

Executive summary:

ELECTROEXTRACTION has proposed a novel primary recovery strategy, which targets intracellular derived products, which were biosynthesized in several microbial hosts, namely yeasts, algae, and bacteria. Protein electroextraction was introduced in industrial practice as an easy, selective, and economic method for intracellular product release with minimal product damage. This novel processing-route was developed on the basis of: a) protein liberation by cell envelope (electro) permeabilization (i.e., "electroextraction") and b) direct product sorption (e.g. Utilizing a finite bath or a fluidized bed contactor).

This approach has resulted in an increased final product quality (e.g., due to mild treatment, selective isolation) and increased process yields (e.g., due to the limited number of downstream operations). The proposed strategy has worked well with yeast as expression hosts of natural or recombinant proteins and enzymes. Electroextraction was non-applicable to other microbial hosts like bacteria - due to microbial lyses, or algae - due to the existence of a very rigid cell wall. Therefore, this innovative primary recovery of (labile) proteins produced by industrial yeast expression systems has translated into increased bioprocess efficiency and reduced operational costs.

Project Context and Objectives:

A group of SMEs participating in the consortium was focusing in Yeast Biotechnology, i.e., the production of valuable biological products from yeast as a factory. In many cases yeast are able to synthesise bio products, which accumulate in the intracellular space. Gentle liberation of those (labile) products as enzymes and recombinant proteins poses technological difficulties. The project proposed a new - highly efficient - method for intracellular protein recovery from yeast, the electroextraction.

The SME participants covered a wide range of bio manufacturing like production of homologous enzymes or particular systems of interest including recombinant tagged proteins or the production of recombinant protein from *S. cerevisiae* and *P. pastoris*. Therefore the whole group was interested in developing a horizontal processing technology which allowed them to become more competitive in their respective fields. The creation of a technology platform for the production and further downstreaming of intracellular yeast products was of help. All SME participants recognized that a major obstacle to become competitive is the elevated costs associate with product downstream processing.

The activities of the Bulgarian biotechnological company ROMB were related to development of productive technologies for the recovery exo- and endo- microbial substances; this company was interested in introducing an innovative technology to lower the production costs for endogenous proteins.

The Spanish company BIOMEDAL as well as the German company 42LS were interested in a cost-effective microbial based protein expression and purification systems. BIOMEDAL already had a bacterial expression system in the market (CASCADE) and a tag for scalable single step protein purification (LYTAG). They were particularly interested in the development of such expression-purification systems in yeast and the assessment of the purification of LYTAGged proteins for further pilot scale protein purification.

Phytolutions, a German company, activities were utilizing yeast technology for expression of recombinant proteins with pharmaceutical applications. An efficient easy, scaleable and low-cost release and purification of these proteins was a prerequisite for commercialization.

The French company BETatech faced a different situation. This SME participated in the electronic system design and manufacturing business with applications in many fields already. Their goal was access to novel markets and more specifically to the biomedicine and

biotechnology sectors. BETAtch required a close collaboration with SMEs in the mentioned sectors, as well as, the expertise provided by the RTD performers to fulfil the planned objectives. BETAtch added to the consortium an electronic / electrical engineering component, which was synergistic to the expertise of the other SMEs within the consortium.

On the basis of the above considerations the project served the various participating SMEs in solving a particular processing need while concomitantly addressing a collective effort that is, establishing electroextraction as a general method for selective product released and further primary capture. This was of prime importance since the efficiency of the manufacturing processes remains a major limiting factor to improving health and wellbeing and to economic development.

ELECTROEXTRACTION brought an element of solution to this problem, and followed a trend by which SMEs have historically explored the less travelled roads of innovation until larger groups adopted them for the common good.

The collaboration of R&D actors from different European countries led to a successful implementation of 'Electroextraction'. The consortium involved an international research team, which developed the flow method for protein electroextraction (CNRS-Toulouse, FR and Sofia University, BG). A broad applicability of this method was assessed with industrial yeasts among the ones utilized for production of homologous or heterologous bio products. SME's had first-hand access to know-how and already developed prototype apparatuses to explore and optimize the electro permeabilization method with their own yeast system(s). The results enabled an overall picture on general technical efficiency of the electro induced protein liberation and to reveal all potential drawbacks connected with the implementation of this new method in practice. A close collaboration between R&D and SME parties was the basis for the identification of potential bottlenecks and to help in actual implementation in the industrial scenario.

Similarly, the industrial partners had the possibility to acquire expertise and knowledge provided by the Downstream Processing Laboratory at the JACOBS University Bremen gGmbH (DE). This was an additional asset since there is a lack of such specialists at the European level. The introduction of a new protein release method in practice required, in addition to the optimization of the relevant operational parameters, an efficient integration into the whole downstream processing train. Of prime importance were considerations regarding subsequent product isolation and further purification. The project proposed a bioprocess intensification strategy based on fast product release after cell envelope electropermeabilization and immediate product sequestration on a solid phase. Therefore, adsorbent-screening experiments must be conducted for each of the proposed products. Further lab scale testing followed after which the type of processing operation was defined, pilot scale evaluated, and eventually implemented in the future by end-users.

These new results will ongoing stimulate the European bio industry to become more competitive with the US-bio industry. This in turn has impact on the development of new bio products by access to the genome of various organisms with consequent faster benefit to European citizens. For example, European health could benefit by having access to medicines with higher efficiency, potency and safety. Not only the health sector but also the food and chemical industries can benefit from developments in horizontal technologies like bioprocessing. ELECTROEXTRACTION was dedicated to solve a Long-term technological challenge and to assist small and medium-sized enterprises (SME) participants in acquiring technological know-how and accessing an international network for their medium to long-term business development.

Within the project, the SME participants were the direct beneficiaries of the project results. They outsourced most of the research and development activities to RTD performers. As a consequence, SMEs received the technological know-how they need to develop a novel bioprocessing strategy and improved pieces of equipment. SMEs developed their activities by buying knowledge from the RTD participants who sold their expertise and work in the field of electroextraction and further down streaming of yeast products. Partial R&D activities were undertaken by the small and medium-sized enterprises (SME)s with their own resources to define initial specifications. small and medium-sized enterprises (SME) participated also in validating and testing of the acquired technology. The intellectual property rights and knowledge developed during the project is now property of the small and medium-sized enterprises (SME) participants. Moreover, the proposed research investment fitted into the overall business strategy of the SMEs involved. 'Electroextraction' was assisting companies in acquiring technological know-how and accessing international networks for their medium to long-term business development. The consortium had decided to follow the default regime, which gave full ownership of all project results ("foreground") and IPR to the small and medium-sized enterprises (SME)s.

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This approach has resulted in an increased final product quality (e.g., due to mild treatment, selective isolation) and increased process yields (e.g., due to the limited number of downstream operations). The proposed strategy has worked well with yeast as expression

hosts of natural or recombinant proteins and enzymes. Electroextraction was non-applicable to other microbial hosts like bacteria - due to microbial lyses, or algae - due to the existence of a very rigid cell wall. Therefore, this innovative primary recovery of (labile) proteins produced by industrial yeast expression systems has translated into increased bioprocess efficiency and reduced operational costs.

These achieved improvements and novel approaches to bioprocessing will help SMEs to better transfer a platform technology to potential end-users in the pharmaceutical and chemical sectors across Europe.

