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Research article

Enhancing γ-decalactone production by protoplast fusion of *Yarrowia lipolytica*

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Abstract: *γ-Decalactone is an aroma compound with a pleasant peachy odour, it can be produced by Yarrowia lipolytica, but the yield is commonly poor. Therefore, the present study aimed to use protoplast fusion performed between different superior mutants of Y. lipolytica to improve γ-decalactone production. Data showed that 45 fusants obtained from all three crosses, the cross 1 enhanced the lipase relative production (C/G) up to 106.24% for the fusant C1/9 over the original strain. Furthermore, the highest record of the lipase production was 9.50% based on the lipase relative production (C/G), with 227.82% more than the original strain, which obtained from the fusant No. C2/3 (cross2). Moreover, the highest record of the lipase production was 10 based on the lipase relative production (C/G), with 239.81% more than the wild type strain, which obtained from the fusants C3/2 and C3/14 (cross3). Finally, the highest record of the γ-decalactone production was 108.78 mg/L with 618.07% more than the wild type strain, which obtained from the fusant C3/14.*

Keywords: *Yarrowia lipolytica, protoplast fusion, γ-decalactone.*

Introduction

Lactones are widely distributed in foods and beverages. They have been found in the aromas of more than 120 foodstuffs, which explains their importance in the aroma industry. An increasing demand for natural products has resulted in the use of biotechnological processes for the production of these lactones. This has led to numerous patents being taken out, and nowadays the biotechnologically produced lactone family is mainly represented by γ-decalactone, but also to a smaller extent by γ-dodecalactone and γ-octalactone [1]. γ-Decalactone has an oily-peachy, extraordinarily tenacious odour and a very powerful, creamy-fruity, peach-like taste in concentrations below 5 ppm [2]. It is used in the formulation of fruit aromas such as strawberry, apricot and peach, but is also present in many fermented products such as bread and whiskies [3].

With its tempting aroma and low odour threshold (0.088 ppm in water) [4], γ-decalactone is recognized internationally as a safe food additive [5] and obtained the characteristics of the universal application in the fragrance industry [6]. The discovery of its accumulation during ricinoleate catabolism stimulated studies on the processes of production reviewed in [1,7]. As a consequence of these studies, γ-decalactone is now one of the aroma compounds most produced through biotechnology. As a second consequence, its price, which was close to $12 000/kg in 1986, has fallen to under $ 100/kg [8]. However, the demand is very high and the low price stimulates research to improve the biotransformation.

γ-Decalactone can be produced from methyl ricinoleate, the major component of esterified castor oil, through a degradation pathway that is present in several yeast species, the most efficient species is *Yarrowia lipolytica* [7]. It is considered as a model organism for the metabolism of hydrophobic compounds and therefore, it has been extensively studied [9].

Yarrowia lipolytica has been used in the industrial production of γ-decalactone, but the yield of this biotransformation is commonly poor**.** Apart from β-oxidation activity, another factor influencing the yields is the toxicity of the lactone. The mechanisms involved in this toxicity have been characterized showing that the carbon-lateral chain of the lactone interacts with membranes, increasing their fluidity and decreasing their integrity [10].

Current literature did not reveal any use of protoplast fusion techniques to improve γ-decalactone production by *Y. lipolytica*. Therefore, this study was initiated to take advantage of the protoplast fusion technique in *Y. lipolytica* to obtain γ-decalactone overproducing strains.

Experimental

Materials

The original strain of *Y. lipolytica* ATCC 20226 was obtained from the culture collection of the ATCC, USA and the superior mutants (Nos., 4/12, 12/8, 8/12 and 10/12) of *Y. lipolytica* were obtained from the previous study [11].

Methods

Growth Conditions

Y. lipolytica strains conserved at 4^{o}C on YPD-agar media [12]. For cultivation, the strains were grown at 30^{o}C and 200 rpm in 50 mL YPD or production media in 250 mL flasks.

Production Medium

The composition of the medium for lipase and γ-decalactone production as mentioned in [13] with some modification was as following: castor oil 20 g/L, yeast extract 3 g/L, peptone 3 g/L, casein 3 g/L, yeast nitrogen base 3 g/L. The media was adjusted at initial to be at pH 6.0.

Protoplast formation

The different superior mutants were grown in 50 mL of protoplasting medium as described by [14] and incubated on a rotary shaking incubator (150 rpm) at 30°C for 18 hrs. Cells were collected by centrifugation and washed twice with sterile distilled water. The washed cells were resuspended in the pre-treatment solution and the suspension was incubated for 20 min at 32°C with gentile agitation. After incubation, cells were centrifuged again and resuspended in a protoplasting buffer containing snail enzyme (1%, w/v) and incubated under shaking (120 rpm) in a water bath at 35°C. Cells were checked periodically, using phase-contrast microscope for the formation of protoplasts. The conversion of cells into protoplasts was completed within one hour of incubation.

Porotoplast fusion

Protoplast fusion could be carried out between selected isolates which differ in either their resistance to heavy metals or lipase production levels as follow: protoplasts from different cultures were mixed, centrifuged at 2500 rpm for 5 min and the supernatant was removed. Two mL of polyethelene glycol (PEG) solution were added and mixed gently with protoplasts [15]. The mixture of protoplasts was incubated up to 30 min at 30°C. Then, the mixture was diluted with 0.65 M KCl. Samples of 0.1 and 0.5 mL of the dilutions were overlayed on the surface of (SG-CAA) media [16] containing tributyrin (0.5% v/v) for selection of high-producing lipase colonies and 0.65 M KCl for maintain the protoplast cells without burst. Plates were then incubated (3 days, 30°C) and the growing colonies (fusants) with high clearing zones were transferred on slants for further studies. Fusants with high clearing zones were then retested on the same plates and selected of the superior fusants for lipase and γ-decalactone production.

Extraction and analysis of γ-decalactone

To quantify lactones during the methyl ricinoleate biotransformation, 1.5-mL samples were taken and poured in 4-mL glass. Then 10 µL 36% HCl was added to stop metabolism and to reach complete lactonization of 4-hydroxy acids. An internal standard (undecanoic γ-lactone, Sigma-Aldrich), 10 µL solubilized in absolute ethanol, was added to reach a final concentration of 100 mg/L. The sample was then extracted with 1.5 mL diethyl ether inverting the vial every second for 90 s. After 15 min, the ether phase was extracted and analyzed by gas chromatography (Chromatograph Hewlett-Packard HP5890, Agilent technology series; Lyons, France), with a DB-5 capillary column (Agilent, 60 m × 320 µm × 0.25 µm) with He as a carrier gas at a linear flow rate of 1 mL/min. The split injector (split ratio, 1:10) temperature was set to 250°C and the flame ionization detector, to 300°C. The oven temperature increased from 60°C to 145°C at 5°C/min, and finally at 2°C/min to reached 215°C [17].

Results and Discussion

Response of superior mutants to different heavy metals

In order to investigate the effect of protoplast fusion on γ-decalactone production, six induced mutants which exhibited the highest γ-decalactone production were selected to be used as parental isolates for protoplast fusion crosses. Moreover, an additional marker, i.e. antifungal and heavy metals resistance or sensitivity were determined for the selected mutants.

Data presented in Table 1 showed the resistance or sensitivity to benomyl (B), cycloheximide (C), Griseofulvin (G), nystatin (N), miconazole (M) and some of heavy metals: selenium (Se), barium (Ba), mercury (Hg), arsenate (As), cadmium (Cd), cobalt (Co) and cesium (Cs). Results showed that, although the original strain was resistant to all antifungal agents, most of the selected mutants exhibited the same antifungal response except the mutants Nos., 4/12, 12/8 and 8/12 proved to be sensitive to N. On the other hand, the original strain and all mutants were resistant to Ba and Cs. However, most of the selected mutants exhibited different response to the other heavy metals.

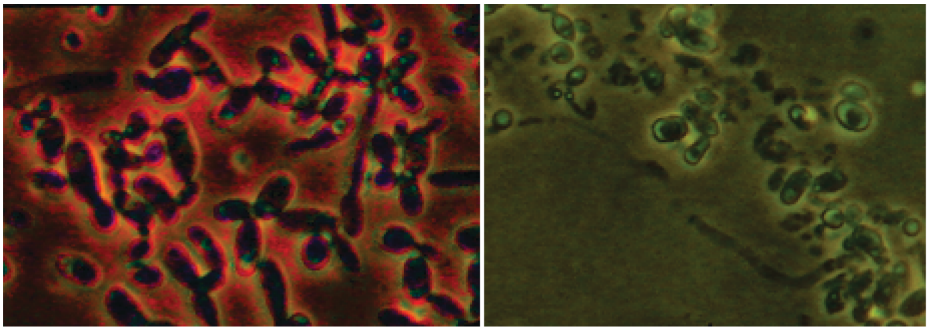
Table 1. Antifungal and heavy metals response of the original strain (W.T) and the superior mutants selected for protoplast fusion

Strain No.	G	N	C	B	Se	Ba	Hg	As	Cd	Co	Cs
W.T	+	+	+	+	+	+	−	−	−	−	+
4/12	+	–	+	+	+	+	–	–	–	–	+
12/8	+	–	+	+	–	+	+	–	+	+	+
8/12	+	–	+	+	–	+	+	+	–	+	+
10/12	+	+	+	+	+	+	+	+	–	+	+

However, three mutants (12/8, 8/12 and 10/12) were selected and introduced subsequently into protoplasting, fusion and regeneration. Two mutants, i.e., 12/8 and 8/12 were used as parental strains for cross1 where As and Cd were used as selective markers. Mutants Nos., 12/8 and 4/12 were used for cross 2 where Se and Cd were used as selective markers. Finally, 4/12 and 12/8 were used for cross 3 where Se and As were used as selective markers.

Protoplast fusion

Among the wide variety of strain improvement protocols, protoplast fusion seems to be an efficient way to induce genetic recombination. This method has also been proved to be valuable in the development of new industrial yeast strains. However, under the conditions mentioned under materials and methods, protoplasts can be isolated as shown in Figure (1/B). On the other hand, the addition of polyethylene glycol to the protoplast suspension resulted in intensive agglutination which leads to the formation of large aggregates which is a precondition for fusion. Furthermore, the presence of calcium ions is normally an important requirement for fusion of the aggregated protoplasts [18].



A **B**

Figure 1. Micrographs represent formation of yeast protoplasts (B) in comparison with the original parent (A)

Anyhow, when the PEG-treated protoplast suspension was embedded into solid regeneration medium, some of them increased in volume and reverted to normal cells. Cell clones with normal cell morphology and prototrophic properties were selected after twice repeated single-colony isolation and storage on slant agar medium at 4°C for further genetic analysis.

