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PROCESSES FOR A SUSTAINABLE ECONOMY

The challenge for industrial biotechnology is to develop sustainable and environmental friendly processes based on renewable sources. Innovative approaches to solving environmental and energy problems for the realization of a sustainable bio-based economy are required. The following article presents few examples and applications in this regard.

On the agenda of every conscious government, humanitarian organization, of every economic entity and analyst there are major issues or concerns regarding the entire humanity that we can not avoid facing anymore. An economic growth to be sustainable and socially responsible needs to take into account resource availability and effects on the environment.

Fossil fuels, besides their use to produce energy, act as building blocks or reagents for producing several materials. There are many nonfuel uses for petroleum, including various specialized products for use in the textile, metallurgical, electrical, and other industries.

With regards to fossil reserves, we are now faced with the paradoxical situation that while crude oil (petroleum) is being consumed faster than ever, the "proven oil reserves" have remained at about the same level of thirty years ago as a consequence of new oil findings. Nevertheless, these "proven oil reserves" are located in increasingly difficult to reach places. Therefore, the cost for extracting the crude oil rises continuously. More important in this respect, the world's crude oil reserves will not last forever.

What can be done? The innovative approach of Industrial Biotechnology

Industrial Biotechnology (IB) uses renewable raw materials as starting product and microorganisms (genetically modified or not) and their enzymes to make useful chemical compounds and biomaterials. While their application in the production of fine chemicals and pharmaceuticals is already well established (e.g. insulin, interferons, erythropoietin, hepatitis B vaccine, vitamin B12, etc.), it is now increasingly being applied to produce bulk chemicals such as biofuels (e.g. ethanol) and

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bio-plastics. These technologies present the inherently advantages of a clean process that has reduced water and energy consumption, less or no waste generation and less CO_2 generation. Additionally, the costs of new biotechnological processes could be 40% lower than those of conventional processes and could imply up to 70% savings on capital equipment (www.suschem.org/).

The biorefinery concept

To explain the process by which biomasses are transformed into energy and products, the concept of "biorefinery" has been introduced.

A biorefinery (Fig. 1) is a fully integrated manufacturing facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass, with minimal waste and emissions. A biorefinery is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum. Biorefineries have been identified as the most promising route to the creation of a new biobased industry.

The starting products used in a biorefinery, all renewable, are starches, lignocellulosic materials, oils and proteins, urban and agro-industrial wastes.

Industrial biotechnology and metabolic engineering

Evolution has produced a huge variety of organisms living in radically different environments. These organisms make biochemicals through a series of enzyme catalyzed reactions referred to as pathways. In particular, some of these organisms have evolved metabolic pathways leading to the synthesis of potentially useful compounds that are difficult to produce by chemical industry or that are environmentally harmful to manufacture. It has to be reminded that the fundamental basis of evolution is the need to survive and reproduce, not to produce potentially important and commercially valuable products. Indeed, interesting metabolites are very often produced by wild type organisms in such low concentrations that biotechnological exploitation is, at least today, impractical. Metabolic pathway manipulation for improving the properties and productivity of microorganisms is an old concept. Cell metabolism can be manipulated changing the external or the internal environment of the cell. Cell performance under changing the external environment has long been practiced by choosing operating conditions during batch processes to improve growth and productivity. Traditionally internal changes have been achieved by random mutagenesis and selection, searching for metabolites that render the cells more active for the desired metabolic process. This approach made use of chemical mutagens and creative selection techniques to identify superior strains. There are many examples of this strategy in the area of antibiotics, amino acids, vitamins, alcohol, solvents and others. However, despite widespread acceptance of many successes, mutagenesis remains essentially a random process. Thanks to recombinant DNA-technology, one can now specifically intervene into the genetic material of these microorganisms. The metabolism of microorganisms can be modified or even completely changed (so-called "metabolic engineering") or genes from higher organisms (plants and animals) or other microorganisms (yeast, bacteria, virus, algae) can be inserted into microorganisms and

