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Large Scale Bioprocess for the Production of Optically Pure *L*-Carnitine

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Summary. A competitive production method using the biotransformation of 4-butyrobetaine to enantiomerically pure *L*-carnitine was developed and scaled-up by Lonza. The process produces *L*-carnitine in 99.5% yield, and >99.9% enantiomeric excess (*ee*). Continuous and discontinuous processes were developed but the fed-batch process was found to be economically the most favourable process mode.

Keywords. *L*-Carnitine; Biotransformation; Enzymes; Large scale production.

Introduction

Functional foods contain ingredients that are believed to provide health benefits beyond their basic nutritional value. The vast majority of such health-enhancing substances are of plant origin. But there are some exceptions to this, one being *L*-carnitine, an active component discovered in meat extract 100 years ago. Numerous clinical studies have reported upon the beneficial effects of *L*-carnitine supplementation. Inspite of this *L*-carnitine has only received media coverage over the last 20 years. The reason that *L*-carnitine came to the media's attention at this time was the exceptional performance of Italian endurance athletes at the Olympic Games in the eighties and during the soccer world championship in the early nineties. *L*-Carnitine assists endurance athletes because it is involved in the metabolism of fatty acids and accelerates muscle recovery after intense exercise. *L*-carnitine can also be converted *via* lactonization or olefinization into polymerizable basic molecules for application in cosmetics, pharmacy, and medicine [1].

This paper will describe how and why *L*-carnitine is produced biotechnologically and what steps are necessary to develop such a process. Lonza produces *L*-carnitine in different forms (Fig. 1). The key ingredient in each case is the *L*-carnitine free base.

Lonza developed a chemical as well as a biotechnological process. However, only the biotechnological process is presently being used for production. The 100%

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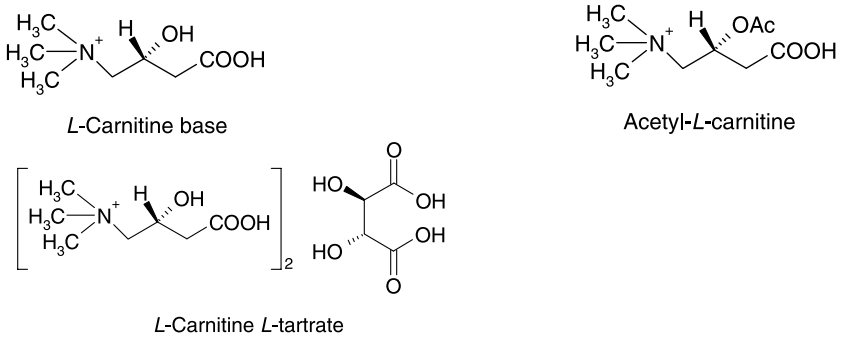

Fig. 1. Examples of different forms of *L*-carnitine; the *L*-carnitine base (molecular weight 161.20) is the biotechnologically produced starting material, used for the other formulations, such as for example of acetyl-*L*-carnitine or the *L*-carnitine *L*-tartrate salt

pure and natural *L*-carnitine is the starting material for all *L*-carnitine derivatives. *L*-carnitine free base is a crystalline, white powder which is extremely hygroscopic and suitable for all liquid formulations. *L*-Carnitine salts such as *L*-carnitine tartrate are free flowing which makes them suitable for use in solid products such as tablets and capsules.

Results and Discussion

How can L-Carnitine be Produced?

D-Carnitine has not only been described as physiologically inactive, but appears to inhibit the *L*-carnitine transport systems. Results of experimentation with racemic and pure *D*-carnitine showed, that *D*-carnitine is not considered safe for human consumption and only physiological *L*-carnitine should be used by food manufacturers [2]. For this reason the optical purity plays a key role in the choice of manufacturing process for *L*-carnitine. There are four different possibilities to produce optically pure *L*-carnitine. These four options are

1. Asymmetric chemical synthesis
2. Extraction from the natural chiral pool
3. Biotransformation of an achiral precursor
4. *De novo* biosynthesis by fermentation