Protoplast fusion and lipase production

This research was mainly planned aiming the combination and improvement of the genes responsible for lipase and γ-decalactone production through protoplast fusion of *Y. lipolytica*. After different crosses of protoplast fusion between superior UV-mutants, the obtained fusants were tested for lipase activities on SG-CAA medium plates containing tributyrin (0.5%, v/v) and screening about 150 fusants colonies, only 45 were characterized as high lipase producer mutants (Tables 2, 3 and 4). These fusants were tested based on both clear zone (C) and growth zone (G) of some superior *Y. lipolytica* fusants to evaluate their lipase productivity (Figure 2).

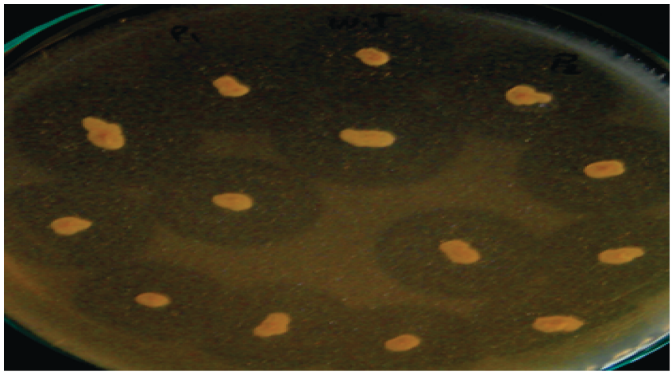


Figure 2. Photograph of some superior *Yarrowia lipolytica* fusants for lipase production in comparison with their original strain

The results presented in Table (2) showed that the cross1 enhanced the lipase relative production (C/G) up to 106.24% for the fusant C1/9 over the original strain. The next highest producer fusants were C1/14 and C1/4 which exceeded their original strain by 96.64 % and 91.85 %, respectively.

On the other hand, the lipase relative production (C/G) enhanced up to 34.38% for the fusant C1/9 over the high first parent (P1).

Table 2. Lipase production measured as both clear zone (C) and growth zone (G) of some *Y. lipolytica* fusants obtained after cross 1

Mutant No.	C	G	C/G	C/G % to W.T	C/G % to P1
W.T	25	6	4.17	100.00	65.16
P1-8/12	32	5	6.40	153.48	100.00
P2-12/8	30	5	6.00	143.88	93.75
C1/1	38	5	7.60	182.25	118.75
C1/2	41	7	5.86	140.53	91.56
C1/3	38	5	7.60	182.25	118.75
C1/4	40	5	8.00	191.85	125.00
C1/5	38	7	5.43	130.22	84.84
C1/6	38	6	6.33	151.80	98.91
C1/7	41	7	5.86	140.53	91.56
C1/8	37	5	7.40	177.46	115.63
C1/9	43	5	8.60	206.24	134.38
C1/10	38	7	5.43	130.22	84.84
C1/11	33	7	4.71	112.95	73.59
C1/12	40	6	6.67	159.95	104.22
C1/13	35	6	5.83	139.81	91.09
C1/14	41	5	8.20	196.64	128.13
C1/15	38	5	7.60	182.25	118.75

Also, data in Table 3 clearly showed that all induced fusants were more productive than the original strain. Furthermore, the highest record of the lipase production was 9.50% based on the lipase relative production (C/G), with 227.82% more than the original strain, which obtained from the fusant No. C2/3. The next highest producer fusants were C2/10 and C2/4 which exceeded their original strain by 96.64 % and 91.85 %, respectively. On the other hand, the lipase relative production (C/G) enhanced up to 34.38% for the fusant C1/9 over the high first parent (P1).

On the other hand, 15 selected fusants obtained from cross 3 in the presence of Se and As. Results in Table 4 showed that all of the fusants exhibited more lipase production than its wild type (W.T). Increasing lipase productivity is ranging from 127.82 to 239.81% higher than the wild type. The highest record of the lipase production was 10 based on the lipase relative production (C/G), with 239.81% more than the wild type strain, which obtained from the fusants C3/2 and C3/14. However, the tested fusants from cross 3 proved to be higher effective for increasing lipase production than fusants obtained from cross 2.

Table 3. Lipase production measured as both clear zone (C) and growth zone (G) of some *Y. lipolytica* fusants obtained after cross 2

Mutant No.	C	G	C/G	C/G % to W.T	C/G % to P1
W.T	25	6	4.17	100.00	57.52
P1-4/12	29	4	7.25	173.86	100.00
P2-12/8	30	5	6.00	143.88	82.76
C2/1	35	5	7.00	167.87	96.55
C2/2	32	5	6.40	153.48	88.28
C2/3	38	4	9.50	227.82	131.03
C2/4	41	5	8.20	196.64	113.10
C2/5	36	6	6.00	143.88	82.76
C2/6	40	6	6.67	159.95	92.00
C2/7	39	5	7.80	187.05	107.59
C2/8	37	5	7.40	177.46	102.07
C2/9	32	4	8.00	191.85	110.34
C2/10	34	4	8.50	203.84	117.24
C2/11	38	5	7.60	182.25	104.83
C2/12	33	6	5.50	131.89	75.86
C2/13	35	7	5.00	119.90	68.97
C2/14	40	7	5.71	136.93	78.76
C2/15	38	5	7.60	182.25	104.83

Table 4. Lipase production measured as both clear zone (C) and growth zone (G) of some *Y. lipolytica* fusants obtained after cross 3.

Mutant No.	C	G	C/G	C/G % to W.T	C/G % to P1
W.T	25	6	4.17	100.00	57.52
P1-4/12	29	4	7.25	173.86	100.00
P1-8/12	32	5	6.40	153.48	88.28
C3/1	33	5	6.60	158.27	91.03
C3/2	40	4	10.00	239.81	137.93
C3/3	32	6	5.33	127.82	73.52
C3/4	38	7	5.43	130.22	74.90
C3/5	40	5	8.00	191.85	110.34
C3/6	37	5	7.40	177.46	102.07
C3/7	38	5	7.60	182.25	104.83
C3/8	36	4	9.00	215.83	124.14
C3/9	40	6	6.67	159.95	92.00
C3/10	31	5	6.20	148.68	85.52
C3/11	38	4	9.50	227.82	131.03
C3/12	39	6	6.50	155.88	89.66
C3/13	40	6	6.67	159.95	92.00
C3/14	40	4	10.00	239.81	137.93
C3/15	37	5	7.40	177.46	102.07

In general, protoplast fusion between higher lipase production mutants proved to be effective to achieve superior lipase production fusants [19]. Therefore, protoplast fusions have been used successfully to enhancement the lipase production. The recombinants strains can be obtained by this technique [20-22].

From the above results, it appeared that the protoplast fusion was a good strategy for improving the lipase productivity of *Y. lipolytica* and the selection of the highest lipolytic zone with tributyrin (0.5% v/v) can be successfully used as a selection procedure to improve the lipase synthetic capacity of the original strain. These results are in accordance with those of Mansour et al. [23], they treated *Y. lipolytica* by UV-mutagenesis to obtain mutants with high ability to hydrolyze olive oil. Some induced mutants over yielded their original strain in oil hydrolysis. The promising mutant No.15 was used in protoplast fusion with the parental strain to obtain high lipase productive fusants which able to lactonization of 4-hydroxydodecanoic acid to form γ-dodecalactone.

Protoplast fusion and γ-decalactone production

Data in Table 5 presents the γ-decalactone productivities of some superior *Y. lipolytica* fusants obtained after different crosses of protoplast fusion. Results clearly showed that all fusants were more productive than the wild type (W.T). Furthermore, the highest record of the γ-decalactone production was 108.78 mg/ L with 618.07% more than the wild type strain, which obtained from the fusant C3/14. The next highest producer fusants were C1/9 and C3/7 which exceeded their original strain by 595.17% and 467.84%, respectively. Meanwhile, the rest fusants produced γ-decalactone higher than the wild type strain but lower than the superior fusant C3/14.

Table 5. γ-Decalactone production of some superior fusants obtained after different protoplast fusion crosses

Parents & fusants	γ-decalactone (mg/L)	% to W.T	Parents & fusants	γ-decalactone (mg/L)	% to W.T
W.T	17.60	100.00	C2/4	37.24	211.59
C1			C2/7	59.09	335.74
P1-8/12	80.79	459.03	C2/9	59.88	340.23
P2-12/8	69.43	394.49	C2/10	51.75	294.03
C1/1	17.76	100.90	C2/11	54.06	307.16
C1/3	19.34	109.87	C3		
C1/4	17.68	100.45	P1-4/12	54.00	306.82
C1/9	104.75	595.17	P2-8/12	80.79	459.03
C1/14	73.06	415.11	C3/2	25.07	142.44
C1/15	67.89	385.74	C3/5	76.53	434.83
C2			C3/7	82.34	467.84
P1-4/12	54.00	306.82	C3/8	58.46	332.16
P2-12/8	69.43	394.49	C3/11	42.60	242.05
C2/3	84.36	479.32	C3/14	108.78	618.07

These results are in accordance with those of Mansour et al. [23]. They used the promising mutant No.15 in protoplast fusion with the parental strain to obtain high lipase productive fusants which able to lactonization of 4-hydroxydodecanoic acid to form γ-dodecalactone.

In the construction of novel strains, protoplast fusion allows the combination of characteristics present in the parents. Although the isolation of a strain displaying specific traits may be a matter of good fortune. The advent of more specific means of manipulating genes has meant that protoplast fusion is no longer the principal method for strain improvement or construction. However, it allows the creation of strains embodying a range of characteristics from the parents or displaying polygenic traits that would be difficult to construct using more refined methods. Although the concentration of γ-decalactone obtained in the present study was lower than one reported production by Pagot et al. [24]. They were able to obtain γ-decalactone in high yields from ricinoleic acid methyl ester. At the end of the growth phase they transferred the concentrated biomass into an uracil limited medium where the biotransformation took place, after 75 hrs the culture broth yields 9.5 g/L of the product. This variation in γ-decalactone yield compared to the present study, the yields of γ-decalactone in all protoplast fusions (< 1 g/L) (Table 5), may be due to the incubation time. However, our results in accordance with Cardillo et al. [25] they used *Aspergillus niger*, *Pichia etchellsii* and *Cladosporium suaveolens* to produce γ-decalactone from castor oil. Common to all these processes is that the yields of γ-decalactone in all the fermentations with these yeasts were always very low (< 1 g/L).

Conclusion

It can be concluded that protoplast fusion between some superior *Y. lipolytica* mutants proved to be an effective technique to enhance γ-decalactone production. In a next step, fed-batch and continuous processes will be investigated in our laboratory for γ-decalactone production by selected *Y. lipolytica* fusants.