Project Results:

ELECTROEXTRACTION has proposed a novel primary recovery strategy, which targets intracellular derived products, which were biosynthesized in several microbial hosts, namely yeasts, algae, and bacteria. Protein electroextraction was introduced in industrial practice as an easy, selective, and economic method for intracellular product release with minimal product damage. This novel processing-route was developed on the basis of: a) protein liberation by cell envelope (electro) permeabilization (i.e., "electroextraction") and b) direct product sorption (e.g. utilizing a finite bath or a fluidized bed contactor).

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Jacobs University, Bremen, Germany

Jacobs University has effectively and successfully engaged in several tasks, which were needed in order to fulfil various technical objectives, as follows:

a)The development of fermentation routines so as to obtain sufficient amounts of several types of biomass to proceed with further down streaming of the targeted products. Cells producing specific intracellular products were utilized for (electro) liberation experiments or were processed according to standard industrial techniques (e.g. bead milling and high pressure homogenization) [WP#2] / [M# 3]. Fermentation strategies were coupled to media design in order to obtain optimized cultivation conditions for the selected yeast strains.

b)The production of enough amounts of intracellular products by utilization of the above mentioned cultivation strategies. Fermentation vessels up to 30l - 40l were employed [WP#2]. Appropriate biochemical and analytical tools were developed or adapted for the study of the systems involved [WP#3].

c)The project has optimized intracellular product (proteins or enzymes) liberation in the continuous mode, by designing and applying a continuous-flow (electro) pulsation technique with the aid of especially designed flow-chambers and power generators. Besides, the

optimization of product liberation by standard methods was required to define the project baseline and to provide a contingency plan for those microbial hosts not amenable for (electro) permeabilization. Therefore, French Press and bead Mill disruption was employed in every case [WP#3].

d) Direct contact methods were established to accelerate product liberation and to stabilize the released product by adsorption on a solid phase. Several adsorbents were tested via semi-quantitative screening. Those selected were further evaluated by isotherm and breakthrough experiments so as to design the best type of contactor required for product capture [WP#5]. The ultimate goal was to integrate the new technology into existing downstream processing platforms.

e) Two prototypical case-examples were worked out more intensively, namely the liberation and capture of a NATURAL enzyme and liberation and capture of a RECOMBINANT protein, both produced by yeasts [WP#5 / WP#6]. Dovetailing of protein liberation and capture has rendered an integrated approach to downstream processing of intracellular proteins from yeast.

Jacobs University has also engaged in the implementation of several dissemination actions, including internal training and human resources exchange [WP# 9]. This has allowed reaching suitable end-users, to promote the utilization of the novel technology, to train human resources in the new methods, and to positively impact on the performance of the European Biotechnology industry (with special emphasis on small and medium-sized enterprises (SME) actors). A positive social impact is therefore also foreseen.

JU has closely worked (has been subcontracted) by the following SME partners:

-BIOMEDAL SL / SPAIN in relation to executing the tasks described in WP # 2, WP #3, WP #5, and WP #6 of the Description of Work (Annex I - DoW), according to a contract celebrated by the mentioned parties.

-PHYTOLUTIONS GmbH / GERMANY in relation to executing the tasks described in WP # 2, WP #5, and WP #6 of the Description of Work (Annex I - DoW), according to a contract celebrated by the mentioned parties.

-42 LIFE SCIENCES / GERMANY in relation to executing the tasks described in WP # 2, WP #3, WP #5, and WP #6 of the Description of Work (Annex I - DoW), according to a contract celebrated by the mentioned parties.

TECHNICAL REPORT ON CASE EXAMPLES: OPTIMISED PROCESSING

Strains. *Kluyveromyces marxianus* var. *bulgaricus* NBIMCC 1984, wild strain was provided by ROMB Ltd, Sofia, Bulgaria. Biomedal, Spain provided *Saccharomyces cerevisiae*, a recombinant strain.

Shake-Flask Cultivation. *Kluyveromyces marxianus* strains were grown in 1% yeast extract, 2% peptone, 2% dextrose (YPD) in 1 litre baffled flasks. Liquid cultures were grown on orbital shaking (220 rpm) at 30°C (New Brunswick Scientific Co., New Brunswick, N.J.). These cultures were grown until the late exponential to stationary phase. Growth was monitored turbidmetrically at 600 nm (Eppendorf Biophotometer). Generated biomass was washed and frozen at -20°C for the further use for conventional lysis where as fresh biomass was utilized for the electroextraction of bio-products.

Shake-Flask Cultivation. *Saccharomyces cerevisiae* a recombinant strain, the inoculum and enzyme production media YNB medium (0.67% yeast nitrogen base and 2% glucose) supplemented with auxotrophic amino acids (50 mg.l⁻¹ L-histidine; 20 mg l⁻¹ L adenine; 70 mg l⁻¹ L-tryptophan, and 20 mg l⁻¹ uracil and sterilized by microfiltration was used to start *S. cerevisiae* PBIVU02 culture. The strain was grown for 16 h in 25-ml flasks containing 5 ml of YNB medium maintained in an orbital shaker (New Brunswick Scientific Co., New Brunswick, N.J (200 rpm) at 30°C. Afterwards, 5 ml of this preinoculum were transferred into 500-ml flasks containing 100 ml of YPD medium (1% yeast extract, 2% bactopectone, and 2% glucose). The inoculum was grown for 24 h in an orbital shaker (200 rpm) at 30°C and then transferred into the bioreactor containing the expression medium YPGal (1% yeast extract, 2% bactopectone, and 4% galactose).

Fermentation routine. Fed-batch fermentation of non-recombinant strain was performed in a 5-L fermenter (NBS BioFlowIII, NJ, USA) utilizing well described procedures in literature. Fermentation was carried out at 30 °C, at an agitation speed of 600 rev./min and aeration rate was set to at 10 l/min. A solution of 600 g/l glucose was added into the reactor using the feedback control system and pH was adjusted to 5.0 by the automatic addition of 25% ammonium hydroxide. Throughout the fermentation 2 ml culture samples were taken and spun at 20 000 × g for 1 min, after which the wet cell pellets were weighed. Fermentation batch was terminated when cultures are in late exponential phase or stationary. Cell wet weights of 200 g/l were reached at the end of fermentation. At the end of fermentation biomass was harvested by centrifuging at 20.000 × g for 15 minutes. The cell pellet used further for various disruption procedures and for experimental routines.

Fermentation routine. Recombinant strain fermentation was carried out in a 20 l bioreactor (HT-Infors, Bottmingen, Switzerland) with a working volume of 10 l at pH 5.5 and 30.0±0.1°C.

YPGal medium and the reaction vessel were sterilized at 121°C for 20 min. The fermenter was inoculated at 5% (v/v) ratio. Oxygen tension was measured as the percentage of dissolved oxygen in the culture medium in relation to air saturation using an oxygen electrode (Ingold, Giessen, Germany). The electrode was calibrated by pumping 100% oxygen from outside. We realized 100% dissolved oxygen at a flow rate of 5 L min⁻¹ (500 rpm), at 30°C before inoculation. During fermentation, air was supplied at a constant flow rate of 5 L min⁻¹. Mechanical agitation, produced by two Rushton turbines, was controlled automatically by setting the percentage of dissolved oxygen in the culture medium at 55%. Samples were collected under aseptic conditions to control fermentation performance and monitor medium composition, biomass, protein concentration, and enzymatic activity.

