria.

Other membrane modifications in gram negative bacteria include changes in membrane phospholipid composition and changes in lipopolysaccharides. Modifications in the membrane polysaccharides may be interesting against solvents with higher logP, but as butanol has one of the lowest this strategy is ineffective.

Under solvent stress, *P. putida* significantly increases the content of cardiolipin and phosphatidylglycerol its membrane [38]. It is possible that the lower transition temperature of these phospholipids allows the membrane to be more stable and less

fluid and thus protecting it against solvents [32]. The enzyme which is responsible for the cardiolipin synthesis in prokaryotes is the cardiolipin synthase, a phospholipase D-type enzyme [39]. These studies of solvent resistance have been done with toluene and xylene, which have higher logP than butanol. Further studies with solvents with lower logP should be done in order to assure the efficiency of this solvent resistance strategy.

Efflux pumps have been studied in both gram positive and gram negative bacteria [40,41]. Their efficiency is limited to solvents with logP higher than 2.69 (toluene) [42]. The strategy is interesting, as the efflux pump actively deploys the solvent from the cytoplasm into the medium, but solvents such as butanol, with a low molecular mass and low logP can easily traverse the membrane, rendering the pump inefficient. Further studies could be done with less permeable modified bacterial membranes and engineered efflux pumps in order to improve the effectiveness of the pump against butanol aggression.

Biohydrogen

Hydrogen (H) is a chemical element present in high quantities in the earth's atmosphere and crust. With an atomic mass 1 it is the lightest element, and the most abundant, constituting 90% of all the atoms in the universe and about three quarters of the universe's mass [43]. However, the atmosphere composition of hydrogen gas (H_2) is almost insignificant. Periodical measures are done by the Earth System Research Laboratory, setting the annual concentration of atmospheric hydrogen within 560 and 430 nmol/mol [44] .

As the concentration in the atmosphere is negligible, it is not profitable to directly extract it from air. The major hydrogen source on earth is water, finding it either in liquid state or in gaseous state in the atmosphere [45]. The cost of electrolysing water is high, because the electricity is in the first stance produce by a non-renewable source. Nowadays the major non-electrolytic sources of hydrogen are natural gas and coal [46].

Hydrogen is highly flammable and can be used in modified combustion engines, although in this way it is more commonly burned mixed with natural gas [47]. It is a very

promising fuel due to the almost non-existent emissions when combusted and water being its only exhaust. The main research of hydrogen engines is centred in fuel cells. Those are composed of a Proton Exchange Membrane (PEM) where the hydrogen is injected producing electricity, which can be used to power an electrical car. The leading countries and states in hydrogen energy research are Iceland, Vanuatu and Hawaii [48]. They all share a common trait: the availability of geothermal energy. By using electricity obtained through this method to electrolyse the water, hydrogen can be obtained without the previous disadvantages.

It would be interesting to bioproduce hydrogen using bacteria in order to bypass the availability of a renewable electricity source. There has been much research done on the topic, defining two major groups of possible pathways for hydrogen generation: light-dependent and light-independent. The most remarkable light-dependent pathways are direct or indirect photolysis of water and photo-fermentation. The main light-independent pathway is dark fermentation.

Direct photolysis is carried out by algae and cyanobacteria. When in anaerobic conditions, or in a proton excess situation, the surplus of protons can be derived into hydrogenases, which use them to produce pure molecular hydrogen. Cyanobacteria have two photosystems: I and II [49,50]. Those are responsible of harvesting the light's energy throughout pigments in order to split water molecules and achieve the necessary electrons and a proton gradient. This in turn, is needed to obtain energy for the reduction of cofactors and ATP synthesis, which are used for the fixation of carbon atoms during the dark phase of photosynthesis. Photosystem II is composed of 20 protein subunits plus its associated lipids and pigment molecules [50]. Photosystem I is composed by 12 proteins and their associated cofactors, lipids and pigments [49]. Both of these systems are on the thylakoid membrane of cyanobacteria. Thylakoids are situated just underneath the plasma membrane, as cyanobacteria don't have cytoplasm subdivisions such as chloroplasts. Associated in function to these photosystems is the hydrogenase enzyme. Its purpose is to relief proton stress in high energy or anaerobic situations, and in nitrogen fixator cyanobacteria it also helps assimilating this compound. The enzyme itself, denominated *hoxEFUYH* in non-nitrogen fixator bacteria, is composed by two subunits, and its expression is regulated by a high number of genes

[51]. It's found in a cluster of five genes, with a different pattern of expression depending on the situation. For its correct assembly it needs two specific endopeptidases. When it comes to its expression, there is at least one regulator gene characterised: LexA.