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Research article

Microbiological contaminants in cosmetics – isolation and characterization

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Abstract: *Cosmetic industries are not obliged to produce sterile cosmetics. Nevertheless, they are liable to assure safety of the product to the potential consumer. The purpose of the study was isolation and identification of microorganisms with the ability to survive and develop in cosmetics. Five cosmetics applied for facial skin and one cosmetic for body care were tested for the presence of contaminating microbiota. Eight microbial strains were isolated from three cosmetics, from which seven were derived from cosmetic applied on the facial skin. One strain was isolated from body care cosmetic. The recovered microbial strains were characterized and identified to the species level as Pseudomonas aeruginosa, Serratia liquefaciens and Candida parapsilosis. The isolates were opportunistic pathogens and may cause skin irritation and infections, especially via wounded epithelium in immunocompromised consumers. Moreover, due to application area, they pose a health risk to the consumer due to easy access to the eye area as well as nasal and oral cavities through usage of cosmetic preparation.*

Keywords: *cosmetics, microbiological contamination, Pseudomonas, Serratia, Candida.*

Introduction

According to the Council Directive (76/768/EEC), established in 1976, a cosmetic product is any substance or preparation, which can be applied onto different parts of the human body (e.g. nails, face, hair, teeth). Its role is to keep body in a good condition, change its appearance as well as remove body odours via perfuming, cleansing or protection [1]. Depending on the application area, cosmetics may be categorized as cosmetics for skin, hair-scalp and oral care as well as fragrances [2]. Cosmetic ingredients govern wide range of products ranging from oily materials, surface active agents, polymers, ultraviolet absorbents to fragrances and vitamins [2, 3]. Companies producing cosmetics are obliged to ensure safety of the product sold, nevertheless, cosmetics are not liable to be sterile [1]. Their microbiological load is strictly controlled at various

manufacture stages and during shelf-life. Cosmetics might contain microbes, due to impurity of raw material and might be contaminated during usage [4, 5]. Microbial spoilage can not only alter physical properties of the product such as colour, taste, odour and viscosity, but also deactivate crucial constituents depriving cosmetic of its features [6, 7]. Microbiological contaminants may produce endotoxins and metabolites causing irritation and allergic reaction of the skin [7]. They can be also pathogens causing hazard to the human health [8].

Microorganisms can survive in environment that fulfil their physical and chemical requirements for proliferation and further development. Most important physical requirements include suitable temperature and pH of the environment. Considering chemical ones, microorganisms require presence of moisture, available and easily metabolized nutrients as well as oxygen [5]. Almost all cosmetics fulfil all of the hereinbefore described requirements for microbial growth. They are rich in a free water, having pH close to neutral. Consumers keep them at home, at room temperature, which is an optimum for proliferation. Most of the cosmetics are found in the bathroom, where the temperature and humidity are high [4]. Depending on the type of closure, different amount of oxygen may access the cosmetic preparation. Composition of cosmetics varies from product to product. The specificity of cosmetic application requires that its ingredients are nourishing and easily assimilated. Hence, such components as proteins, minerals, vitamins and glycerine are easily metabolized source of nitrogen, carbon, hydrogen as well as micro- and macro-elements, necessary for microbial development [4, 5].

The SCCP (Scientific Committee on Consumer Products) in "Notes of Guidance for the testing of Cosmetic Ingredients and their Safety Evaluation" stated that even cosmetics applied by children under three years old, around the eye area and on mucous membrane may be contaminated by saprophytic microorganism up to 100 CFU (Colony Forming Unit) in 0.5 g/mL of the cosmetic preparation [8]. Anyway, such microorganisms as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus brasiliensis* were restricted by EU Pharmacopoeia as most commonly found contaminants posing microbial spoilage in cosmetics and risk to the consumer health. They may not be found in the given volume of cosmetic sample [9]. In order to protect cosmetic product from microbial spoilage to which cosmetic is subjected during use, preservatives are added. Despite their function, addition of preservatives belongs to the one of the most controversial issues concerning cosmetic industry [10].

According to the RAPEX (the Rapid Alert System for non-food consumer products), 173 cosmetics were recalled from the market, from year 2005 to 2008. Among them, 24 were highly contaminated with undesired microorganisms, especially *Pseudomonas aeruginosa* [11]. As stated in RAPEX Reports, 86 cosmetics were notified in 2012 as health risk to the human, from which 8 cosmetics were categorized as a microbiological hazard. In 2013, 13 cosmetics

(calculated basing on weekly reports) were notified as either contaminated with microbial pathogens or having too high microbiological load [12]. The objective of the study was to isolate and identify microorganisms recovered from cosmetics during their use as well as to assess their potential health hazard to the consumer.

Experimental

Materials

Six commercial cosmetic products, abbreviated from A to F were randomly delivered by consumers after approximately 12 months of usage. These included cosmetic applied on facial skin (No A – Anti-Wrinkle Cream, B – Anti-Eye Wrinkle Cream, C – Moisturizing Cream, E – Anti-Redness Cream and F – Moisturizing Cream) and used for body care (No. D – Moisturizing Cream after Shaving and Depilation). Agar slants of nutrient broth supplied with glucose as carbon source (peptone 5.0 g/L, yeast extract 2.0 g/L, meat extract 2.0 g/L, sodium chloride 4.0 g/L, glucose 10.0 g/L, agar 20.0 g/L, distilled water up to 1 L) were used for isolates activation before identification tests.

Methods

Isolation of microorganisms

Depending on the type of cosmetic closure, a plate method with samples seeding or printing were applied to recover microorganisms. A sterile Dilution liquid (peptone K 5.0 g/L, NaCl 8.5 g/L, distilled water up to 1 L, pH 7.2±0.2) was used to dilute cream sample. Recovery of microorganisms from cosmetics was performed on Tryptic Soy Agar with Neutralizers (TSA, Merck KGaA, Darmstadt, Germany), from which single strains were isolated and cultivated on a Plate Count Agar (PCA, Merck KGaA, Darmstadt, Germany) medium. All samples were incubated at 30^{o}C for seven days. 24-hour activated cultures were used for all further analyses and identification.

Identification of isolates

Identification of recovered strains included macroscopic and microscopic characteristics as well as biochemical profiling. Isolated microorganisms were analyzed microscopically. API® tests (bioMerieux, Warsaw, Poland) were chosen and performed for their biochemical profiling. API® 20E set included the following biochemical tests for active enzymes or fermentation/ oxidation reactions: 1 – β-galactosidase, 2 – arginine dihydrolase, 3 – lysine decarboxylase, 4 – ornithine decarboxylase, 5 – citrate utilization, 6 – hydrogen sulphide production, 7 – urease, 8 – tryptophan deaminase, 9 – indole production, 10 – acetoin production, 11 – gelatinase, 12 – fermentation/oxidation of glucose, 13 – fermentation/oxidation of mannitol 14 – fermentation/oxidation of inositol, 15 – fermentation/oxidation of sorbitol, 16 – fermentation/oxidation of rhamnose, 17 – fermentation/oxidation of sucrose, 18 – fermentation/ oxidation of melibiose, 19 – fermentation/oxidation of amygdalin, 20 – fermentation/ oxidation of arabinose. API® 20C AUX set consisted

of biochemical tests for assimilation of the following compounds: 1 – D-glucose, 2 – glycerol, 3 – calcium 2-ketogluconate, 4 – L-arabinose, 5 – D-xylose, 6 – adonitol, 7 – xylitol, 8 – D-galactose, 9 – inositol, 10 – D-sorbitol, 11 – methyl-αD-glucopyranoside, 12 – N-acetyl-glucosamine, 13 – D-cellobiose, 14 – D-lactose, 15 – D-maltose, 16 – D-sucrose, 17 – D-trehalose, 18 – melezitose, 19 – D-raffinose. Recorded results were compared with API® database of reference strains, basing on which the isolated microbial strains were identified to the species level.

Results and Discussion

Contaminants in cosmetics

Microorganisms were isolated from two cosmetics among six investigated (a face care cosmetic – seven isolates and a body care cosmetic – one isolate). Considering morphological features of the recovered strains, seven ones (apart from strain F VII) were either straight rods or curved coccobacilli. All bacteria were motile and did not form special arrangements (Table 1). They were Gram-negative, and gave a positive result in catalase test. Considering strain F VII, the no motile budding cells were yeast species (Table 1).

API® tests are fast and accurate method to perform biochemical profiles of isolated microorganisms and basing on the obtained results to identify them (Table 1, Figure 1, 2, 3). The obtained results for all isolates, except from strain D I, isolated from Moisturizing Cream after Shaving and Depilation, may be considered as accurate, their percentage of identity with official reference strain was higher than 75% (Table 1).

The growth characteristics of isolates confirmed the result of the test. However, the accuracy of identification of strain F III isolated from facial cream was not so high. Compatibility to *Serratia liquefaciens* should be taken into account. The cause of the obtained result may be a wide diversity of environmental species. It should be noted that *Serratia liquefaciens* altogether with *Pseudomonas aeruginosa* were isolated from the contaminated contact lenses of the patient suffering from red eye, which indicates a probability of their coexistence in the skin [13].

In case of identity results for microbial strain D I, the API® database indicated its compatibility to *Pseudomonas aeruginosa* and *Providencia stuartii* with identity equal to 28.0% and 22.9%, respectively. The growth characteristics of the recovered strain may differ the general characteristic features of the *Providencia* and *Pseudomonas* genera due to the wide diversity of species isolated from natural environments, therefore, the obtained result may be considered as reliable.

Despite the fact that seven distinct strains were investigated, they belonged to two species *Pseudomonas aeruginosa* (four strains) and *Serratia liquefaciens* (three strains). Nevertheless, they differ in their biochemical profiles within species (Figure 1, 2), which is also reflected in their identity comparing to the reference strains. It may be resulted from the biochemical diversity of both

species due to wide range of their primary environmental habitats from which they were transferred to the cosmetic environment.

Identification of strain F VII, isolated from Moisturizing Cream, as *Candida parapsilosis* belonging to the yeast species is considered as reliable and accurate one due to very high identity percentage (96.1%) with the official reference strain from API® database (Table 1, Figure 3).