Total vs. Selective Product Liberation: Bead-Milling. The cells in late exponential phase was harvested by centrifugation at 3,200 g for 5 min at 4°C, the resulting pellet was washed with deionised water following centrifugation. After washing, cell pellet was re-suspended in lysis buffer [20mM phosphate buffer approximately pH 7.5, 1mM PMSF] to final concentration of 50% (w/v). This cell suspension was subjected to lyses. Cell disruption was performed by mixing the cell suspension with glass beads (0.75 mm in size) by overhead stirrer homogenizer. The ratio of the wet cell weight to glass beads was 1:4. The total suspension was mixed with the overhead stirrer for about 90 minutes.

Total vs. Selective Product Liberation: Enzymatic release. The cells in the exponential phase are harvested and utilized for the release of protein by enzymatic lysis. Washed yeast cells were resuspended in the lysis buffer 20mM phosphate buffer approximately pH 7.5, 1mM PMSF to final concentration of 2% (w/v). To this suspension 1 mg/ml of Beta-glucanase (Sunson Industry Group Co., Ltd., China) is added. The total suspension is incubated at 37°C, 250 rpm under orbital shaking. After 3 hrs total protein released was estimated by Bradford method.

Total vs. Selective Product Liberation: Electroextraction. The cells in the exponential phase was harvested and utilized for electroextraction. Washed cell were re-suspended in pulsation media to wet weight of 10 %. Electro pulsation was done in continuous mode at 2 ml/min. Optimized pulse duration, pulse number, frequency and field intensity were used depending on the strain. After electro pulsation the cells were incubated in post pulsing medium for around 6 h. The protein released was estimated by Bradford method.

Dovetailed process: Expanded bed adsorption. Expanded bed adsorption (EBA) is an integrated process for the capture of the recombinant protein. EBA combines clarification, concentration and partial purification into single unit operation thus reducing the process time and cost. Direct sequestration of realized product on the solid adsorbent could be achieved with EBA technology. The bioprocess intensification and integration are

implemented for the current capture of native enzymes / protein isolated after cell disintegration either by mechanical or enzymatic or by electro-permeabilization means. Disrupted cells are directly fed onto EBA column for the capture of the realized protein. For capturing Streamline DEAE beads are utilized.

The expansion factors, flow rate, bed height, protein concentration and wet weight of biomass in load are optimized. Product binding and elution were also optimized. As it already know that the performance of EBA is negatively affected with cell or cell debris interaction with adsorbent beads. Several biomass adsorbent inhibitors were screened in order not comprise the product binding capacity and hydrodynamic bed stability during anion exchange expanded bed adsorption. To clearly estimate the degree of interaction and aggregation of non-recombinant cell debris Residence time distribution (RTD) are performed. RTD can be performed as described before (Fernandez-Lahore 2002, J. Chromatography A). The degree of interaction and aggregation cells onto the solid phase is estimated utilizing the sophisticated tools developed by (Vennapusa et al 2008, J. Chromatography A). Expanded bed adsorption process design developed here in can be applied or extended later to the pilot scale process.

Product capture: Binding and Elution. Expanded bed sorption performance was performed utilizing Streamline materials in an UpFront EBA column (40 cm in height and 1 cm inner diameter). The mobile phase was a 20 mM TRIS buffer (pH 7.4; Conductivity 2 mS/cm). EBA was performed manually by connecting the column to the peristaltic pump. The desired flow rate was adjusted during process operation. Superficial velocity was 152 cm/h. Naked or PVP (biomass interaction inhibitors) -covered beads were utilized (Vennapusa et al 2010, J. Biosci Bioengg). Biomass concentration in the load was approximately 10.0 % on wet basis. The bound protein / enzymes were eluted with step gradient by increasing sodium chloride concentration from 0 to 500 mM in equilibration buffer depending on the product type. Total protein concentration and enzyme activity in the load, flow through and elute was monitored off-line with sophisticated analytical tools developed with in the project. The protein concentration, enzyme activity and specific activity were compared between the conventional lyses and electroextraction. The electroextraction yielded an enzyme with relatively high specific activity with a ≥ 85 % of yield.

Process analytics: SDS PAGE analysis. SDS polyacrylamide gel electrophoresis 1-D gel electrophoresis was performed in 12.5% v/v SDS vertical slab in a Hoefer SE260 gel electrophoresis unit in 0.75 mm thick 10 cm X 10.5 cm gels at 25 mA per gel. The gels were stained with Coomassie Blue. A Prestained SDS PAGE protein marker (Serva, 6.5 - 200 KDa) was included along with the chromatographic fractions obtained after expanded bed adsorption. The purity of the samples was quantitatively determined by SDS page. Depending on the product molecular weight different percentages of SDS gel were casted.

Additional work performed by Jacobs University has resulted in benefit for the SME parties other than the described in the preceding paragraphs:

1) Polysaccharides (PSS) were obtained from several algae cultures by (electro) pulsation-assisted or by chemical treatment. Such PSS showed antiviral (anti-phage) activity and could be utilized in the dairy industry as protecting agents during yogurt or cheese manufacturing. PHYTOLUTIONS is willing to fill a patent on these procedures.

2) Phycoerythrin (PE), which is one of the most commonly used fluorescent dyes for FACS analysis, was extracted by mechanical disruption from algae sources (*Porphyridium* spp. Cultures) and further purified utilising JACOBS proprietary methods. PHYTOLUTIONS is evaluating the convenience of manufacture and commercialize such high-added-value product that sells at USD 50 / mg.

3) Cloning and periplasmic expression of DNA Polymerase I (polA / JW3835) in *E. coli* so as to attempt a selective liberation. Electro pulsation resulted in bacterial lyses and therefore, mechanical disruption i.e. high-pressure homogenization was implemented instead for more efficient processing. The functional characteristics of the recombinant enzyme will be tested by 42 LIFE SCIENCES, which could result in cooperation beyond the scope of the present project.

Institute de Pharmacologie et de Biologie Structurale, Toulouse, France

IPBS made a design of a whole electroextraction system for continuous electric field treatment. They developed pulsing chambers for continuous flow operation and integration of the pulse generator.

The technical objectives were:

a) Biological [WP#3] and engineering [WP#4] aspects of the electroextraction process should be optimised. This requires the study of factors affecting protein liberation under electrical pulsation in aqueous media, as well as, the design and testing of continuous electropulsation cells to treat large amounts of feedstock [M#5, 6, 7, 9, and 10].

b) To reinforce the utilisation of technical and scientific methods and tools, dissemination action will include internal training and human resources exchange, according to WP# 9.

c) Training activities are considered under WP#10 CAPACITY BUILDING.

This report covers the deliveries 5, 9, 10, 12, 13, 15, 16 and 21 (a and b)

Introduction

The basic concept in Flow Electroextraction is to alter the wall and membranes of yeasts by delivering calibrated electric pulses on a colonized aqueous flow. This is protected by United States Patent Application 20040097715, Kind Code A1, Electropulsing process for producing proteins. Teissie, Justin et al. May 20, 2004. This is obtained by a proper design of the pulsing chamber and by an adjustment of the flow rate with the pulse frequency. There is a need to deliver a given number of pulses during the residency of the yeasts in the pulsed volume. The pulsing chamber is a resistive load, which value is dependent on its geometry and on the conductivity of the solution. The critical field is dependent on the geometry of the chamber.