Nature is intrinsically enantioselective as primary and secondary metabolites exist in only one optical form. The catalysts of nature, enzymes, are endowed with a high substrate-, regio-, and enantionselectivity. Consequently if optical purity is of importance as in the production of *L*-carnitine then biotechnological production methods appear to have a 'natural' advantage over chemical synthetic methods. Extraction from the natural chiral pool is always an option, albeit often not an economical one. A rich natural source of *L*-carnitine is meat, but extraction is not a commercially viable option for cost reasons. Moreover, animal derived raw materials should be avoided because of potential microbial, viral, and prion cross-contaminations.

The chemical synthesis of *L*-carnitine usually starts with the cheap raw materials such as epichlorhydrine and trimethylamine, followed by a separation of the racemate by, *e.g.*, fractionated cristallisation [3]. The chemical synthesis of enantiomerically pure *L*-carnitine was commercially less attractive, at the time Lonza started to develop the bioprocess. This argument holds true for the biological racemic resolution as well, where *L*-carnitine can be obtained, *e.g.*, by the selective hydrolysis of racemic carnitine amide by *Pseudomonas* [4] and racemic acetyl-*D*, *L*-carnitine [5] or acetyl or betaine esters using bacterial betaine ester hydrolases [6]. Several chemical and biological [7] methods to recover and convert the wrong enantiomer (*D*-carnitine) exist but they are not economically feasible. For these reasons Lonza chose to develop a biotechnological production process which starts with the achiral substrate 4-butyrobetaine and results in a 99.5% yield of pure *L*-carnitine at high concentrations. The high yield of *L*-carnitine also simplifies the down stream processing and reduces costs.

Biotechnological L-Carnitine Production

Some 20 years ago, the scientists at Lonza came to the conclusion that the most attractive process for the production of *L*-carnitine is a biotransformation analogous to the natural process, which also takes place in the human body.

The first task was to find bacteria that transformed an achiral precursor 4-butyrobetaine into chiral or optically active *L*-carnitine at very high concentrations. *Hans Kulla* and his team at Lonza [8] were able to isolate and cultivate a microorganism from soil which contained the necessary enzymes involved in the degradation of 4-butyrobetaine *via* *L*-carnitine-CoA and the tricarboxylic acid pathway. Logically the wild type organism only produced traces of *L*-carnitine as overproduction of a particular metabolite is contra to the well balanced physiology of any microorganism. Therefore the enzyme, carnitine dehydrogenase which metabolizes the accumulated *L*-carnitine had to be inactivated by classical methods. The resulting strain metabolised 4-butyrobetaine to produce *L*-carnitine analogous to the β-oxidation of fatty acids (see Fig. 2) and accumulates large amounts of pure *L*-carnitine in fermentation solutions.

This strain was subjected to a classical strain development program to obtain a production strain with high productivity, a high precursor uptake rate, and a high *L*-carnitine tolerance. Once this goal was reached the following steps were still necessary to realise a scalable process. Lonza evaluated several different process options (Table 1), and they were all tested in the larger scale. Despite the fact that the continuous process [9, 10] had a much higher volumetric productivity compared to the fed-batch culture ($130\,\mathrm{g\,dm^{-3}\,d^{-1}}$ *vs.* $30\,\mathrm{g\,dm^{-3}\,d^{-1}}$), the fed-batch process was the process of choice.

The fed-batch process costs were 40% lower than that of the continuous process due to the $\sim$99.5% bioconversion of 4-butyrobetaine compared to only 91% in the continuous process. The lower bioconversion yield resulted in additional unit operations in the down stream processing to separate the 4-butyrobetaine from the product and therefore led to higher costs.