This strategy can represent a great challenge for Synthetic Biology to replicate, because it doesn't only involve the genes which codify for the photosystem components and the hydrogenase, but also the whole of the genes which generate the thylakoid structures and the pathways and expression regulators of each of them. A good way of characterising the dispensable genes could be the one used by Venter et al. while developing the Minimal Genome Project [52]. A transposon was used to disable random genes of the bacteria, monitoring the effect of each dysfunction within the whole bacterial metabolism. If the impaired gene doesn't affect the bacterial function being monitored, the gene can be omitted.

Photofermentation is another possible approach to hydrogen production. Certain strains of purple bacteria can generate this compound from organic substrates using a single photosystem fixed to the interior of the plasma membrane [53]. Its full structure has not been completely elucidated, but some studies have determined that of its reaction centre, which is the part of the photosystem where the light energy is turned into chemical energy [54]. This photosystem can't break the water molecule by itself, but in anaerobic conditions and with the adequate substrate, short chain organic acids, as an electron donor, the reaction can take place. The bacteria uses the surplus of protons from the gradient to generate energy in order to transfer the electrons to the ferredoxin, capable of catalysing the formation of molecular hydrogen in nitrogen absence conditions.

This method of hydrogen obtainment has certain advantages over direct photolysis [55]. First of all, the yield of hydrogen production is high per substrate converted. Secondly, the reaction takes place when the bacteria is supplied with organic substrates. These can be found in waste from industrial processes, and therefore the process of hydrogen synthesis could at the same time help in bioremediation. The main disadvantage is that the products of the photofermentation aren't only water, but also CO_2 from the acid degradation, not being completely environmentally-friendly.

Another advantage, from the Synthetic Biology perspective, is that the pathways involved are much simpler than the ones of direct photolysis, being easier to attempt its replication in a synthetic organism. If the goal is to succeed in this creation, further research on the genes implicated in these pathways should be done.

Both direct photolysis and photofermentation require large infrastructures so the bacteria receive direct sunlight. A solution for this problem is dark hydrogen fermentation, carried out by heterotrophic anaerobic bacteria. The excess of protons product of the degradation of organic compounds is channelled into a hydrogenase which renders molecular hydrogen [56]. Therefore, the only required genes for hydrogen synthesis are the ones controlling the expression of the hydrogenase, rendering this process as very interesting from the Synthetic Biology perspective. This technique wouldn't require the development of novel infrastructures, as it could work in already existing bioreactors, not needing direct solar input, and being fed with diverse organic substrates [57].

Many studies have been carried out in order to better comprehend and improve dark fermentation. Strict anaerobes such as certain strains of thermophilic bacteria can obtain a maximum yield of 4 H_2 per glucose, while facultative anaerobes only reach a mere yield of 2 H_2 per glucose [58].

Degradative genes

One of the objectives of this work is to determine which degradative pathways could be added into a synthetic organism. It would be interesting to control the origin of the bacteria's energy and carbon source, as it can help degrading either toxic compounds or improve the yield in pre-existing feedstock degradation.

While synthetizing ethanol out of sugar cane, the plant is crushed and the sugar extracted [59]. The fermentation is carried out by certain strains of yeast, consuming the monosaccharides and starch while producing ethanol at a maximum concentration of 15%. The cellulosic fibres are treated with bacterial cellulases, partially hydrolysing the fibres into glucose, which is also fermented. Then the resultant ethanol is distilled

into its final concentration. Although the yield is high, there are still a lot of drawbacks to ethanol production. First of all, the only part which is used is the stem, the leaves are burned away first, before harvesting the canes. Secondly, the stem contains a high quantity of lignin, a complex aromatic polymer, which can't be used for ethanol synthesis and is expensive to deal with. The stem contains as well much fibre, that, although it is processed by cellulases, great part is discarded.

Synthetic bacteria which follow similar pathways in butanol or hydrogen production should be able to use the whole or most part of the plant directly, and not only sugar cane, but all types of agricultural feedstock. Some authors believe in the possibility of engineering plants in order to make them optimal for biofuel production, minimizing the proportion of lignin or changing its structure [60]. This would suppose the usage of culture land solely for this purpose, instead of recycling the waste produced by other cultures. Enzymes capable of lignin degradation have been reported in fungal species of the genre basidiomycetes [61]. The characterized enzymes are laccase, aryl-alcohol oxidase and a manganese dependant peroxidase. A bacteria capable of expressing these should be able to degrade lignin and metabolize its components. In order to completely degrade agricultural feedstock, synthetic bacteria should be also provided of cellulases.