Design of the pulsing chamber

The pulses must be monitored on line to control that a proper delivery is always present. The square waved pulse technology was chosen, where the field E (i.e. the voltage U delivered by the generator) was constant during the pulse duration T .

We evaluated the field distribution with different applicator geometries by a simulation using Comsol. The most homogeneous distribution was present with the plane parallel electrode configuration. The pulsed volume is limited to the part that is just between the applicators. The field E in case of plane parallel electrodes (at a gap distance d) is: $E = U/d$

The resistance with plane parallel electrodes is: $R = d/(\epsilon S)$

S being the section of the applicators (parallel to the flow), ϵ the conductivity of the solution

The electric charge that is delivered during one single pulse is UT/R and must be provided continuously by the generator. The limit is therefore the power supply integrated inside the generator. This was one of the key improvements of the system during the program. A 50 W was used rather than the 12 W initially present. Due to the associated Joule heating in the circuits, it was decided not to increase any further the power supply.

The current was limited to 6 amps for safety and reliability reasons (again the problem of the energy dissipation). In fact this can be enhanced but may affect the lifetime of the generator. This 6 amp limit was another limiting parameter in the design of the chamber. The maximum voltage that can be delivered was $U = RI$

The frequency of the pulses was controlled by the flow rate Q , the volume ($Vol = Sd$) of the chamber and the number of pulses N that need to be applied to obtain an effective extraction. There is a problem due to the fact that the flow is not uniform in the chamber

This means that the time of residency is not the average one (Vol/Q) everywhere in the pulsed volume

To get the proof of concept of an effective flow electroextraction, we decide to optimize the existing system present at the beginning of the project (called Betatech S20). It was clear that if this set-up was successful, it will be easy to increase the flow by an Array of chambers. This strategy will limit the power needed per chamber. To increase the power needed with a large number of such chambers, an array of pulse generators will be used with less power required per unit. The advantage to work under these conditions (low current) is to limit the Joule heating, that is not needed in the extraction process but will increase the cost of use. To keep Q high, we need to increase the achievable frequency F . This was attempted by increasing the power supply as mentioned above.

The conductivity of the buffer is another parameter controlling the electrical requirements of the set-up. Yeasts are in suspension in water. A careful and repetitive wash brings an initial low conductivity. But Electroporation means ion leakage from the cytoplasm, i.e. a higher conductance. When working with Pulsing chambers in series, we must select a conductivity associated to the total leakage of the cytoplasm for safety reasons. For 20% Vol/Vol , we get a final conductance of 1 mS/cm.

Electropulsator

A Betatech S20 Electropulsator was used. The voltage can be as high as 2000V, pulse duration can be selected between 5 and 50 ms and the adjustable pulse frequency was up to 1 kHz. It can be used in a continuous delivery mode to work on a flow of cells. The pulse rise-time was about 0.5 μ s without any load and the decay under the control of the load.

A major concern in continuous pulse delivery is the occurrence of electrochemical reactions on the surface of the applicators. Previous studies in our group did suggest that alternating polarities of the successive pulses should prevent the associated corrosion. A pulse inverter was added but working at a 2 Hz frequency. Symmetrical pulses were triggered.

We observed the dependence of the duty cycle (delay between pulses) as a function of the applied voltage (field) and pulse duration.

These results were obtained with the low power supply design. As the delay was rather high (less than 10 pulses per second with $E = 3\text{ kV/cm}$, $T = 2\text{ ms}$), it was decided to increase the

power of the electropulsator up to 50 W to reach a high frequency needed to get a high flow rate. This duty cycle was under the control of the buffer conductivity.

It was then decided to increase the power of the generator by coupling 2 S20 (with the 50 W power supplies) in a master slave configuration

The sequence was the following: 1 Pulse delivery by the master 2 Polarity inversion 3 Pulse delivery by the slave (on command from the master after a delay of 15 ms) 4 Polarity inversion. The repetition could be obtained every 32 ms if the pulse duration was 1 ms.

Flow pulsing chambers

A very flexible design of the pulsing chamber was obtained inserting a succession of applicators and spacers in a cylindrical holder. One economical advantage was that the production can be obtained at a low cost. Taking into account the field that is needed for protein extraction from Yeasts (3 kV/cm) and the specifications of the S20 (2000 V max), the gap between the electrodes was designed to be 0.6 cm. The length of each pulsing applicator was 1.5 cm and the width 0.6 cm. This means that the cross section for the flow of the yeast suspension was 0.36 cm².

Flow processing

After preliminary trials with dummy loads to validate the system on the long term use, suspensions of double washed yeast in pure water were pulsed. Previous experiments predicted that pulse duration of 2 ms was needed. We observed that a repetitive period of 65 ms can be used while keeping stable the delivery of the electric pulses on the long term (hours). It was checked that under these conditions the use of a bipolar sequence was preventing the formation of rust from the stainless steel electrodes while this was the case in the unipolar configuration

Electroextraction of pilot scale volumes of yeasts (*Saccharomyces Cerevisiae*):

Experimental protocol

The process protocol starts with an overnight culture (37°C, agitation) of 30mL, then 270mL of YPD broth are added the next morning, and the culture is incubated, under agitation, at 37°C, until yeasts reach a late log growth phase (turbidimetry measurement every 30minutes). At the end of the culture, yeasts are washed two times with water and resuspended in water at 15.108 cells/mL. We obtained more or less 100mL of yeast suspension in water that way (i.e.a 3 times reduction in volume to work on a concentrated

sample). Afterwards, the suspension is pulsed by the system described above which allows us to deliver bipolar pulsations. The pulse suspension is then diluted in a buffer with a higher ionic content containing Glycerol (0.105 Phosphate, 0.3 M glycerol, 1 mM DTT). The system is then incubated overnight at room temperature and the leaked protein content is assayed.

Flow electroextraction

The best parameters to obtain the highest protein leak (evaluated by a Bradford assay) were: 3kV/cm; 30 or 25 unipolar pulses/cell; pulse duration=2ms; Period=36 or 72ms respectively; Flow rate=1.4mL/s, 15.108 cells/mL. Thus, by comparison to a classical chemical lysis we obtained 92.6% of total protein leak with electroextraction (R=30). Then, a bipolar pulsation flow method was assayed. This prevented the generation of electrochemical species by the electrodes and increased the safety of the pilot. By delivering to each cell 12.5 or 15 bipolar pulses (25 or 30 pulses delivered per cell during their residency in the pulsing chamber) of 2ms duration at a period of 79 or 65 ms respectively (field strength =3kV/cm), Flow rate=1.4mL/s, 15.108 cells/mL, 100% of the total amount of cytoplasmic proteins was recovered by comparison with chemical lysis.