Microbial production strains are susceptible to gradients in large bioreactors, the cooling capacity can be limiting, and a precise maintenance of the physicochemical

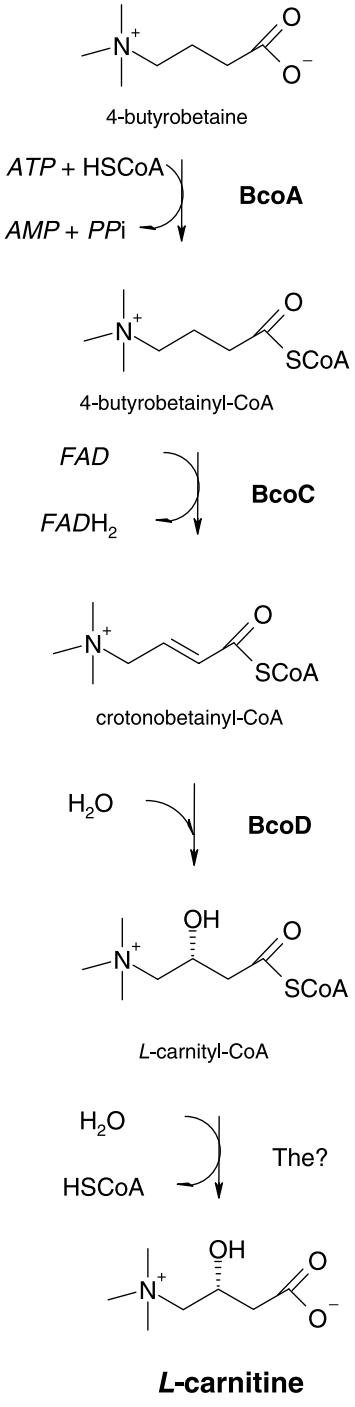

Fig. 2. The conversion of 4-butyrobetaine to *L*-carnitine; the thioesterase (The) is only postulated, since the reaction could also be spontaneous; BcoA: 4-Butyrobetainyl-CoA-Synthetase, BcoC: 4-Butyrobetainyl-CoA-Dehydrogenase, BcpD Crotobetainyl-CoA-Hydrolase, The: Thioesterase

environment is a prerequisite in high performance fermentation. Extensive scale-up studies with a suitable production strain were needed to reach a commercially viable process. Scale-down experiments and computer simulation were used to

Table 1. The economics for the bioproduction of optically pure *L*-carnitine were tested with four different process modes; the fed-batch process gave the most attractive cost benefit ratio, when the sum of all aspects are considered (productivity, purification yields, process reliability, material costs *etc*.)

	Qp / mol dm^{-3} h^{-1}	Y / %	\$ / A.U.
Continuous process with cell recycling	0.034	91	100
	0.0019	99.5	105
Continous two-stage process with cell recycling	0.0155	99.5	75
Fed-batch process	0.0075	99.5	60

Qp Volumetric productivity; Y Bioconversion yield of 4-butyrobetaine; \$ Cost estimation for the biotransformation and isolation of pure *L*-carnitine; A.U. Arbitrary units

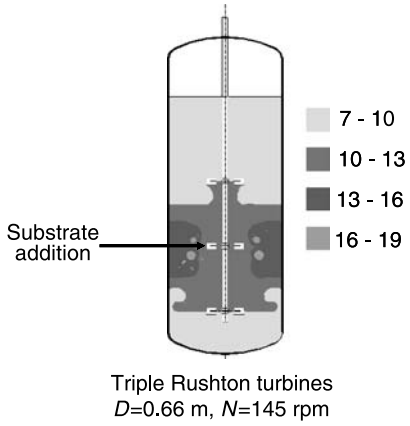

Fig. 3. Computer simulation of substrate distribution in g/m^3 in a 15 m^3 fermentor

study, *e.g*., gradients with different impeller designs, impeller positions, feed dip-pipe design, and dip-pipe positions. Figure 3 shows such a computer simulation of the substrate concentration range in g/m^3 in a 15 m^3 stirred tank fermenter. We studied such substrate gradients for different positions of dip pipes and different impellers in order to find the optimal configuration. The most promising configurations were tested in large scale, to define and validate the final and optimal design criteria. Similar experiments were carried out to investigate oxygen gradients.