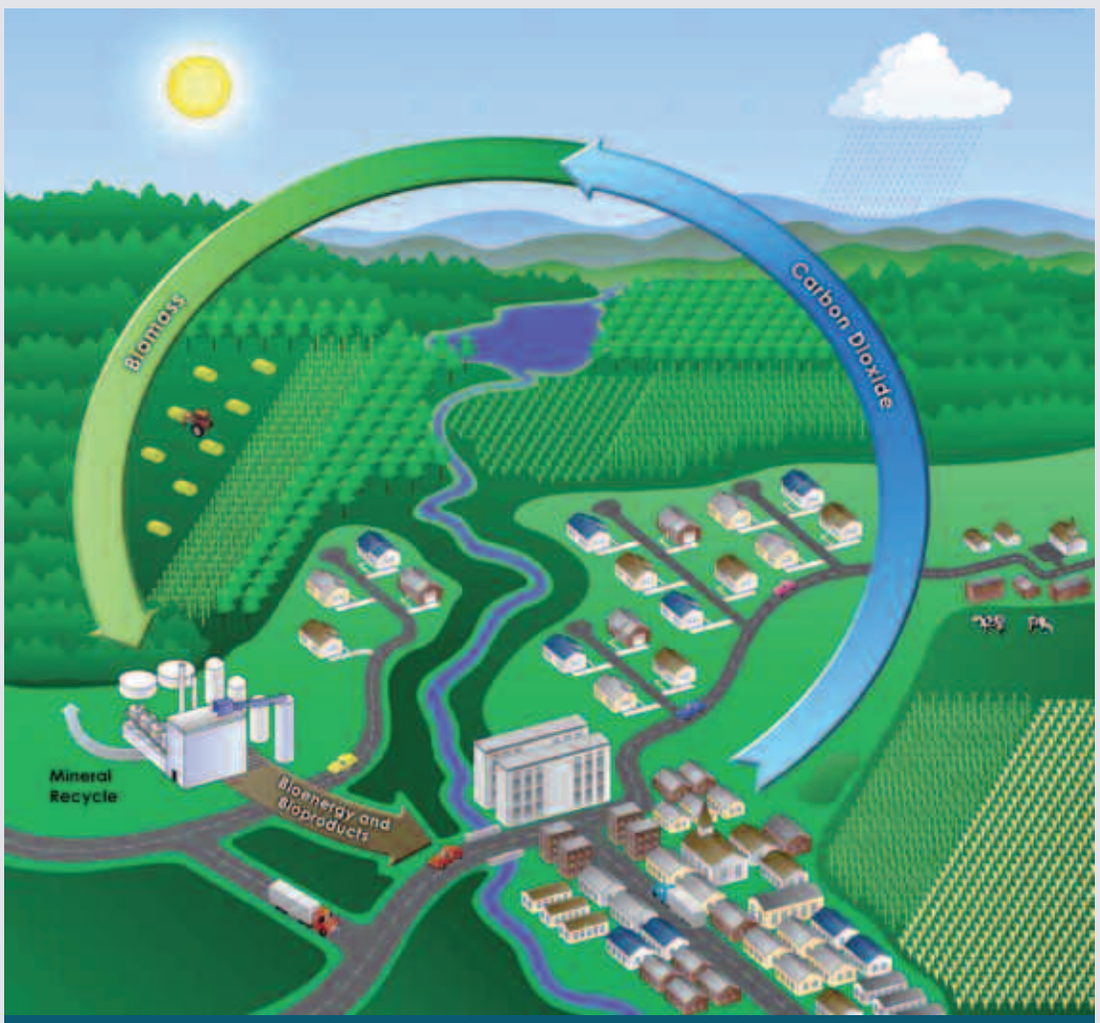


Fig. 1 - Idealized biorefinery concept (Adapted from ORNL Review www.ornl.gov/info/ornlreview/v33_2_00/bioenergy.htm)