Large volume process

Those parameters were checked on large volumes as needed on a pilot. A production of 2L gives 500mL of yeast suspension in water at 15.108 cell/mL. It was treated under N=30 bipolar pulses/cell of 2ms duration under a field strength of 3kV/cm, with a period of 65ms. The 500mL passed through the pulsing chamber in 6 minutes in a continuous treatment. This confirms that this flow process method of electroextraction is efficient on large volumes at a pilot scale

Load in yeasts

We then tested the flow process method with a higher concentration of yeasts. The culture of yeasts was resuspended at 30.108 cell/mL in water. The 3 KV/cm, 2 ms pulsing conditions were delivered on 1.4 ml/s flow. We were expecting a better recovery in protein (twice what was got under the low load, i.e. 10 mg of protein in 100 ml of suspension). This failure may be due to the effect of a high concentration on the field distribution. The mean distance between each cell is only 7 m. Such a proximity is known from previous studies of our group on mammalian cells to affect the field distribution (in silico evaluation) and electroporation (experimental assay). The conclusion is that the working protocol should mention 15 108 yeast per ml as the upper limit.

Protein size distribution

It was checked that there is no degradation of total protein overnight after electroextraction. In order to do that, a SDS-PAGE gel was run using supernatants obtained 2h and 18h after extraction, as well as negative unpulsed controls of yeasts in water. The silver staining method was used to reveal this gel because it is the most sensitive one.

The same bands are present between the negative control and the samples, with a bigger intensity for the samples. They are reflecting the excretion system. Several new bands were specifically present in the pulsed samples. Their intensity was larger at 18h in agreement with previous studies telling that the electroextraction was a slow process. But the pattern at 18h was the same as at 2h confirming the lack of degradation during the electroextraction mediated leak.

Growth phase

Tests were realized in stationary growth phase, to see if, with the same conditions, it is as efficient as in exponential growth phase. The positive control was a mechanical lysis using glass beads ($d = 450\text{--}600\mu\text{m}$), because the chemical lysis is inefficient along the stationary growth phase. We obtain the better results with $N=35$ bipolar impulsions/cell, but only 77.3% of protein leak of the total amount of cytoplasmic proteins was recovered by comparison with mechanical lysis. So we can conclude that the flow process method of electroextraction is more efficient in exponential growth phase than in stationary growth phase.

Conclusion

The pilot is able to extract proteins at a flow rate of 1.4 ml/sec at 15×10^8 yeasts per ml with no time limit. Therefore:

- a) The system is amenable for continuous operation thus permitting the treatment of volumes in the range of mL to dozen L per hour; the demo unit is treating about 4 l/hour. An array of 10 units will give the treatment of a culture of 400 L in about 3 hours (due to the concentration associated to the washing step)
- b) A single passage through the pulsing chamber is enough for inducing cell envelope alteration necessary for cytoplasmic protein release;
- c) No other chemical must be added and thus potential product contaminants are not present;
- d) Electric parameters limit the heating during pulsation to avoid the use of cooling system;
- e) The method showed a fractionation capability and preserves the biological activity of the released intracellular products. This is shown by the PAGE results.

Sofia University, Sofia, Bulgaria

During the project Electroextraction Sofia University participated in the realisation of work packages 2, 3, 6, 8 and 9. According to the tasks foreseen in these work packages, the team of Sofia University has developed and has delivered to the partners in the project protocols for laboratory scale in flow electroextraction of total protein, beta-galactosidase and superoxide dismutase from non-recombinant yeast and protocol for in flow electroextraction of *E. coli* beta-galactosidase from two recombinant strains *S. cerevisiae* (D5). All expected results (M5, M6, M7, and M8) were achieved. Furthermore, the team of Sofia University developed protocols for in flow electroextraction of proteins, and enzymes from non-recombinant yeast strain up to debits of 5 - 6 l/h, with specific activity of electroextracted enzymes 2-9 times higher than the specific activity of mechanically obtained lysate (M9).

Additionally, on demand of Phytolutions - a partner entering later in the project, a research was performed on the influence of electric field on isolation of chlorophyll and carotenoids from *Nannochloropsis*, and polysaccharides from *Porphyridium*. A protocol for chemical extraction of phycoerythrin from *Porphyridium* without cell disintegration was developed. Different dissemination activities according to WP 9 have been organized - training in the electroextraction technique of scientists and students from ROMB, Phytolutions, Jacobs University and Sofia University as well as Workshop at SU with participation of scientists and representatives of industry (D21b). Articles on electroinduced extraction of enzymes and proteins from yeast, as well as electroinduced extraction of pigments and polysaccharides from algae are in state of preparation. An economic analysis of the developed procedures was realized together with ROMB (D19-20).

Main achievements

The main task of the team of Sofia University in this project was:

1. Development of protocol for in flow electroextraction of total protein and enzymes from non-recombinant and recombinant yeast strains representing interest for the Bulgarian company ROMB and the Spanish company BIOMEDAL.
2. Evaluation the possibility for enhanced extraction of pigments and polysaccharides from microalgae representing interest for the German company PHYTOLUTIONS.

The experiments have been carried out with the following yeast strains:

- Saccharomyces cerevisiae* (baker's yeast) - commercialized product
- Kluyveromyces lactis* var. *lactis* (NBIMCC - 2209) industrial strain for production of proteinase and beta-galactosidase
- Kluyveromyces lactis* var. *lactis* (NBIMCC - 199) industrial strain for production of beta-galactosidase and protein - application for nutritive purposes
- Kluyveromyces marxianus* var. *bulgaricus* (NBIMCC - 1984) industrial strain for production of thermo-stable superoxidedismutase- patented by ROMB
- Saccharomyces cerevisiae* W303-1A -LYTAG -beta-Galactosidase, producing E.coli beta-galactosidase - (Biomedal)
- and algae *Nannochloropsis gaditana* and *Porphyridium purpureum* (Phytolution)

Electropulsation was performed in batch and in flow mode with the following generators:

A.In batch pulsation was performed with Generator Cytopulse PA 4000 (USA), giving rectangular pulses with maximal output voltage 1100V. Pulsing chambers with standard Al electrodes with 1, 2 or 4 mm gap were utilized.

B.In flow electropulsation. Two generators have been utilized:

- Generator GHT (Betatech - France) with maximal output voltage 2000 V, maximal debit - 4, 8 ml/min.
- Generator Hydropulse mini (GBS-Elektronik, Germany), 2500 V output voltage, maximal debit - 90 ml/min.

The chambers for in flow treatment, with stainless steel electrodes - 3 and 4 mm apart were produced by Bulgarian company Mehel. Flow control was performed with peristaltic pump.

Electroextraction of total protein from non-recombinant yeast.

Bulgarian company ROMB is interested in obtaining total protein from yeast which can be cultivated on whey. Whey is a waste product from the dairy industry. It represents a cheap fermentable source for cultivation of yeast such as *K. fragilis* and *K. lactis* utilized for production of total protein and different enzymes.

According to our previous data (Anal. Biochem. 315 (1), 77, 2003), the maximal yield of total protein obtained after in flow pulsation was about 50-60 % after 6 hours postpulse incubation. The main purposes of the present study were to increase the quantity of protein liberated and to shorten the time of incubation after pulsation.

For realization of this goal we performed experiments with different yeasts and scanned high range of electric field parameters: field intensity, number, duration and frequency of pulses. The influence of composition and pH of medium, and the presence of reducing agent, increasing the cell wall porosity were also investigated.