Lonza operates 15, 50, and 75 m^3 fermenters for the production of *L*-carnitine. The photographs in Fig. 4 show the dimension of the 15 and 75 m^3 scale. The baffles, visible in both photographs, are designed as heat exchangers, to remove the heat which is generated by the production organism during fermentation. The evolution of heat is a consequence of the thermodynamics of the overall microbial activity. The rate of microbial heat formation Q_W shown in Eq. (1) can be so high, that if not removed it raises the temperature in the fermenter beyond the optimum range. About 50–60% of the available substrate enthalpy is given off as heat! These internal cooling elements are needed, as the metabolic heat generation during fermentation can exceed 40 kcal h^{-1}.

$$Q_W = Vo^X \mu^X X / Y_H \quad (1)$$

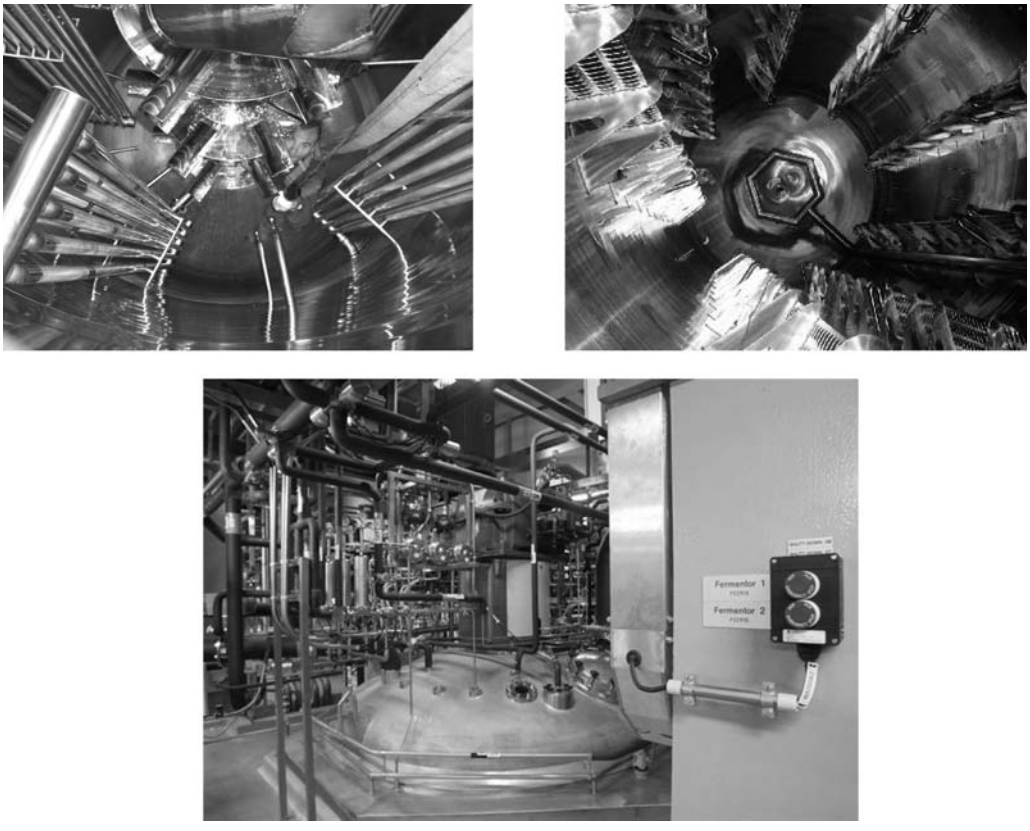

Fig. 4. The top left picture shows the interior of a 15 m^3 fermentor, and the top right picture shows the interior of a 75 m^3 fermentor with the shaft and turbines removed for better view; the photograph below shows a 75 m^3 fermentor with the 500 kW motor for the stirrer; the two pictures give an impression of the dimensions of the vessels, and the corresponding issues with regard to mass, heat, and momentum transfer in the liquid during fermentation

Q_W = rate of microbial heat formation [kcal $\times$ h^{-1}]; Vo = working volume of fermentor [dm^3]; μ = growth rate of microorganism [h^{-1}]; X = biomass [g/dm^3]; Y_H = microbial heat formation coefficient [$g_{Biomass} \times$ kcal].