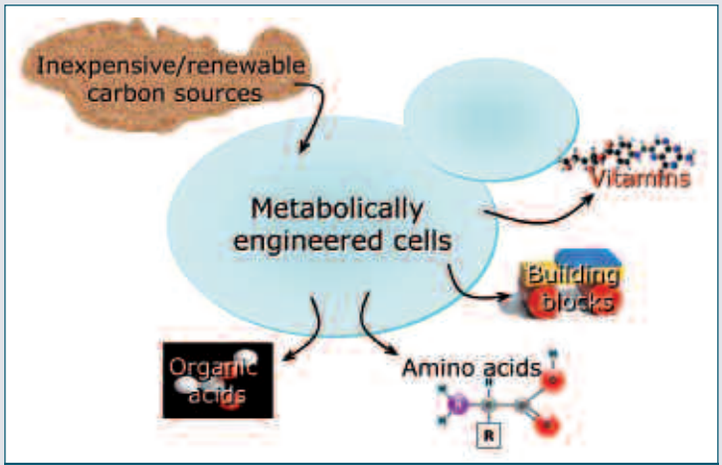


Fig. 2 - Schematic representation of the development of cellular-based bioprocesses: biomasses are transformed in chemical compounds of interest through metabolic engineering of (well-known) microorganisms

brought to expression. Thus, new gene products can be made or new metabolic pathways can be created to produce chemical substances via industrial fermentation processes. The ability to select mutants or to develop organisms by means of rDNA technologies to enable fast and environmental friendly productions of these products has the obvious potential to revolutionize the biotechnological industry.

The research team of Industrial Microbiology of University of Milano-Bicocca is involved in the above mentioned research activities and applications (Fig. 2). In the last years, the team has developed, patented and licensed the technology for the production of lactic and ascorbic acids from recombinant yeast hosts.

Lactic acid production from recombinant yeasts

L-Lactic acid, first discovered by the Swedish chemist Scheele (1780), has been traditionally used as a food preservative and food flavouring compound [1]. It also finds applications in cosmetics and pharmaceuticals. The world-wide production of lactic acid (currently an estimated 250,000 t/year) [2] is rapidly increasing, mainly as a result of the growing market for polylactic acid. It is expected that this biodegradable polymer, produced from renewable resources, will replace various petrochemical-based polymers in applications ranging from packaging to fibers.

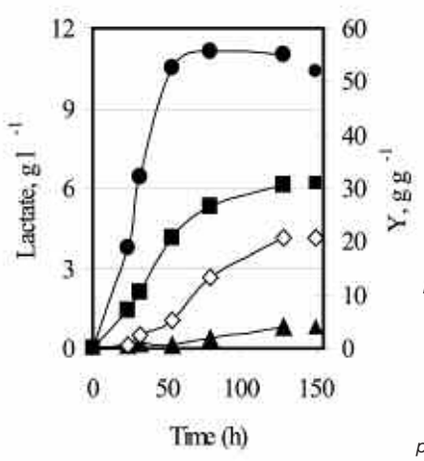
This carboxylic acid is currently mainly produced using lactic-acid bacteria, such as various *Lactobacillus* species, via an anaerobic fermentation that operates optimally at pH values where the salt of the organic acid rather than the free acid is formed, although free lactic acid is preferred for most industrial processes [1]. Different research teams have been involved in the production of lactate from metabolic engineered yeasts such as *Saccharomyces cerevisiae* [2-9], *Kluyveromyces lactis* [10a,b, 11], *Torulaspora delbrueckii* [10b] and *Zygosaccharomyces bailii* [12]. The use of microorganisms like yeasts, that are more tolerant to low pH values than the current producing organisms, could strongly decrease the amount of neutralizing agents required and lower the cost of down-stream processing. Pyruvate is the end product of glycolysis; it can be further metabolized either by the pyruvate dehydrogenase complex (Pdh, EC 1.2.4.1) to acetyl-coenzyme A or by pyruvate decarboxylase (Pdc, EC4.1.1.1) to acetaldehyde and subsequently to ethanol.

We analysed the lactate production from metabolic engineered *Saccharomyces cerevisiae* cells expressing a heterologous lactate dehydrogenase (*LDH*) gene. The *LDH* gene expression in a budding yeast cell introduces a novel and alternative pathway for the NAD^+ regeneration, allowing a direct reduction of the intracellular pyruvate to lactate, leading to a simultaneous accumulation of lactate and ethanol [4].