Table 1. Morphological characteristics and biochemical identification of microorganisms isolated from cosmetics

Strain	Morphological features				Biochemical profiling
	size (µm)	shape	motility	pigment formation	identification (identity*)
Cosmetic D					
D I	0.3×1.0÷1.3	straight rods	no motile	no pigment	*Pseudomonas aeruginosa* (28.0%)
Cosmetic F					
F I	0.3×0.6÷1.3	straight rods	motile	green	*Pseudomonas aeruginosa* (98.2%)
F II	0.3×1.0÷1.6	straight rods	motile	green	*Pseudomonas aeruginosa* (76.4%)
F III	0.3×0.3÷1.0	curved coccobacilli	motile	yellowish	*Serratia liquefaciens*
F IV	0.3×0.6÷1.0	curved coccobacilli	motile	yellowish	*Serratia liquefaciens* (98.1%)
F V	0.3×0.6÷1.6	straight rods	motile	green	*Pseudomonas aeruginosa* (76.4%)
F VI	0.3×0.3÷1.0	curved coccobacilli	motile	white	*Serratia liquefaciens* (98.1%)
F VII	1.3÷1.7×3.0÷5.0	elongated ovoid	nonmotile	no pigment	*Candida parapsilosis* (96.1%)

* – comparing to API® database of reference strains

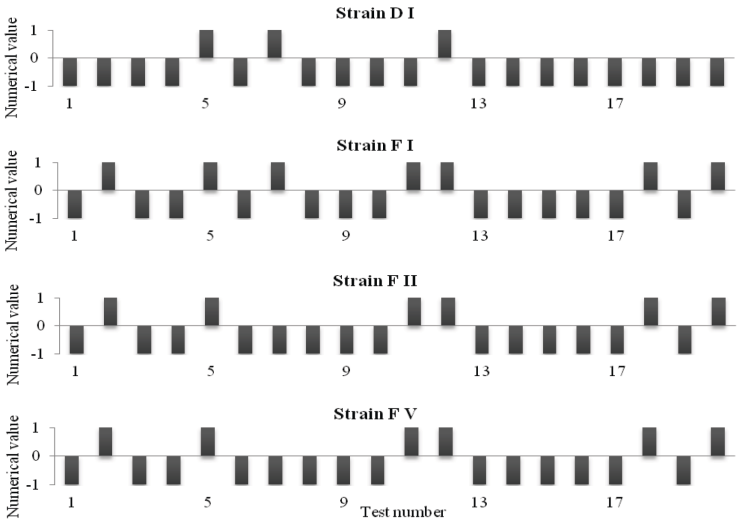

Figure 1. Biochemical profiles of *Pseudomonas aeruginosa* (strains D I, F I, F II and F V) according to API®20E test; "1" – positive, "-1" – negative result

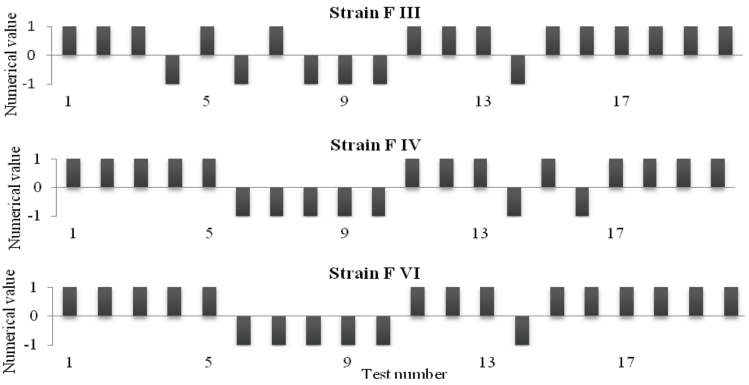

Figure 2. Biochemical profiles of *Serratia liquefaciens* (strains F III, F IV and F VII) according to API®20E test; "1" - positive, "-1" – negative result

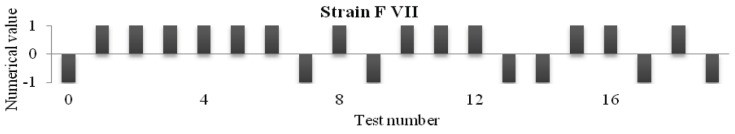

Figure 3. Biochemical profiles of *Candida parapsilosis* (strain F VIII) according to API®20C test; "1" – positive, "-1" – negative result.

Potential hazard of recovered isolates to the consumer's health

Four different microbial species were isolated from the cosmetic environment. Three of them, *Pseudomonas aeruginosa*, *Serratia liquefaciens* and *Candida parapsilosis* were found in the same cosmetic product applied on the facial skin. According to literature, *Pseudomonas aeruginosa* is found in water and soil, some strains were also isolated from skin, animals and plants. It was also isolated from the drainage system and medical equipment [11, 14]. Similarly, *Serratia liquefaciens* was recovered from water, soil, animals, insects and plants [13]. *Candida parapsilosis* is one of the most commonly isolated fungi from the subungual space of hand as well as was also found in other nonhuman environments such as soil, domestic animals, insects [15]. All of the microbial contaminants express similar features: they can survive in harsh conditions, are able to metabolize complex substrates and are resistant to various antimicrobial agents. Since they are ubiquitous in nature, can be easily transported to the cosmetic environment within human hand. *Pseudomonas aeruginosa* belongs to the one of the most commonly isolated bacterium from cosmetic products, whereas *Serratia liquefaciens* was found in antibacterial soap and hand lotion [11, 13]. According to the literature data, *Candida parapsilosis* was recovered from cream [16]. All of the recovered microorganisms are opportunistic pathogens, which basing on the application area may pose a health risk to the consumer due to easy access to the eye area as well as nasal and oral cavities through cosmetic preparation. Abscess formation due to infection was reported for each microorganism. Moreover, eye infections were noted by *Serratia liquefaciens* and *Pseudomonas aeruginosa*, which as well as *Candida parapsilosis* was responsible for fingernails and toenails infections [13, 15, 17]. The immune competent individual is less susceptible to infections with the isolated microorganisms. In contrary, they were reported to cause numerous nosocomial infections of immunocompromised patients, including bloodstream, urinary or fungal peritonitis (in case of *Candida parapsilosis*) infections, which can be lethal [13, 15, 17]. The health risk to potential consumer varies within its own immune system, nevertheless, these microorganisms may cause an infection even in healthy person [18] due to inconsistent skin structure and wounded epithelium as a result of harsh environmental conditions. None of the recovered microbial contaminants should be found in the cosmetic environment.

Conclusions

The performed study confirms that microbiological contamination cosmetic product is a current issue. The steps of microscopic and macroscopic observation of features of recovered microorganisms altogether with biochemical profiling are essential to appropriately identify the microbial strains to the species level. The recovered microorganisms belong to *Pseudomonas aeruginosa*, *Serratia liquefaciens* and Candida *parapsilosis*. They are opportunistic pathogens widely

distributed in nature, which may cause skin irritation and infections, especially via wounded epithelium. Due to application area, they can be a serious threat and the cause of infections of other parts of the body including eye and nails.

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Review article

Succinic acid biosynthesis by *Corynebacterium glutamicum*

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Abstract: *Succinic acid is one of the most desirable raw materials. Currently, it is obtained mainly by hydrogenation of maleic acid derived from the C4 petroleum fraction. Corynebacterium glutamicum is considered as a possible microbiological producer of this acid. Under oxygen deprivation conditions this bacteria secretes L-lactate, succinate and acetate. Succinic acid is an intermediate of the Krebs cycle, in order to achieve high efficiency of its biosynthesis, metabolic engineering of C. glutamicum is required. The best producers described so far are C. glutamicum R ΔldhA pCRA717 (146 g/L) and C. glutamicum BOL-3/pAN6-gap (133 g/L). Succinic acid biosynthesis can also be achieved under aerobic conditions. C. glutamicum ZX1 (pEacsAgltA) produces aerobically 21.7 g/L of succinate. Genetic engineering is also necessary to enable the use of low-cost, waste carbon source such as glycerol or starch. The paper also presents and discusses examples of modifications of bacterial cells, allowing them to use these two carbon sources.*

Keywords: *Corynebacterium glutamicum, succinic acid, metabolic engineering.*

Introduction

Succinic acid (SA, 1.4- butanediolic acid) is one of 12 chemicals that could be produced from sugars, through microbial fermentation [1]. It was estimated that it is a component or substrate for more than 25 mln tons of industrial products per year. It is used for chemical synthesis of 1.4-butanediol, tetrahydrofuran, γ-butyrolactone, adipic acid, succinate salts, N-methyl pyrrolidinone, 2-pyrrolidinone, succinate polyamides. It is also widely applied in the production of coatings, plasticizers, surfactants, detergents or foaming agents, as a component of ion chelators, various green solvents and biodegradable plastics. It could be exploited in food industry as acidulant, flavouring or anti-microbial agent as well as in health-related products (Figure 1) [2-7].

The global market for bio-succinate is currently worth about $ 200 million. It is predicted that its value will reach 496 million dollars in 2016. Rapid increase in demand for this raw material, from current 40 thousand tons per year to more than 2 million tons in 2020 is anticipated [5, 7].

At present, succinic acid is produced mainly by hydrogenation of maleic acid or its anhydride (1), which is synthesized chemically or obtained as a product of oxidation of n-butane (2), derived from the C4 petroleum fraction.

$$C_2H_2(CO)_2O + H_2 + H_2O \rightarrow C_4H_6O_4 \quad (1)$$

$$3CH_3CH_2CH_2CH_3 + 14.5O_2 \rightarrow C_2H_2(CO)_2O + 4CO_2 + 4CO + 14H_2O \quad (2)$$

Cost of succinic acid production is highly dependent on its purity, country of origin, cost of substrates and ranges from 6 to 9 $/kg [8]. The rising prices of the fossil resources have limited the use of petrochemical succinate for wide range applications. Therefore, there is a need to develop a cost-effective fermentation process. Environmental aspects of microbiological production of succinate are also very important. Since its introduction, CO_2 emission will be significantly reduced [9].

Only a few companies produce succinic acid in biotechnological way. BioAmber, which has recently launched production plants in France and Canada is the current market leader. Likewise, other bio-based bulk chemicals such as butanediol and tetrahydrofuran are produced by this company. Its main competitors are Myriant Technologies LLC and Royal DSM NV cooperating with Roquette Frères SA. Probably the biggest plant in Europe will be opened in Italy by Royal DSM NV. It will produce approximately 10.000 tonnes of succinic acid. BASF with the CSM are going to start production of succinate with *Basfia succiniciproducens* in the near future [10-11].

Metabolic basis of succinic acid production by *Corynebacterium glutamicum*

There are a number of microorganisms which could be used in industrial production of bio-based succinic acid. The most frequently mentioned are: *Actinobacillus succinogenes* [5, 12-16] *Anaerobiospirillum succiniciproducens* [17-21], *Mannheimia succiniciproducens* [22-24], *Corynebacterium glutamicum* [25-38], *Escherichia coli* [39-42] and *Yarrowia lipolytica* [43-44]. However, efficient and cost-effective method, which is competitive with the current applied chemical process is still sought. It was estimated that the biosynthesis will be profitable if the concentration of the final product reaches ~ 150 g/L or the productivity of 5 g/L/h [5].