The overall *L*-carnitine process is shown in Fig. 5. Once this process was scaled-up, the waste generation during the chemical and the biotechnological *L*-carnitine production process could be compared. Table 2 shows, that the biotechnological process is a much 'cleaner' process. The difference varies between a several fold reduction of wastewater and salt load, and an order of magnitude of reduction of the waste for incineration.

The quality of the production strain (Fig. 6) in a biotechnological process is not only responsible for the efficiency and cost of fermentation. The performance of the strain will also have a direct influence on the cost and performance of the isolation and purification (down stream processing). The nutrient requirements, the product concentrations, possible by-products produced and other strain related factors will affect the number and type of isolation-purification steps needed. A good production strain is not everything, but everything is nothing without a good production strain. For this reason, the development of bioprocesses for small mole-

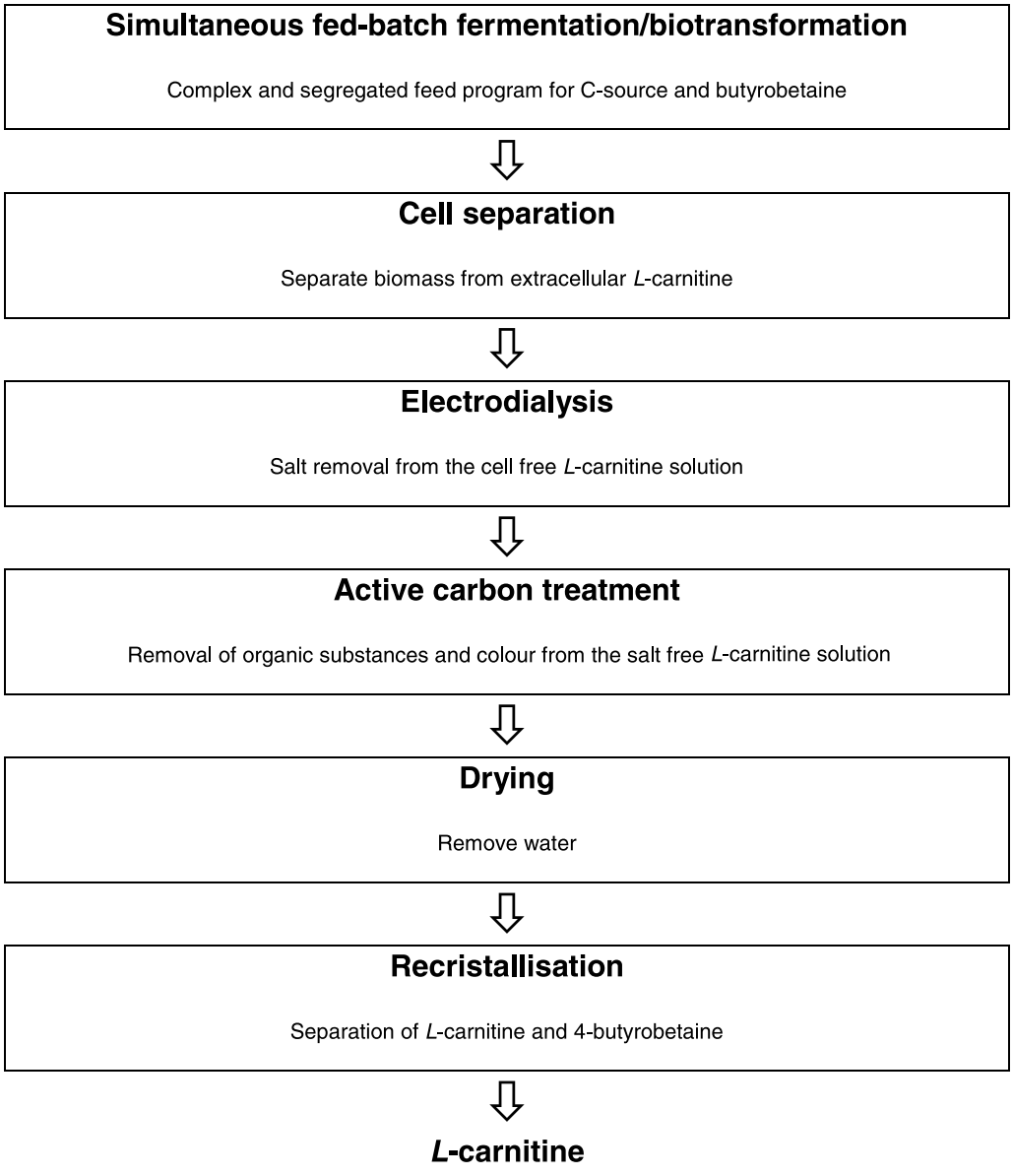

Fig. 5. Because of the very high solubility of *L*-carnitine in water and the similar physico-chemical properties of 4-butyrobetaine, no selective recovery method was available; the steps following the two recristallisation steps are dependent upon the desired final compound

cules focuses mainly on the fermentation and primary recovery after fermentation. Many of the isolation and purification techniques in large scale industrial production of small molecules like *L*-carnitine have been developed and used in chemistry for long time, and can be applied 'immediately'.

Future Developments

How will *L*-carnitine be produced in the future*?* Further process improvements to the existing Lonza route starting from an achiral educt as described above is an

Table 2. A comparison of the waste generation during the chemical and the biotechnological *L*-carnitine process; the basis of calculation were the Lonza chemical- and bio-process [3, 9] described in this paper

	Biotechnological process	Chemical process