Four different *S. cerevisiae* strains were transformed with six different wild type and one mutagenised *LDH* genes, in combination or not with the over-expression of a lactate transporter [13]. Fig. 3 summarises some of the obtained data. The resulting yield values varied from as low as 0.0008 to as high as 0.52 (grams of lactate produced per grams of glucose consumed). In this respect, and to the best of our knowledge, higher redirections of the glycolysis flux have never been obtained before without any disruption and/or limitation of the competing biochemical pathways. This was also implemented by disrupting the *PCD* gene and/or the *PDH* gene in other well known yeast, *K. lactis* [10a,b, 11].

Finally, the physiological consequences of such production were evaluated. Despite yeasts can grow at low pH, a high production of lactic acid reflects on a high accumulation of lactate and H^+ into the cells, that have to spend energy to keep the internal pH at physiological levels. A flow cytometric method to determine internal pH was settled, and subsequently coupled with a sorting system aimed to enrich in subpopulation with the higher internal pH. From these cells single clones were isolated and it could be possible to further increase the production [14].

In conclusion, we proved that the redirection of the pathway towards



Different recombinant S. cerevisiae GRF18U cells were shake-flask grown in glucose 2% wv^{-1} minimal selective medium till stationary phase. Samples were collected at indicated times (h) for lactate (g l^{-1}) and yield (gram of lactate produced per gram of glucose consumed; values are here below reported) determinations. ▲: B. taurus LDH, yield 4%±0.2%; ◇: L. casei LDH, yield 21%±0.4%; ■: L. plantarum LDH, yield 31%±0.5%; ●: Mutated L. plantarum LDH, yield 52%±0.7%

Fig. 3 - Lactate production and yield obtained from recombinant GRF18U host cells

a) Schematic representation of the biosynthetic pathways leading from D-glucose to L-ascorbic acid in plants, underlying activities that are shared with yeasts. The following enzymes are involved: A. L-galactono-1,4-lactone dehydrogenase (1.3.2.3); B. L-galactose dehydrogenase; C. sugar phosphatase (3.1.3.23); D. hydrolase; E. GDP-mannose-3,5-epimerase (5.1.3.18); F. mannose-1-phosphate guanylyltransferase (2.7.7.22); G. phosphomannomutase (5.4.2.8); H. mannose-6-phosphate isomerase (5.3.1.8); I. glucose-6-phosphate isomerase (5.3.1.9); J. hexokinase (2.7.1.1)

b) Comparison of intracellular antioxidant levels measured by spectrophotometric analyses in the control yeast strain (open bar) compared to the engineered yeast strain (full bar). Cells were grown in minimal synthetic medium with 2% Glucose as carbon source and samples were collected after 24 hours from the inoculum

Fig. 4 - Development of a yeast strain capable to convert D-Glucose into L-Ascorbic acid

the lactate production can be strongly modulated by the genetic background of the host cell, by the source of the heterologous Ldh enzyme, by improving its biochemical properties, by modulating the export of lactate in the culture media as well as by improving physiological properties.

The results obtained have been patented and partially licensed, offering, in this way, a clear example of technology transfer [15].

Ascorbic acid production from recombinant yeasts

Vitamin C or L-ascorbic acid is an indispensable important metabolite for different physiological functions and an essential nutrient for animals lacking its biosynthetic pathway like humans. L-ascorbic acid acts as a scavenger of reactive oxygen species, protecting tissues from harmful oxidative products. This capacity leads to an increasing demand as a food additive [16].

L-ascorbic acid is conventionally synthesized by a variety of chemical methods, which are generally variations of the Reichstein process, which utilize glucose as starting material [17, 18].

Novel biotechnological processes, which convert glucose into vitamin C in one step, would be desirable and yeasts, such as *S. cerevisiae*, offer themselves as biocatalysts due to their GRAS (Generally Recognized As Safe) status. However, yeasts lack the ability to produce L-ascorbic acid naturally, producing instead erythroascorbic acid, a structurally but not biologically related compound.

It is possible to take advantage of existing enzymatic activities already present in yeast and complement them with heterologous ones taken from naturally occurring biosynthetic pathways, in order to create a yeast strain capable of producing ascorbic acid. The project presents different challenges since the naturally occurring synthetic pathways see at least 14 steps.