One of the most promising producers of succinic acid is *Corynebacterium* glutamicum. This microorganism was isolated in 1957 by Kinoshita and co-workers. Kyowa Hakko Kogyo Co., Ltd., Tokyo, was searching for microorganisms able to produce and secrete large amounts of glutamic acid. Isolated then the wild strain of *C. glutamicum* has been described as a mesophilic, nonmotile, non-spore forming, Gram-positive, catalase-positive, morphologically variable soil microorganisms [45].

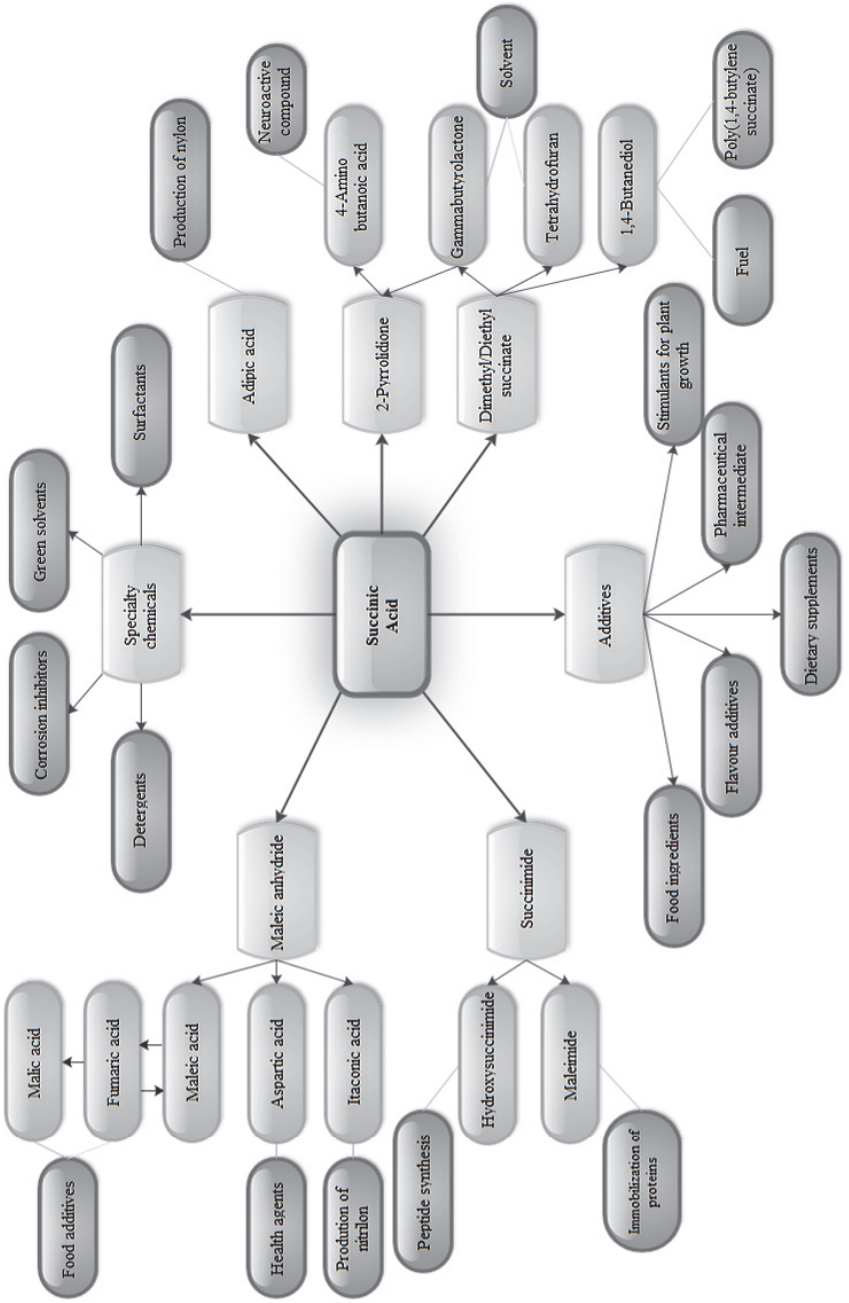

Figure 1. Applications of succinic acid

Due to the simple mechanisms of regulation of the primary metabolites biosynthesis, they are used in the biotechnology industry for the production of amino acids and nucleosides [46]. Knowledge about the genomes of several *C. glutamicum* strains has allowed for a better understanding of their physiology and to extend the range of fermentation products possible to obtain [35, 47, 48]. Modifications of the metabolism allow to produce a wide range of organic acids such as L- and D-lactic acid [49, 50], succinic acid [25-38], pyruvic acid [51], 2-ketoisovaleriate and 2-oxoglutarate [52, 53].

As a result of the oxygen and nitrogen sources (final electron acceptors) limitation, growth of *C. glutamicum* is inhibited [25, 26]. Despite the adverse conditions cells retain the ability to ferment carbohydrates. For the first time this property was described by Dominguez et al. (1993), who placed a thick suspension of *C. glutamicum* cells in mineral medium. After a few hours under oxygen deprivation conditions they observed organic acids formation. L-lactic acid was the main product (15.7 g/L), in addition small amounts of succinic and acetic acid were detected [25]. Preliminary studies in which *C. glutamicum* able to grow at temperatures above 40°C were used, shows that these strains, after 24-hour incubation in mineral medium are able to produce about 20 g/L of lactic acid. Among the fermentation products also acetic and succinic acids were found (unpublished results). This thesis shows that production of succinic acid by *C. glutamicum* requires not only the development of appropriate culture conditions, but first of all the genetic modifications of these microorganisms.

C. glutamicum is able to utilize monosaccharides such as glucose, fructose and ribose, disaccharides: sucrose, mannose, and maltose, alcohols: inositol or ethanol, organic acids: pyruvic acid, propionic acid, lactic acid, acetic acid and gluconic acid, and amino acids: L-glutamic acid and L-glutamine [54]. Different carbon sources derived from the environment are introduced at different levels to the central metabolic pathways such as glycolysis (Embden-Meyerhof-Parnas pathway, EMP), pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA) and glyoxylate cycle [55]. The most important central metabolite is pyruvate. It is produced mainly by glycolysis. It can be converted by lactate dehydrogenase (LDH), pyruvate carboxylase (PC), or pyruvate:quinone oxidoreductase (POQ) to L-lactate, oxaloacetate and acetate, respectively (Figure 2) [56]. Another important central metabolite is phosphoenolopyruvate (PEP). In oxygen deprivation conditions and in presence of CO_2 also phosphoenolpyruvate is carboxylated by PEPC to oxaloacetate. During succinic acid production the metabolism of bacteria requires to be directed towards transformation of pyruvate and phosphoenolpyruvate to oxaloacetate by appropriate carboxylases.

Inui et al. (2004) identified enzymes involved in the biosynthesis of organic acids and established their importance for cell metabolism. In their studies, they have considered: L-lactate dehydrogenase (LDH, gene *ldhA*),

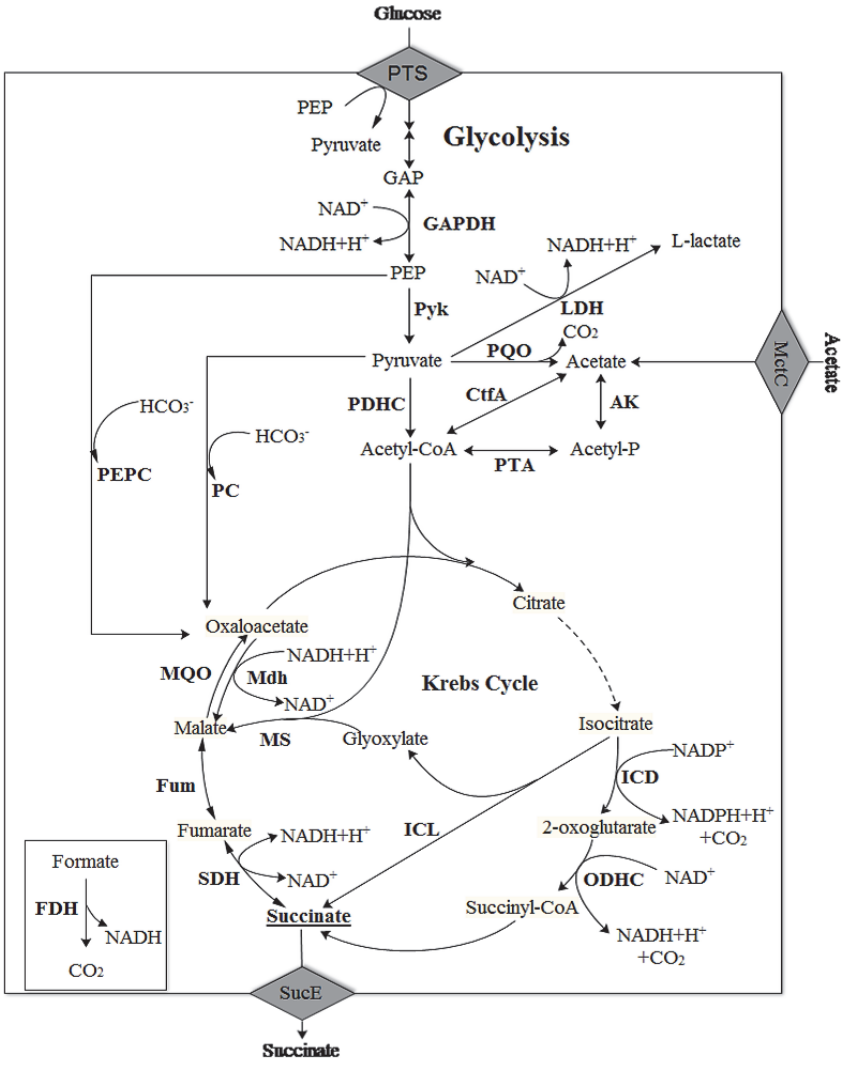

Figure 2. Succinic acid production pathway in *Corynebacterium glutamicum*
AK (*ack*) – acetate kinase, CtfA (*cat*) – CoA transferase A, FDH (*fdh* from *Mycobacterium vaccae*) – formate dehydrogenase, Fum (*fum*) – fumarase, GAP– glyceraldehyde-3P, GAPDH (*gapA*) – glyceraldehyde-3P dehydrogenase, ICD (*icd*) – isocitrate dehydrogenase, ICL (*aceA*) – isocitrate dehydrogenase, LDH (*ldhA*) – L-lactate dehydrogenase, MctC (*mctC*) – monocarboxylic acid transporter, Mdh (*mdh*) – malate dehydrogenase, MQO (*mqo*) – malate:quinone oxidoreductase, MS (*aceB*) – malate synthase, ODHC (*odhA*, *aceF*, *lpd*) – oxoglutarate dehydrogenase complex, PC (*pyc*) – pyruvate carboxylase, PEP – phosphoenolpyruvate, PEPC (*ppc*) – PEP carboxylase, PTA – phosphotransacetylase, PQO (*pqo*) – pyruvate: quinone oxidoreductase, PTS – phosphotransferase system, Pyc (*pyk)* – pyruvate kinase, SDH (*sdhCAB*) – succinate dehydrogenase, SucE (*sucE*) – succinate exporter