Experiments were started by in batch pulsation of baker's yeast, where we established firstly that at suitable combination of electrical parameters (field intensity 3.5 - 5 kV/cm, 10 pulses, and 1 ms duration) up to 100% liberation of total protein is possible. A dependence of protein release on pH of medium was established. Maximal yield was registered 6 hours after pulsation. The same efficiency was obtained by in flow pulsation with slight change of the parameters as follows:

A. Baker's yeast - optimal conditions: 3.5-4.5 kV/cm, 10 pulses, 1 ms duration, 2.4 - 4.8 ml/min, 2 Hz. After pulsation of cells (10% wet weight suspension) and incubation in 250 mM potassium phosphate buffer (PPB) pH 7 for 6 hours, at room temperature about 100% release was obtained

B. *K.lactis* 2209 - optimal conditions for 90-100% total protein liberation: field intensity 3.5 kV/cm, pulse duration 1.25-1.35 ms, 2.4 - 4.8 ml/min, 10 pulses, 2 Hz, cell concentration - 10 % wet weight. After pulsation cells were diluted in 250 mM PB, pH 7 and incubated for 6 h at room temperature.

C. *K. lactis* 199 - with this strain a large scale of debits were tested: 2.4 - 27 ml/min.

Best experimental condition was: 10 pulses, 15 Hz, 1.5-1.75 ms, postpulse incubation - 250 mM PPB. The addition of reducing agent - dithiotreitol (DTT), which increases the cell wall

porosity, accelerates the protein liberation, and shortens the time of incubation with 2-3 hours. The efficiency is preserved up to cell concentration 20% wet weight.

Conclusions

A suitable electrical conditions for high efficiency (90-100%) total protein extraction by in flow process can be found for different yeast strains. The efficiency can be preserved at least up to debits of 27 ml/min. The time needed for maximal protein recovery can be shortened to 2-3h in presence of low concentrations of reducing agent.

Electroinduced extraction of enzymes from non-recombinant yeast.

A. Extraction of beta-galactosidase from K.lactis 199

Beta-galactosidase produced from K.lactis and K.marxianus is an industrial enzyme with wide applications, including food industry and medicine.

The protocol optimization was based on testing different electrical parameters at flow rates from 4.8 to 27 ml/min, and cell concentration varying from 7.5 to 25% wet weight. For increasing the liberation of proteins we studied also the influence of the presence of reducing agent, the composition and pH of the medium. These experiments were performed with generator Hydropulse mini. Different combinations of electrical parameters, giving maximal yield for only 3 hours of incubation were established.

Different approaches have been tested for increasing the specific activity in the extract. As a result, we established a very simple way to augment the specific activity and factors of purification from 2 to 4.6 were registered. Briefly, after pulsation the cells were incubated for 1 h in PPB with pH 8, followed by centrifugation. The supernatant was removed and cells are diluted in buffer, optimal for beta-Gal release. The reducing agent which increases the cell wall porosity was added at different concentrations. The efficiency of beta-Gal extraction is preserved for cell concentration up to 20% wet weigh. When increasing cell density, the optimal results were registered at lower intensities. Over 20% wet weight the efficiency of enzyme liberation decrease.

Conclusions

In flow electropulsation is a suitable method for fast, high efficiency extraction of beta-galactosidase from K. Lactis. In dependence on the conditions of treatment, the specific

activity (SA) ranges from 0,94 U/mg (90% recovery of total enzyme activity) to 2,7 U/mg (corresponding to 55% recovery). The specific activity of cell lysate (mechanical disintegration) is 0,58 U/mg, i.e. a factor of purification in order of 1,62 to 4,6 can be achieved without additional procedure of purification. Commercial products with specific activity in the range of 1000 - 5000 units/gram exist on the market. That means the enzyme produced by electropulsation correspond to the quality of some commercial products, without need of additional purification.

B. ELECTROEXTRACTION of thermostable superoxide dismutase from *K. marxianus* var.bulgaricus

Superoxide dismutase is applied in medicine and cosmetics for removing the generated superoxide anion radical. All these applications suppose a high stability and preservation of its activity, including high temperature. The potential role of yeast as producer of SOD is well recognized. The production of thermostable SOD represents an interest for the firm ROMB. All experiments have been carried out in flow mode with debits up to 90 ml/min. No reducing agent was used. Optimal field intensity was in the range of 4-4.5 kV/cm, in dependence of pulse duration. Maximal liberation (85% from total) was obtained 4-5h after pulsation. After 2 hours of postpulse incubation the recovery of enzyme was about 67%, and the specific activity - 124 U/mg. The increase of debit up to 90 ml/min did not reduce the efficiency of extraction. For obtaining the same yield, an increase of the pulse number from 10 to 13 was sufficient.

Conclusions

The enzyme recovery obtained by ELECTROEXTRACTION, 2 h after pulsation, corresponds to the recovery obtained after thermal treatment and dialysis in the published procedure (J. Chromatography B, 877, 3529-36, 2009), the specific activity obtained by ELECTROEXTRACTION being higher. According to the procedure for large scale production of SOD, there are 2 steps of mechanical disintegration, 1 step of separation and 2 steps of membrane filtration for obtaining about 67 % recovery with specific activity about 100U/mg. An eventual application of ELECTROEXTRACTION instead mechanical disintegration will make unnecessary the mentioned steps, leading to a reduction of the costs of extraction/purification with 25-30%.

Electroinduced extraction of *E coli* beta-galactosidase from *Saccharomyces cerevisiae* W303-1A.

The Spanish firm BIOMEDAL provided for this study two strains *Saccharomyces cerevisiae* W303-1A (pMAB10-LacZ-5), and *Saccharomyces cerevisiae* W303-1A [pBIVU02-1] expressing LYTAG - beta galactosidase fusion protein from *E coli*.

Maximal recovery by in flow treatment (about 45-50% from total) was obtained after 20h postpulse incubation. The most probable reason for the need of this long postpulse incubation is the large molecular mass of the enzyme, which in its native form is about 465 kDa. In order to increase the specific activity in the extract, we applied the same approach as with *K. lactis* beta-galactosidase. Immediately after pulsation, the cells were diluted in PPB pH 8. After 2 h of incubation this buffer was substituted with PPB pH 7, and the cells were incubated further for 18h. This approach enabled us to eliminate during the first incubation about 60% from total protein and to increase respectively the specific activity in the extract.

Conclusions

This is the first study on electroinduced liberation of recombinant proteins from yeast; furthermore these are the first data on liberation of so large protein (465 kDa). Despite the extremely stable cell wall of the recombinant strains the ELECTROEXTRACTION provokes about 50-60% liberation of total protein 2h after pulsation, and about 70-80% by longer incubation - results very close to those obtained with non-recombinant strains. It seems highly probable that the recovery of smaller recombinant proteins can be performed with an efficiency, similar to that obtained with SOD (32 kDa) and beta-galactosidase from *K. lactis* (250 kDa).

Extraction of pigments and polysaccharides from Algae

The German company Phytolutions was interested in conducting experiments on electric field effect on pigments, lipids and polysaccharide extractions from microalgae *Nannochloropsis* and *Porphyridium*.

A. *Nannochloropsis*.

Nannochloropsis gaditana stands out as an important source of pigments of great commercial value - chlorophyll a, beta-carotene, violaxanthine. The isolation of chlorophyll and carotenoids is performed by using different solvents - acetone, methanol, dimethylformamide, and ethanol. Efficient extraction with ethanol needs long incubation times - about 24 h at 40°C and several consecutive extractions.