The engineered yeast complemented with 5 genes cloned from *A. thaliana* (*AtME*, *AtVTC2*, *AtMIP/VTC4*, *AtLGDH*, *AtAGD*) and then further optimized by substitution with one endogenous activity (*ScALO*, Fig. 4a) is able to produce ascorbic acid by direct conversion of D-glucose (Fig. 4b).

An interesting characteristic acquired by the host yeast strain during ascorbic acid production is an increment of its robustness. Stress can damage subcellular components and can induce apoptosis (programmed cell death), cell necrosis and cell lyses. These effects are often mediated by the generation of Reactive Oxygen Species (ROS) (for recent reviews see: [19, 20]).

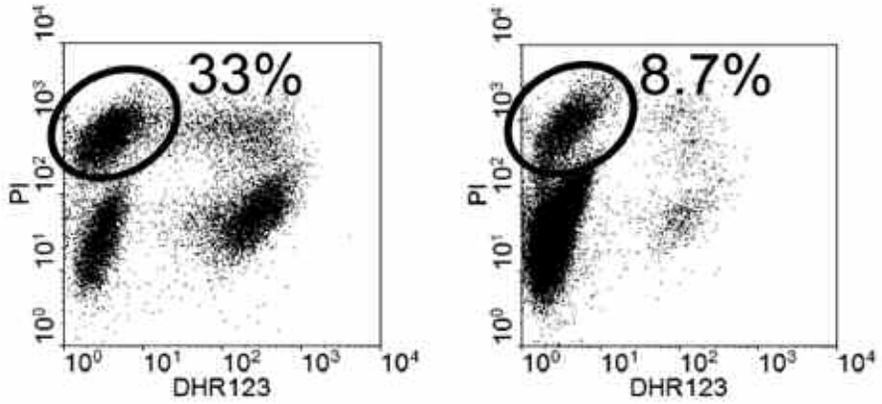
Experiments were performed that clearly correlated the ascorbic acid production with both a reduction of ROS generation and an improvement in the viability of the producing strain in respect to the non-producing one, when an oxidative stress was imposed (Fig. 5) [21].

This method can be used for further investigations either on strains with improved ascorbic acid production as well as strains growing under different stressing conditions [22].

These results are important in prospective of an industrial production process, wherein stress on the organism used as means for production, typically leads to lower or zero production of the product, productivity, yield of the product, or two or more thereof.

Conclusions

The ability to produce numerous compounds and chemicals using renewable resources and microorganisms will offer alternatives to the use of petroleum, with clear environmental benefits. The task at stakes is huge and requires a sincere collaboration between public and private research facilities, universities, and governments. Knowledge in



Wild type (GRF18U, left picture) and recombinant strains ([AtME, AtVTC2, AtMIP/VTC4, AtLGD, AtAGD, ScALO], right picture) grown in minimal glucose medium added with H_2O_2 3.0 mM were analyzed after DHR123 and PI staining (rodamine signal is reported in the abscissa and PI signal on the ordinate axes). The numbers in the circle represent the percentage of dead or severely damaged cells in the analysed population

Fig. 5 - Flow cytometric analysis of wild type and vitamin C producing yeasts under oxidative stress

genetics, biochemistry, molecular biology, fermentation technologies, organic chemistry and process engineering need to be integrated. Public education policies that will help the commercialization of industrial biotechnology products will have to be designed.

It is therefore evident the need of adequate funding to promote R&D and collaborative research of selected laboratories and policy initiatives to realize a sustainable biobased economy. Together with the benefit of reducing emissions of carbon dioxide and airborne pollutants, soil erosion, and protecting water supplies and quality, the implementation of Industrial Biotechnology processes will open up new technologies, industries, and export opportunities and it will stimulate growth, especially in rural, farm and forest economies.

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RIASSUNTO

Processi per un'economia sostenibile

Le biotecnologie industriali devono superare la sfida di sviluppare processi sostenibili e ambientalmente compatibili basati su risorse rinnovabili e che abbiano prodotti finali biodegradabili. A questo scopo sono richiesti approcci innovativi per risolvere i problemi ambientali ed energetici. In questo articolo vengono presentati esempi e applicazioni in questo campo.