phosphoenolpyruvate carboxylase (PEPC, gene *ppc*), pyruvate carboxylase (PC, gene *pyc*), phosphoenolpyruvate carboxykinase (PEPCK, gene *pckA*) fumarase (FUM, gene *fum*) and isocitrate lyase (ICL, gene *aceA*) [26]. Bacterial strains with induced deletion of certain genes were constructed. Comparison of enzyme activity, maintaining the balance of NAD^+ and NADH, and the glucose consumption rate between wild-type and recombinant strains, with induced deletion of certain genes can lead to some interesting conclusions. The *ΔldhA* recombinant showed no lactate dehydrogenase activity, therefore it can be deduced that there are no LDH isoenzymes in the genome of *C. glutamicum*. Elimination of lactate dehydrogenase effectively blocked the biosynthesis of lactic acid, but also it disturbed balance between NAD^+ and NADH. In consequence glycolysis and glucose consumption is inhibited and expected increase in the concentration of succinic acid is not observed. Constructing of *C. glutamicum* strains: *Δppc*, *Δpyc*, *ΔppcΔpyc*, *ΔpckA* allowed to determine the importance of enzymes involved in anaplerotic reactions which direct the carbon flux into the reducing arm of the TCA cycle. In absence of PEPC activity, radical reduction in the amount of succinic and lactic acid is noted. Also the ratio between NAD^+/NADH has been disturbed. The lack of this enzyme inhibits the subsequent reactions in TCA cycle reductive arm in which NADH is oxidized. This causes inhibition of GAPDH and following on from this the whole glycolysis. Deletions of other genes did not significantly affect the metabolism. The carbon source consumption rate and concentrations of co-substrates were similar to wild-type strain. Studying *ΔldhAΔppc* double mutants it was observed that the lack of activity of PEPC and LDH seriously impairs cell functioning. In limited oxygen conditions PEPC activity is critical for the regulation of the very significant metabolic node. This enzyme is responsible for providing the substrate – oxaloacetate for the reductive TCA cycle arm, whose function includes regeneration of NAD^+. The proof this statement strains with fumarase deletion were constructed. Lack of this enzyme also disrupts glycolysis [26].

An important factor, influencing the course of the metabolism, is the oxygen concentration in the fermentation medium. Its limitation affects the proportions between the available cofactors. Elevation of the amount of oxidized dinucleotide induces increase in the activity of certain enzymes involved in glycolysis, for example: glyceraldehyde-3P dehydrogenase (GAPDH), triosephosphate isomerase (TPI), phosphoglycerate kinase (PGK) and already mentioned anaplerotic enzymes responsible for the biosynthesis of lactate and succinate, such as phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) [26, 49, 50].

In *C. glutamicum* cells, glycolysis is regulated by a kind of a cascade involving enzymes responsible for the oxidation and reduction of cofactors. Carbon dioxide is formed by the oxidation of pyruvate to acetyl-CoA and acetate or isocitrate to 2-oxoglutarate. It is used for carboxylation of pyruvate or phosphoenolpyruvate whereby oxaloacetate, the substrate for the reductive arm

of TCA cycle is formed (Figure 2). Nicotinamide adenine dinucleotide (NADH – reduced form, NAD^+ – oxidized form) is a cofactor and component regulating multiple metabolic pathways. In the tricarboxylic acid cycle oxaloacetate is converted to malate by malate dehydrogenase, the reaction requires presence of the reduced form of NADH or NADPH, whereas conversion of fumarate to succinate by succinate dehydrogenase involves the MQH_2 availability. Regeneration of the oxidized menaquinone requires the presence of NADH. The basic way of sugars assimilation is glycolysis. The activity of enzymes involved in glycolysis depends on maintaining the balance between the cofactors. The oxidized form of NADH is used by GAPDH, the excess of the cofactor reduced form inhibits the metabolism of sugars. Fermentation process can be directed by deletion of certain genes, introduction of more active isoforms of enzymes or providing additional, specific substrates for the anaplerotic reactions responsible for the regeneration of the co-substrates, but all this changes may affect the activity of the glycolytic pathway [26, 57].

Genetic modification of *Corynebacterium glutamicum* towards overproduction of succinic acid

The first papers concerning the production of organic acids by *C. glutamicum* were focused on the main product of fermentation – L- lactic acid. Wild strains of these microorganisms possess only L-lactate dehydrogenase, so that it is possible to obtain high purity L-lactate (> 99.9%) [26]. As it was already mentioned, in oxygen deprivation condition succinic acid is produced in small amounts, therefore metabolic paths have to be directed toward reducing arm of the Krebs cycle, where succinate is synthesized. Inui et al. (2004) used *C. glutamicum* R, which was proliferated to a high density, then biomass were separated and placed in the fermentation medium, supplemented with sodium bicarbonate as an additional source of CO_2 for PEPC and PEP. Under these conditions, activation of the reductive arm of the TCA cycle, an increase in the amount of NAD^+ and intensification of glycolysis were observed [26]. In another experiment the same group used thick suspension of bacterial cells (30 $g_{d.w.}$/L) in fed batch fermentation. As a result 52 g/L L-lactate, small amount of succinate (1.18 g/L) and acetate were received [27]. The addition of CO_2 source (sodium bicarbonate) to the fermentation medium leads to an increase in the total concentration of organic acids, and changed the proportions between them. The more bicarbonate is added the more glucose is transformed into succinic acid. In fed batch fermentation in the presence of 400 mmol bicarbonate 90 g/L L-lactate, 22.7 g/L succinate and 3 g/L acetate were obtained [27].

In oxygen deprivation conditions L-lactic acid is generated as a main product. Pyruvate is formed as the final product of glycolysis and is a key central metabolite used for synthesis of succinate, lactate and acetate as well as L-alanine or L-valine (Figure 2) [48, 57].

Table 1. Biotechnological production of succinic acid by different bacterial species

Stain	Medium	Succinic acid titre g/L (mmol/L)	Succinic acid yield mol succinate/mol substrate (g/g)	Productivity mmol/L/h (g/L/h)	By-products	References
***C. glutamicum* R *ΔldhA* pCRA717**	Mineral, with glucose and bicarbonate	146,3 (1240)	1,40 (0,92)	27 (3,1)	acetate	[27]
C. glutamicum* BOL-3/pAN6-*gap	Mineral, with glucose bicarbonate and formate	133,8 (1134)	1,67 (1,09)	21 (2,48)	2-oxoglutarate, acetate, fumarate, malate	[30]
C. glutamicum* BL-1 pVWEx1-*glpFKD	Mineral, with glycerol	9,3 (79)	0,21 (0,27)	3,6 (0,42)	acetate	[31]
***C. glutamicum* BL-1/pAN6pyc^{P458S}ppc**	Mineral, with glucose	10,6 (90)	0,45 (0,30)	0,8 (0,09)	2-oxoglutarate, acetate	[29]
***C. glutamicum* ZX1** (p*EacsAgltA*)	Mineral, with glucose	27,1 (241)	0,63 (0,42)	3,55 (0,32)	pyruvate	[64]
C. glutamicum* ATCC13032/pCC-*pgsA-amyA	Mineral, with starch	14 (119)	1,33 (0,88)	-	lactate, acetate	[65]
***A. succinogenes* FZ53**	Complex, with glucose	105,8 (897)	1,26 (0,83)	0,18 (1,3)	formate, acetate	[10]
***M. succiniciproducens* LPK7**	Complex, medium with glucose	52,4 (444)	0,35 (0,76)	15 (1,75)	acetate, formate, lactate	[28]
***E.coli* AFP111(p1rc99A-*pyc*)**	Complex with glucose and bicarbonate	99,2 (837)	1,68 (1,10)	11 (1.30)	acetate, pyruvate	[39]

For this reason Litsanov et al. (2012) assumed that the key issues relating to the production of succinic acid by *C. glutamicum* are: elimination of lactic and acetic acid biosynthesis, maintaining the appropriate balance between the $NADH/NAD^+$ and provide access to large amounts of CO_2 [30, 35].

C. glutamicum R *ΔldhA* pCRA717 was devoid of LDH activity and exhibiting an overexpression of native pyruvate carboxylase (PC). It was used in two-stage batch fermentation previously proposed by Okino et al. (2005). The biomass of bacterial cells were placed in mineral salts medium the concentration of about 50 g/L. Using the periodic addition of glucose and sodium bicarbonate, after 46 hours of fermentation 146 g/L succinic acid was obtained in a yield of $Y_{P/S}$ 1.4 mol succinate per mol of glucose (Table 1). Unfortunately, also the by-products were formed, the main of them was acetic acid (1.8 g/L) [27].

Litsanov et al. (2012) used *the C. glutamicum* ATCC 13032 to construct a new strain with repressed expression of the main enzymes responsible for the biosynthesis of lactic and acetic acid. Production of L-lactate was stopped due to deactivation of *ldhA* gene. Blocking the expression of several genes encoding acetylo-CoA:CoA transferase (*cat*), pyruvate:menaquinone oxidoreductase (*pqo*), fosfotransacetylase and acetate kinase (*pta-ackA*) caused a reduction of acetic acid biosynthesis. The new strain *C. glutamicum ΔcatΔpqoΔpta-ackAΔldh* was called BOL-1 [30].

As previously mentioned, the bottleneck of succinic acid biosynthesis is carboxylation of pyruvate and phosphoenolpyruvate. Plasmid pAN6-*pyc*^{P458S} contains homologous pyruvate carboxylase which activity is increased by exchange of the proline at the position of 458 to serine [59]. Gene *pyc*^{P458S} under the control of a strong, constitutive promoter *Ptfu* (encoding translation elongation factor Tu) [60] was integrated into the BOL-1 bacterial chromosome, in previously deleted coding region of the gene *pta-ack*. As a result strain *C. glutamicum ΔcatΔpqoΔpta-ack::*P_{tfu} *pyc*^{P458S}*ΔldhA* (BOL-2) was created [30].