Taking into account that the chlorophyll and carotenoids are utilized in food industry and cosmetics we chose in our experiments the ethanol as solvent because it is not toxic.

Cells were treated with intensities from 6 - 7.33 kV/cm, 10 pulses with duration from 1.25 to 2 ms, 8 Hz, debits up to 14,4 ml/min.

Control and pulsed cells were diluted in Ethanol (100%) and incubated at 4°C for different periods (1h - 24h). The electrical conditions with most pronounced effect on pigment extractions are 7 kV/cm, 11 pulses with duration 1.5 ms, 9Hz. At this condition there is a decrease of the volume of electroporated cells - about 30-35% in comparison with the control native cells, which can be a result of loss of cytoplasmic content (water).

Conclusions

There are no published data up to now on electric field effect alone or electric field combined with solvent for Nannochloropsis. We established that at definite electrical conditions an increased liberation of chlorophyll a during the 1st extraction (ratio 1:4), and of carotenoids (ratio 1:2.7) take place.

Electroinduced liberation of polysaccharides from Porphyridium

The unicellular red microalga *Porphyridium cruentum* can synthesize and secrete sulphated polysaccharides. Their use has been suggested in cosmetics, health food, as well as in medicine as antiviral, antiradiation and antioxidant medication. The present study aimed to check the possibility for electroinduced enhancement of polysaccharide release. Cells were treated in flow with debits up to 27 ml/min, field intensities between 5 and 6.5 kV/cm, pulse duration 1.5-1.75 ms. We established that the treatment of suspensions over 40 mg/ml leads to an attachment of the cells to the positive electrode and at the high voltages provoke short circuit. This is probably due to the high negative charge of cell surface because of the number of sulphate groups. At definite combinations of electric parameters liberation of polysaccharides in the medium was obtained. The electron micrographs demonstrate a clear difference in the surface of control and pulsed cells

Conclusions

The rectangular electric field pulses applied in flow mode can provoke an additional release of polysaccharides, and considerable changes in the cell surface of *Porphyridium*

Potential Impact:

ELECTROEXTRACTION brings an element of solution to this problem, and follows a trend by which SMEs have historically explored the less travelled roads of innovation until larger groups adopt them for the common good. Protein-based and antibodies showed sales of \pm USD 40 B in 2003, with as of 2004 close to 100 products been approved by at least one national regulatory agency. Biopharmaceuticals have been the fastest developing product-line in modern health industry and in 2004, over 200 are in clinical trials and at least 200 are known to be at pre-clinical stages. Therefore, these (bio) products are climbing fast toward as much as 35% of the world pharmaceutical market in the coming 10 years -- this would mean sales of more than USD 200 B. The youth of biopharmaceuticals (first recombinant drug on the market: insulin, 1982) explain why its manufacturing processes are still so inefficient. Companies certainly saw and continue to bring significant productivity gains during fermentation or cell cultivation. But it is the downstream processing part of bio product manufacturing cycle the one recognized as a major bottleneck. This pushes-up the cost and price of drugs, slows down their development and limits their accessibility to the community of patients. Besides recombinant polypeptides as bio therapeutics pharmaceuticals can be targeted in an indirect way by this project e.g., the production of protein leads for drug discovery and the manufacture of biotransformation agents to produce active drugs. The relevance of the project is now crystal clear.

Modern biotechnology heavily depends on the availability of efficient processes, which should be able to generate competitive products or services in terms of quality and cost. A critical assessment of current bioprocess technology will reveal that fermentation procedures are already in a phase of technological maturity. However, product recovery and purification (also referred as downstream processing) still poses a number of important challenges. For example, downstream operations cost usually represents 50-80% of the total processing cost. Thus, it comes as no surprise that optimization of the later steps is considered as a central element in appropriate process design. As bio-product mass production tends to be hindered by high costs of goods and process materials the development of innovative, cost-effective downstream processing systems is mandatory.

The ELECTROEXTRACTION consortium has focusing in the production of several bio-products either natural or recombinant all of which have market potential. These valuable biological products -which accumulate in the intracellular space-, were produced from yeast, bacteria, and algae. Gentle liberation of those (labile) products as enzymes and recombinant proteins poses technological difficulties that were adequately tackled by the project. Therefore, the group has developed a horizontal technology allowing them to be more competitive in their respective markets. Adoption of such technology will also help in establishing a platform for the production and further down streaming, particularly of intracellular yeast products. All

the Biotech SME participants have recognized that a major obstacle to become competitive is the elevated costs associate with product downstream processing.

More specifically, a positive impact of the project can be observed at the level of the individual SME partners:

a) The activities of the Bulgarian biotechnological company ROMB are related to development of productive technologies for the recovery exo- and endo- microbial substances; this company is interested in introducing an innovative technology to lower the production costs for endogenous proteins. ROMB demonstrated a suitable processing route based on natural enzyme(s) extraction from industrial yeast strains by electroextraction and negative chromatography.

b) The Spanish company BIOMEDAL was interested in a cost-effective microbial-based protein expression and purification systems. BIOMEDAL employed a proprietary bacterial expression system (CASCADE) and a tag for scalable single-step protein purification (LYTAG). They were particularly interested in the development of such expression-purification systems in yeast and the assessment of the purification of LYTAGged proteins for further pilot scale protein purification.

c) The German Company 42 LIFE SCIENCES obtained a highly pure enzyme preparation that could allow them to reduce costs of manufacturing of their diagnostic line of products.

d) The German firma PHYTOLUTIONS is utilizing algae technology for expression of recombinant proteins with applications in the food, cosmetics, and pharmaceutical sectors. An efficient easy, scaleable and low-cost release and purification of these proteins is a prerequisite for commercialization, a result obtained by the company during the project development.

e) Improvements and novel approaches to bioprocessing were achieved thus, helping SMEs to better transfer a platform technology to potential end-users in the pharmaceutical and chemical sectors across Europe. BETAtch has accessed novel markets and more specifically the biomedicine and biotechnology sectors. BETAtch has required a close collaboration with Biotech SME partners in the mentioned sectors, as well as, the expertise provided by the RTD performers to fulfill the planned objectives. BETAtch has built up an (electro) pulsation system, which is ready for commercialization.

On the basis of the above considerations this project serves the various participating SMEs in solving a particular processing need while concomitantly addressing a collective effort that is, establishing electroextraction as a general method for selective product released and further primary capture. This is of prime importance since the efficiency of the manufacturing processes remains a major limiting factor to improving health and well-being and to economic development.

The successful implementation of " ELECTROEXTRACTION" has required collaboration of Research and Development (R&D) actors from different European countries. The proposed consortium involves the international research team, which developed the flow method for protein electroextraction (CNRS-Toulouse, FR and Sofia University, BG). A broad applicability of this method was assessed with industrial yeasts among the ones utilized for production of homologous or heterologous bio products. This means the SME's have had first-hand access to know-how and already developed prototype apparatuses to explore and optimize the electroporabilization method with their own yeast system(s). The expected results has enabled to obtain an overall picture on general technical efficiency of the electro induced protein liberation and to reveal all potential drawbacks connected with the implementation of this new method in practice. A close collaboration between R&D and SME parties proved to be an adequate basis for the identification of potential bottlenecks and to help in actual implementation in the industrial scenario.