Other interesting feedstock for bacteria are industrial and human waste and certain contaminated waters and soils. This would render a double benefit, by generating biofuels and by helping with bioremediation.

Enzymes such as esterases and lipases are present among many species of microorganisms. They are of particular interest because of their capacity to degrade plastic polymers. Esterases break up esters into an alcohol and an organic acid, which can be further catabolized, while lipases degrade aliphatic molecules. The fungus *Yarrowia lipolytica* has been reported to be capable of degrading contaminants such as aliphatic and aromatic molecules, organic pollutants, trinitrotoluene and metals [62]. Strains of the bacteria *Comamonas acidovorans, Paenibacillus amylolyticus, Acidovorax delafieldi and Leptothrix sp.* are capable of degrading propylene and polyurethane by expressing certain enzymes, such as the PUR esterase from *A. delafieldi* [63,64].

Contaminated water contains toxic substances of diverse origins. Organic solvents present in polluted waters can be degraded by bacterial enzymes. *Bacillus pallidus* is able to use isopropanol and acetone as only carbon source [65]. Another strain of *Bacillus, B. licheniformis,* is able to produce esterases which help the bacteria to degrade the solvent in its medium [66]. Other microorganisms are capable of metabolizing petroleum and its by-products. Species of bacteria and fungus are currently commercialized for bioremediation in oil contamination [67].

Screenings among microorganisms should be done in order to further characterize new degradative enzymes.

Discussion

Both hydrogen and butanol bioproduction seem viable ways of overcoming the depletion of fossil fuel fuels. Each of them have certain drawbacks and advantages over the other. Butanol, due to its chemical properties, is a fuel that can be directly used in gasoline engines, without further machinery modifications. Hydrogen, in the other hand, is a gas, and therefore it would require a higher level of control when being burned. This, added to its high flammability, make it riskier to use than butanol in combustion engines. It is well a minor problem, as the main use of hydrogen as a fuel relies in chemovoltaic cells, which generate electricity by conducting the gas through a PTM, with no combustion being involved in the process. The fact that hydrogen is used in PTMs and not directly combusted means that the whole of the machinery dependent on petroleum should be adapted to electricity. The transition would imply a high economical investment. Hydrogen storage has to be done correctly and cautiously, avoiding the contact with oxygen. An unexpected solution for this problem relies in the bacteria used for its synthesis. Most are strict anaerobes, or only synthetize hydrogen in such conditions, which assures that the gas never comes in contact with oxygen.

The arguably major drawback to butanol is the effects over the climate change. When burned, the exhaust gasses are CO_2 and water vapor. Hydrogen's only exhaust is pure molecular water. Butanol would be a good solution for the power crisis, but would continue to contribute affecting one of the main environmental issues according to the

UN: global warming. A possible solution would be creating a bacteria which could synthetize butanol directly from solar energy, being photoautotrophic. This, even though it is still a very intangible idea nowadays, would cause a lower impact on climate change, as the CO_2 emissions would be partially countered by the synthesis of the compound.

Still, butanol could be synthetized out of organic solvent waste, as the synthetic bacteria are already adapted to life in mediums saturated with it, and therefore contribute to bioremediation, helping to get rid of toxic compounds. Hydrogen produced by photofermentation can also help in this process, as the bacteria who carry out the process use short chain organic acids as electron donors. The waste water product of these two methods should still be treated, because the process generates unwanted products. To avoid almost completely the residue generation, direct water photolysis should be considered. Its only feed is water, sunlight and CO_2, with minor quantities of other substrates, significantly decreasing the amount of rests.

By knowledge of the pathways and enzymes involved, and the replicability of these in a synthetic organism, butanol is the closest and easiest solution. The full pathways and enzymes are known from years past, and many metabolic engineering experiments have been done in order to improve the yield and resistance of the production. It could represent an immediate solution for the depletion, although hydrogen is much more promising in terms of environmental friendliness and future sustainability.

Certain problems remain to be unsolved for butanol synthesis. Its high toxicity still doesn't allow to highly concentrate it. This could be solved by synthetizing the compound in continuous cultures, where the product is promptly deployed from the medium [68]. Another possible strategy would be using semipermeable membranes, which allow butanol traversing, retiring in from the culture [69]. A remarkable solution for making the synthesis more profitable is adding a second pathway to the bacteria, allowing it to produce another easy to extract compound with economic interest. This is the case of riboflavin, co-expressed in *Clostridium acetobutylicum*, which doesn't influence the yield of butanol production, but increases the economic benefit of the process [16].