Strains *C.glutamicum ΔldhA*/pAN6-*pyc*^{P458S}, BOL-1/pAN6-*pyc*^{P458S} and BOL-2 were tested for their ability to the production of succinic acid. To provide adequate amount of CO_2 for carboxylation, 200 mM bicarbonate was added [30]. After 21 hours fermentation with *C. glutamicum ΔldhA*/pAN6-*pyc*^{P458S} succinic acid concentration reached 15.3 g/L and $Y_{P/S}$ of up to 1.16 mol of succinic acid per mol of glucose. Aside from succinate small amounts of acetate (3 g/L), pyruvate, malate, fumarate and 2-ketoglutarate as by-product were formed. BOL-1/pAN6-*pyc*P^{458S} produced 90% less acetate than the reference strain. Glucose consumption and production of succinic acid (13.6 g/L) was also about 38% lower. *C. glutamicum* BOL-2 showed similar characteristics to BOL-1/pAN6-*pyc*^{P458S}, however the rate of glucose consumption and the production of succinic acid were more than 80% higher than during fermentation with strain BOL-2 [30].

Efficient succinic acid biosynthesis requires the presence of NADH, but the excess inhibits one of the enzymes involved in glycolysis. Formate dehydrogenase,

encoded by a gene *fdh* derived from *Mycobacterium vaccae* was introduced under the control of a strong promoter *Ptfu* into *C. glutamicum* cells. This enzyme catalyses oxidation of formate to carbon dioxide, donating the electrons to NAD^+ (Figure 2) [60]. A single copy of the *fdh* gene was incorporated into BOL-2 bacterial chromosome, in a place of the locus *Δpqo*. As a result *C. glutamicum Δpqo::*P_{tfu}*fdhΔpta-ack::*P_{tfu}*pyc*^{P458S}*ΔldhA* (BOL-3) was obtained. Constitutively expressed *fdh* gene allows to provide additional portions of CO_2 and NADH. Carbon dioxide is a substrate for appropriate carboxylase during biosynthesis of oxaloacetate. Additional portions of NADH are used in the reductive arm of the TCA cycle, which is responsible for NAD^+ generation. In result glycolysis should be stimulated, but to high FDH activity is the reason for the inhibition of glycolysis. In further modifications of the strain BOL-3 homologous gene *gapA* encoding glycerol-3-P dehydrogenase (GAPDH) was used. Overexpression of the gene *gap* was achieved by introducing it in pAN6-*gap* plasmid. Thus, increased specific activity of GAPDH compensated the inhibition of this enzyme caused by an excess of NADH. Strains were tested for their ability to production of succinic acid and formation of undesirable metabolites, such as acetic and lactic acid. Fermentations were carried out in a medium containing bicarbonate and sodium formate. If the tested cells had no FDH activity, formate caused decrease in the amount of succinate and glucose consumption rate. After 3 hours of fermentation strain BOL-3 reached stationary phase, which lasted for several hours and then again started to consume glucose and generate succinic acid. This phenomenon could be explained as the effect of formic acid. In undissociated form it penetrates the cytoplasmic membrane and distorts ion gradient which is pH depended [30, 61]. Finally, 16.6 g/L succinate was obtained with yield $Y_{P/S}$ 1.26 mol succinate per mol of glucose. The concentration of TCA cycle intermediates such as malate and fumarate increased while the amounts of undesired metabolic products such as acetic acid were reduced. Fermentation using strain BOL-3/pAN6-*gap* had a slightly different course. During the fermentation stationary phase was not observed, after 20 hours 17.8 g/L succinic acid and the yield $Y_{P/S}$ 1.41 mol succinate per mol of glucose were obtained. Again, in the fermentation broth malate and fumarate were identified. BOL-3/pAN6-*gap* strain was also tested during the fed-batch fermentation. Production medium contained sodium formate and bicarbonate, which were added periodically. The cells remain metabolically active for more than 2 days. After addition of the last formate portion, fermentation was stopped. Its high concentration of about 35 g/L probably has toxic effects on cell metabolism. After 53 hours of fermentation 133 g/L succinic acid were formed, at this time the cells used 679 mmol/L glucose and 772 mmol/L formate (Table 1). The yield of product relative to substrate reached: 1.67 mol succinate per mol of glucose, this is the highest score achieved so far for the anaerobic fermentation of succinic acid with *C. glutamicum* [30]. Unfortunately, the use of

formate increases the cost of production and purification of succinic acid, and therefore the method may be prohibitive in large scale.

Zhu et al. 2014 presented a dual route for anaerobic succinate production by *C. glutamicum*. In the first step they eliminated genes encoding L-lactate dehydrogenase (*ldhA*), enzymes responsible for acetate synthesis (*pqo, cat, ackA*) and pyruvate carboxylase (*pyc*). Phosphoenolpyruvate carboxylase (*ppc*) responsible for oxaloacetate synthesis and genes coding for enzymes involved in the glyoxylate cycle, such as isocitrate lyase (*aceA*), malate synthase (*aceB*) and citrate synthase (*gltA*) were overexpressed. In order to ensure effective export of succinate to fermentation medium, putative exporter encoded by *sucE* gene was up-regulated. Described strain *C. glutamicum* SA5 was used in dual phase, fed-batch fermentation including an aerobic growth and anaerobic fermentation. Anaerobic phase was carried out with 27.5 g dry cell/L. After 98 h of fed-batch fermentation succinic acid concentration reached 109 g/L, with $Y_{P/S}$ 1.32 mole succinate per mole of glucose. Acetate and small amounts of the TCA cycle intermidients such as α-ketoglutarate, pyruvate, malate and fumarate were formed [37].

Aerobic conditions have many advantages over anaerobic processes, among them is worth to mention about higher biomass generation, faster carbon throughput and product formation. Litsanov et al. (2012) proposed aerobic production of succinic acid. Under these conditions an intensive growth of *C. glutamicum* biomass is observed. To cause inhibition of growth and conversion of carbon sources mainly to organic acids, nitrogen sources have to be limited. The production of succinic acid in aerobic culture was achieved by construction of *C. glutamicum ΔsdhCAB* strain, which was devoid of succinate dehydrogenase (SDH), the Krebs cycle was inhibited at the stage of conversion of succinic acid to fumaric acid. Further modifications were aimed at reducing acetic acid biosynthesis. The mutant *ΔpqoΔpta-ackAΔsdhCABΔcat* was called *C. glutamicum* BL-1. Deletions of the enzymes responsible for the production of acetic acid such as pyruvate: quinone oxidoreductase (PQO*, pqo*), CoA transferase (CoAT, *cat*), phosphotransacetylase (PTA) and acetate kinase (AK), which are under the control of the *pta-ack*, do not eliminate the formation of acetate completely [29]. It is not clear which other proteins are involved in the formation of acetate. Anaplerotic reactions providing oxaloacetate to the TCA cycle have an influence on the succinic acid biosynthesis. To increase its availability strain *C. glutamicum* BL-1/pAN6-*pyc*^{P458S}*ppc* was constructed. Genes *pyc*^{P458S} and *ppc* introduced under the control of a strong promoter triggered a considerable increase in the production of succinic acid. Finally 10.6 g/L of succinic acid were produced with a yield $Y_{P/S}$ 0.45 mole succinate per mole of glucose [29].

Zhu et al. (2013) were engineered *C. glutamicum* ATCC 13032 to increase succinate production and eliminate acetate accumulation. Genes encoding L-lactate dehydrogenase (*ldhA*), succinate dehydrogenase complex (*sdhCAB*) and

enzymes responsible for acetate synthesis (*pqo, pta, cat*) were disturbed. Native promoters of the genes coding pyruvate and phosphoenolpyruvate carboxylase (*pyc*, *ppc*) were replaced by the strong promoter of the superoxide dismutase (*sod*). The resulting strain was named *C. glutamicum* ZX1. To avoid the accumulation of acetate expression of several enzymes responsible for the conversion of acetyl-coA to acetate should be blocked [62-64]. Alternatively, introduction of more efficient acetate assimilation pathway could solve this problem. The assimilation pathway from *Bacillus subtilis*, catalyzed by AMP-forming acetyl-CoA synthetase (*acsA*) was expressed in strain ZX1. New strain ZX1 (pE*acsA*) did not accumulate acetate at the end of fermentation, this time pyruvate was the main by-product. Reduction of its accumulation was obtained by citrate synthase overexpressing. Therefore, the gene encoding the native citrate synthase (*gltA*) was introduced into strain ZX1 using plasmid pE*acsAgltA*. The resulting strain ZX1 (pE*acsAgltA*). To assess the ability of the resulting strain for succinic acid production under aerobic conditions, fed-batch cultures were carried out. Strain *C. glutamicum* ZX1 (pE*acsAgltA*) produced 21.7 g/L succinate with $Y_{P/S}$ 0.63 mole succinate per mole of glucose (Table 1). The major by-product was pyruvate [64].

As it was shown in Table 1 the highest succinic acid, production described so far, was obtained by strains *C. glutamicum* R *ΔldhA* pCRA717 and *C. glutamicum* BOL-3/pAN6-*gap* which produce in oxygen deprivation conditions, respectively 146 and 133 g/L of succinic acid [27, 30], whereas *A. succinogenes* FZ53 produces 105 g/L [12] and *E. coli* AFP111(pTrc99A-*pyc*) 100 g/L [35]. Both of the *C. glutamicum* strains exhibit excellent properties. Alas, they are still not suitable for use in industrial scale. However, the concentration of succinic acid achieved during fermentation with any other microorganism is significantly lower. Likewise, aerobic succinate production involving *C. glutamicum* is not possible. Described above *C. glutamicum* ZX1 (pE*acsAgltA*) synthesized only 21.7 g/L of succinate (Table 1) [64].

Succinate production from alternative carbon sources by *Corynebacterium glutamicum*

The primary carbon source in described process was glucose, additionaly formate or carbonate were added in some cases. To reduce the cost of organic acids production, waste substances from different industries could be used. However, the metabolic abilities of *C. glutamicum* wild strains do not allow using many cheap and available carbon sources. Numerous strains able to utilize alternative substrates, such as starch [65-69], lactose [70-71], and galactose [71], arabinose [72-75], xylose [76-80], cellobiose [80-81]; dicarboxylic acids [82-83] or glycerol [31, 84, 85] have been constructed. We would like to present only the two most interesting examples.

Previously described *C. glutamicum* BL-1 was used to create a new strain able to utilize glycerol, waste substance generated during the biodiesel production.

The gene operon *glpFKD* from *E. coli* encoding proteins responsible for transport and metabolism of glycerol has been cloned into the *C. glutamicum* BL-1. As a result, strain *C. glutamicum* BL-1 pVWEx1-*glpFKD* was constructed. It was able to aerobic production of about 79 mol/L (9.3 g/L) succinic acid from glycerol with a yield $Y_{P/S}$ 0.21 mole succinate per mole of glycerol (Table 1) [31].