Similarly, the industrial partners have had the possibility to acquire expertise and knowledge provided by the Downstream Processing Laboratory at the JACOBS University Bremen gGmbH (DE). This was an additional asset since there is a lack of such specialists at the European level. The introduction of a new protein release method in practice has required, in addition to the optimization of the relevant operational parameters, an efficient integration into the whole downstream processing train. Of prime importance were considerations regarding subsequent product isolation and further purification. In this project we proposed a bioprocess intensification strategy based on fast product release after cell envelope electroporabilization and immediate product sequestration on a solid phase.

ELECTROEXTRACTION has conformed to the objectives of ""Research for SMEs"" since has supported a small groups of innovative SMEs in solving technological problems and acquiring technological know-how. The project perfectly fitted into the overall business and innovation needs of the mentioned SMEs, which were given the opportunity to subcontract research to RTD performers in order to acquire the necessary technological knowledge. The project also rendered clear exploitation potential and economic benefits for the SMEs involved.

Within the project, the SME participants were the direct beneficiaries of the project results. They had outsourced most of the research and development activities to RTD performers. As a consequence, SMEs have received the technological know-how they need to develop a novel bioprocess strategy and improved pieces of equipment. SMEs bought knowledge from the RTD participants who sold their expertise and work in the field of electroextraction and further down streaming of yeast products. The consortium has decided to follow the default regime, which gives full ownership of all project results ("foreground") and IPR to the SMEs.

Partial R&D activities were also undertaken by the SMEs with their own resources to define initial specifications. SMEs had participated in validation and testing of the acquired technology. The intellectual property rights and knowledge developed during the project has been transferred to the SME participants. ELECTROEXTRACTION has assisted companies in acquiring technological know-how and accessing international networks for their medium to long-term business development.

Impact specific to the SME actors

It is currently recognized that there is a lack of progress in the area of bioprocessing at the European level. This situation is further worsened by the fact that (bio) manufacturing capacity is also becoming limited due to the booming of new biotech products expected to enter the market in the near future. A clear example is provided in the field of monoclonal antibody production. As bio product mass production tends to be hindered by high costs of goods and process materials the development of innovative, cost-effective downstream processing systems is mandatory. These new developments will also stimulate the European bio industry to become more competitive with the US-bio industry. This in turn will impact positively on the development of new bio products by access to the genome of various organisms with consequent faster benefit to European citizens. For example, European health will benefit by having access to medicines with higher efficiency, potency and safety. Not only the health sector but also the food and chemical industries can benefit from developments in horizontal technologies like bioprocessing. This project will reinforce European competition in the priority field of biotechnology.

ELECTROEXTRACTION has had a beneficial impact at the level of the individual participating SMEs. ELECTROEXTRACTION was dedicated to solve a LONG-term technological challenge and assist SME participants in acquiring technological know-how and accessing an international network for their medium to long-term business development. Some of these benefits can be summarized as follows:

a)PHYTOLUTIONS. Due to the known problems of recovery of intracellular located proteins from algae, an improved method leading to high quality products with lower production costs has greatly improved the competitiveness of the company. Especially in the growing market of the so-called white biotechnology -- namely the production of biologic products for industrial use in contrast to therapeutic proteins for medical use -- requires cost effective production methods. Here, environmental friendly production processes on a biological basis have to compete with traditional, mostly chemical based processes. Processes enabling the production of quality products for lower unit costs will develop areas in which biotechnology will be competitive.

b)BIOMEDAL. Biomedal develops innovative technologies and services to optimize recombinant protein expression and purification in microorganisms. Biomedal technology platform comprises six patent and patent applications and cooperation with different R&D projects in the field of development of innovative research and production genetic tools. This company has clearly benefited from the project since new competencies were introduced, particularly those related to the future implementation of Biomedal genetic systems to the bioprocess field.

c)ROMB is a Bulgarian company, which main activities are focused on obtaining biotechnologically natural active substances (BAS) of microbial origin. Since this category of products has limited added value potential, improved processing technologies are able to promote a major impact in the economy of the company. This has implications for the local economy and favors the creation of job posts in a developing economic system. These technical advances also ensure the production of BAS to meets the European requirements for quality and safety. ROMB has business partners from Germany, France, USA, Italy, etc., but it wants to enlarge its infrastructure with new sections and expertise. The new technology has allowed ROMB to produce more economically the enzymes for which they have already market as well as eventually to increase the quantity and the range of the endogenous yeast products they can offer to the market.

d)BETATECH. BETatech is specializing in electronic industrial systems but has had no previous markets in the Biotech sector. Once established the parameters of a generators needed for recovery of recombinant native proteins on small, middle or large scale (in dependence of the need and capacity of the SMEs) these generators can be placed on the market. The chambers with the most suitable parameters can be also commercialized. So the proof-of-concept expected under this program will resulted in an immediate marketable new technology tool. As result new or more affordable products have become available for application fields such as red biotechnology (e.g. pharmaceutical products) and white biotechnology (industrial enzymes, environmental processes, renewable biomaterials).

e)42LS has participated in the project to gain intellectual property in the development of recombinant proteins from yeast and bacterial cells. They will profit by the new and cheaper products derived by control of the related chemical processes. The new technology will rationalize their existing conventional enzyme production enabling higher quality of their products under ecologic and environment friendly conditions. In this way they hope to be enabled to compete at the international markets.

It can be concluded that ELECTROEXTRACTION has benefited the participating SME by helping them to acquire new technical tools. This was assured on the basis of a strong internal cooperation within the consortium, which has favoured knowledge exchange between SMEs and R&D partners. All activities within the consortium were open to all partners letting them to have a global overview of project development and needs. At the end of the project, participating SME have acquired developed protocols/ready to use for extraction /sorption at laboratory and a system able to operate at pilot scale. Most importantly, they are in position to further apply and optimize the new technology for systems not tested during the project.

All the previous considerations have stressed the importance and potential impact of ELECTROEXTRACTION at the consortium level and in relation to European and worldwide markets. Aiming to introduce a new method for intracellular protein recovery, as well as new technology for more facile and economical bio product processing from yeast, this project address directly Community objectives under the Framework Programme 7 (FP7), as follows:

- a) Food, Agriculture and Fisheries, and Biotechnology. Therefore the project contributed to the EU objective of building a European Knowledge Based Bio- Economy (KBBE). More specifically ELECTROEXTRACTION meets the priority under the topic "life sciences, biotechnology and biochemistry for sustainable non- food products and processes".
- b) Nano sciences, Nanotechnologies, Materials and new Production Technologies. This theme aims at improving the competitiveness of European industry and generates the knowledge needed to transform it from a resource- intensive to a knowledge-intensive industry. ELECTROEXTRACTION met this priority under "Integration of technologies for industrial applications - focusing on new technologies, materials and applications to address the needs identified by the different European Technology Platforms".
- c) ELECTROEXTRACTION was in frame within the Capacities Program as designed to help strengthen and optimize the knowledge capacities that Europe needs if it is to become a thriving knowledge-based economy. The project aimed to strengthen the competitiveness of European industry by targeting high tech SMEs.

List of Websites:

<http://www.electroextraction.org>