The current work focuses on fuel production throughout synthetic organisms, but it only regards the fuels as an energy source, not in all of its diverse applications. While speaking of fossil fuel depletion, not only will the power sources be affected, but a wide range of activities will be forced to change. Petroleum is currently used in chemical industry, producing solvents, mineral oils, lubricants, etc. Materials industry also depends on crude oil, it is used in resins synthesis, asphalt, and plastic materials production. A rough 5% of the total petroleum is consumed by the plastic industry in the US, the major oil consumer worldwide [70]. These problems should also be taken into consideration in order to overpass the depletion of fossil fuels.

Another observation to be done would be the resistance of the enzymes in the synthetic bacteria to the compounds it produces. A bacteria which synthetizes butanol should have solvent resistant enzymes. The position of these in the bacteria, whether it is excreted or membranous, should also be taken into consideration. Solvent resistant enzymes are present in extremophile microorganisms which thrive in mediums saturated with these compounds. These stable enzymes can be identified by performing a screening for the microorganisms that produce them [71]. A strain of *Pseudomonas aeruginosa* is able to produce lipases which are functional under concentrations up to 25% (v/v) of organic solvents [72]. Proteases active in the presence of butanol were also identified in *Pseudomonas aeruginosa* [73]. Solvent stable esterases have been reported in *Bacillus licheniformis* [66]. The most promising microorganisms for resistant enzyme characterization are thermophiles, as the thermophilic capacity of enzymes seems to be correlated to solvent tolerance [74].

If the enzyme isn't found in known organisms, modifications can be done to them in order to improve their stability in organic solvent presence. Many strategies have been proposed in order to stabilize these, but much of those methods have been focused on modifying the enzyme after the expression, such as fixating them in solid structures, entrapping them in micelles, or chemical and physical modifications [75]. Solvents directly affect the enzymes by altering their structure, disrupting the hydrophobic core and denaturalizing the protein or its active site, or not allowing the conformation changes needed for the reaction to take place [74]. Changes in the protein sequence can be done by direct mutagenesis or random modifications, characterizing afterwards the

most efficient and stable proteins. Modifications such as increasing the overall hydrophobicity of the enzyme, or making them more compact seem to improve their resistance by minimizing the penetration of the solvent. In certain enzymes, disulfide bonds have proven to improve the overall stability.

Something to take into consideration while creating an artificial organism are the ethical issues. In the 26th of March 1975 the Biological Weapons Convention entered in force, by which production, development and stockpiling of these was banned worldwide [76]. By this, Synthetic Biology can't be focused in the production of bioweapons. Still, a bacteria which synthetizes compounds such as biofuels could be seriously harmful for the environment if there was an accidental release. To debate the ethical issues of Synthetic Biology the European project SYNBIOSAFE was created in January 2007, and developed until it's conclusion in December 2008 [77]. The project aims to promote a socially acceptable development in the area, promoting biosafety and ethics debate. It states a basis for the correct development and research in synthetic organisms, minimizing the social and environmental repercussions.

Finally, why should synthetic biology be considered as a plausible solution for the upcoming power crisis? There are other projects in different science areas, such as ITER nuclear fusion project, aiming to provide clean energy worldwide by this method [78]. It plans to start its activities by 2030 with a prototype, which will connect fusion power into the grid by 2040. The project started on 2008, and it's expected to spend 32 years in completing the first phases of the project. It would be a reasonable long term solution for the energy crisis, but wouldn't be ready by the time it will occur. Engineered microorganisms with the capability of synthetizing biofuels are already being used for biofuel synthesis in prototype plants. They represent a shorter term response, but this can be finally refined by fully synthetic organisms, having then a complete and highly refined control over the artificial lifeform. Craig Venter et al. were able to synthetize a whole minimal bacterial genome in 2008 [79], the next step could be to synthetize the genome of a bacteria with functional interest.

Conclusions

There is a huge amount of bibliography on the subject of Synthetic Biology as well as bacterial fuel synthesis and compound degradation, with a great number of characterized genes and metabolic pathways.

It is advantageous to use Synthetic Biology in creating a new organism instead of using Genetic Engineering on a preexisting one, as it has a much higher level of control over the design. It allows to prescind from all the genes which interfere with the desired bacterial function, adding only the required ones.

The ability of creating a synthetic organism that fulfills the purpose postulated in this work relies on the full comprehension of each particular gene and its function within the whole.

It could be possible to create a synthetic organism that incorporates the needed pathways in order to synthetize butanol or hydrogen, although further research needs to be carried as to establish the exact set of genes with compose and are needed to replicate the pathways.

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