Waste for incineration[1]	0.5	4.5
TOC in waste water[2]	360	750
Waste water[3]	40	220
Salts[1]	0.8	3.3

[1] Tons per ton of *L*-carnitine; [2] kg per ton of *L*-carnitine; [3] m^3 per ton of *L*-carnitine

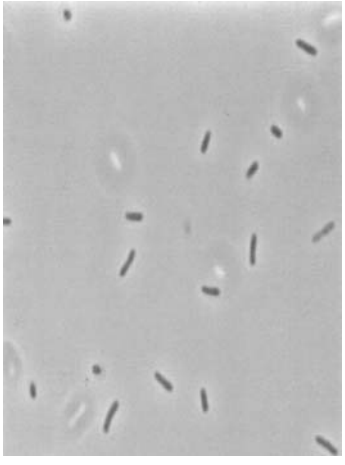

Fig. 6. Photograph of the *L*-carnitine production strain, a bacterium isolated from soil samples; the production strain is a natural soil isolate and not genetically modified; it is taxonomically related to *Agrobacterium* and *Rhizobium*; the strain was assumed to belong to a new, yet unidentified genus; the bacterium is a non-sporulating, *Gram* negative, and motile microorganism, which metabolises substrates aerobically (chemoorganotrophs)

ongoing process. The use of a recombinant production strain where the productivity is increased by a gene dosage effect [11, 12] is another possible further improvement. Other more radical changes would require a very large research effort to develop a process that would compete with the exisiting process. *De novo* synthesis would be an option although it has never been observed in prokaryotes. The biosynthesis of *L*-carnitine in eukaryotic organisms was first elucidated in the ascomycete *Neurospora crassa* [13]. However *de novo* synthesis using a eukaryotic microorganism has inherent disadvantages. Only very low concentrations of *L*-carnitine have been achieved with fungal processes using complex medium which complicates the work-up. For example, Takeda has patented a process with *Acremonium* that produces only 258 μg *L*-carnitine/cm^3 in 16 days [14]. An extensive strain development process would be necessary to optimise such a strain and develop a process that could compete with the existing process.

As already mentioned, epichlorhydrin and trimethylamine are the cheap starting materials and a racemate separation is used in the final step. But the chemical process still contains some *D*-carnitine and the process itself produces large

amounts of waste (see Table 2). Innovative chemical synthesis solving these problems may replace this synthesis in the future.

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