Biosynthesis of organic acids from starch is possible by using cell surface-engineered *C. glutamicum* ATCC 13032. Gene *amyA*, encoding α-amylase from *Streptococcus bovis* 148, was fused with C-terminal region of an anchor *pgsA* from *Bacillus subtilis*. The construct was inserted into pCC plasmid and cloned into *C. glutamicum* cells. The recombinant strain exhibited the ability to express the PgsA-anchored α-amylase on the cell surface, degraded starch and produced organic acids. Fermentations, with high-density cell suspensions were carried out in three different temperatures: 30, 35 and 40°C. Maximum for organic acid concentration was observed in 35°C. Unfortunately the higher temperature the more significant decrease in activity of the α-amylase was observed. After five 10 h fermentation cycles with biomass recycle, *C. glutamicum* ATCC13032/pCC-*pgsA-amyA* produced 107.8 g/L of total organic acids, including 88,9 g/L (0.99 M) lactate and 14.0 g/L (119 mmol/L) succinate (Table 1) [65].

Conclusion and future prospects

Bio-succinate is a very desirable product due to the large number of applications. For several years, potential producers of this acid have been sought. Among the most frequent mentioned bacterial species, which may be used for the biosynthesis of the acid are *Escherichia coli*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens* and *Corynebacterium glutamicum* (Table 1).

Technologies with *C. glutamicum* have many advantages. Among them it is worth to remark the possibility of using cheap, mineral media which not only reduce the manufacturing costs at the stage of fermentation, but also during isolation and purification of the final product. Lack of cells growth under anaerobic conditions enables the carbon sources to be completely transformed into succinate. There is also the possibility of biomass recycling and using it again in the next cycle of fermentation, which further reduces costs and increases efficiency of the process. However, to achieve effective production of succinate it is necessary to introduce a number of improvements. Succinic acid production with *C. glutamicum* requires high concentration of biomass in the fermentation medium. It may entail technological problems. Therefore, it is necessary to increase succinate production rates. Economical relevant and sustainable biosynthesis of organic acids in an industrial scale are dependent on the use of low-cost carbon sources, in particular from renewable resources. However, *C. glutamicum* cannot utilize many of them. Especially glycerol, starch, whey, straw or hemi- and lignocellulose are widely available and potentially attractive sources of renewable feedstock. Therefore *C. glutamicum* needs to be genetically modified to expand the spectrum of sugars that can be utilized. Other genetic

modifications are necessary to limit the production of metabolic waste products, especially acetate. To make this possible, we need to deepen the knowledge about the metabolism and cell function.

Bio-succinate large scale production can significantly reduce the use of non-renewable carbon sources and greenhouse gas emission, so we believe that worthwhile and competitive technology can be established in the near future.

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Research article

Determination of total contents of transition metals in selected granular black teas marketed in Poland

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Abstract: *The total contents of metals in three granulated black tea samples marketed in Poland were determined. Four elements consisting transition metals (Ni, Cu, Zn and Fe) were analyzed using flame atomic absorption spectrophotometry (FAAS). All examined tea brands contain considerable contents of the studied transition metals. Among the tested transition metals Fe (242-589 mg kg^{-1}) was the most abundant one in the granular black tea imported from India. The highest value of Fe was analyzed in the cheapest tea, the smallest in the most expensive. The other metals are less abundant than Fe, their values varied from 8.50-8.90 for Ni, 7.80-10.4 for Cu and 8.13-12.6 for Zn mg kg^{-1}. The accuracy of the proposed method was assessed by determining the recovery of metals from analyzed tea samples using the standard addition method. Recovery assays of nickel, copper, zinc and iron were demonstrated satisfactory, mean recoveries 99.29, 100.31, 99.94 and 99.79 %, respectively.*

Keywords: *transition metal, granular tea, flame atomic absorption spectrometry.*

Introduction

A variety of chemical elements contained in food and beverages are relatively easy absorbed by the human body. Despite the fact that most of them are very important for human health, they can also be toxic, which usually is the result of the presence of contamination in soil, the application of fertilizers and pesticides. Therefore, it is important to study the daily intake of trace elements in the diet [1, 2].

So far, tea has been reported as one of the most widely consumed beverage in the world, taking the second place, after water, in terms of consumption [3, 4]. This represents over 40% of all beverages consumed in winter and 25% in summer. The annual tea consumption *per capita* in Poland is almost 1kg. Most consumers drink this beverage 2-3 times a day, and about 20% even 4-5 times a day [5]. It is estimated that the tea market in Poland is the third largest among all markets in Europe, after Russia and the United Kingdom. In 2011, black tea (including granular one) and herbal tea made out respectively 69.6% and 15.8% of the Polish tea market. Tea is perceived by consumers as a tasty, healthy and safe beverage [6]. Tea drinking has been also claimed to have a large number of

health benefits, including cancer and heart diseases prevention [7]. These advantages are attributed to polyphenolic compounds occurring in tea [3].

Unfortunately, more often, the presence of contaminants in tea leaves may arise as a result of environmental pollution: the air, water and soil [8]. Significant impact on the presence of harmful elements is the quality of drinking water used for tea brewing [9]. The majority of polluting agents (lead, cadmium, arsenic, mercury or nickel) occurring in tea have deleterious effects on human health despite the fact that only about 40% of them goes to the brew. The current European Union legislation on contaminants in foods does not set maximum levels of harmful elements in tea [9].

The content of trace elements, including copper, iron, nickel and zinc, both in raw materials and final products of the food industry should be controlled due to several reasons [10]. Firstly to judge their nutritional value and secondly to guard against any probable ill-effect. For example, copper can be associated with non-Indian childhood cirrhosis [11] and Wilson's disease [12], iron - Parkinson's disease and Alzheimer's disease [13], nickel – carcinogenicity or allergy [14], zinc – nausea, vomiting or epigastric pain (only with extremely high zinc intakes) [15].

The use of rapid methods for the determination of trace elements in food has significant importance [16-20]. These methods should have a high selectivity, a suitable analytical sensitivity and simplicity of performance, as well as reliability, high precision of determinations made, international recognition and facility of validation. These features dominate in atomic spectrometry methods with particular emphasis on atomic absorption spectrometry (AAS). The large popularity of techniques based on the principles of atomic spectrometry results from the fact that these techniques are accurate, precise, flexible and relatively inexpensive. Atomic absorption spectrometry is the most comprehensive method due to a variety of analytical techniques and relatively well recognized interference occurring in different techniques [21].

The aim of this study was the quantitative determination of total zinc, copper, iron and nickel contents using AAS method in teas which are known and generally available on the Polish market. The obtained mean elemental concentrations were compared with the corresponding values of different countries available in the literature.

Experimental

Materials

Atomic absorption standard solution of Ni, Zn, Cu and Fe at a concentration of 1000 mg/L were obtained from Merck Sp. z o.o. Darmstadt, Germany. Nitric acid applied in digestion process was of analytical reagent grade (Lach-Ner, Neratovice, Czech Republic). Ultrapure water from Elix system (Millipore, Bedford, MA, USA) was used for preparation of working reagents and for sample dilutions. The glassware was cleaned and immersed in 4.5 % nitric acid

(J.T. Baker, Deventer, The Netherlands) for 24 h. Teflon beaker was treated with 4.5 % nitric acid and washed with ultrapure water. To prepare the final standard stock solutions, 1 mL of nickel and iron, 0.5 mL of copper and 0.2 mL of zinc standard solution (1000 mg L^{-1}) was diluted with ultrapure water to 100 mL. Standard working solutions were freshly prepared by diluting the obtained final standard stock solution with ultrapure water to an appropriate concentration before analysis.

Samples

Three, black and granulated tea samples from various trade marks were purchased from stores in Lodz. The selected teas were imported from India. They varied in respect of price. The first sample was the most expensive, followed by the second and the third sample, which was the cheapest. The study samples were collected in the first quarter of 2012. Each package of tea weighed 100 g. Three replicate samples of each tea were quantified using flame atomic absorption spectrophotometry (FAAS).

Methods

Preliminary treatment of samples

The tea granules were crushed using pestle and mortar. The powder was stored at room temperature in dry containers for analysis. To avoid metallic contamination, nonmetallic tools were used during sample preparation.

In these studies 10 ± 0,1 g of tea sample was used. It was the most optimal portion of the tea to determining all tested transition metals. Due to industrial safety 1 ± 0,1 g portions of tea samples were used in a microwave digestion system. Each portion of tea was treated 8 mL of 65 %HNO_3. The vessels were heated in a microwave digestion system according to the manufacturer's instructions (Magnum II, Ertec, Wroclaw, Poland). After mineralization, the liquid residues were placed in a final volumetric flask (100 mL) and diluted with distilled water. A blank mineralization was carried out in the same way.

Measurements

For metal analysis, a GBC 932 AA Spectrometer from GBC (Scientific Equipment Pty Ltd., Dandenong, Victoria, Australia) with deuterium background correction was used. The system was operated via GBC Avanta Ver. 1.33 Software Package. Spectrometer was equipped with hollow cathode lamps and an air–acetylene burner. The instrument parameters are shown in Table 1. All samples were prepared in triplicate and injected consecutively in triplicate. The samples were diluted and measured only in case of analysis of iron. The calibration curve was obtained using three standards and blank. To assess the accuracy of the total procedure, the determined samples were enriched with metal standard solutions and re-analysed.

Results and Discussion

Spectrometric analysis of granulated black teas is relatively inexpensive and the operation is simple, it concerns an analytical method for the determination of the transition metal contents in tea [22]. AAS methods require destruction of organic matter by wet mineralization. In our method we have optimized samples mass to 10 ± 0,1 g in order to the detection limits were exceeded. The executed method, which does not require very complicated pre-analytical manipulation, detects the concentration of metals with good accuracy and precision. The analytical curves of the selected metals, obtained by plotting the mean of absorbance at the appropriate wavelengths (Table 1) against the concentration generated the specific parameters which are shown in Table 2.

Table 1. Instrumental conditions of the flame atomic absorption spectrophotometer GBC 932

	Nickel	Copper	Zinc	Iron
Wavelenght (nm)	232.0	324.7	213.9	248.3
Slit width (nm)	0.2	0.2	0.2	0.2
Lamp current (mA)	4.0	3.0	5.0	5.0

Table 2. Linearity parameters for the determination of selected metals

Parameters	Ni	Cu	Zn	Fe
Linearity range [μg mL^{-1}]	2.5 to 10	1.25 to 5.0	0.5 to 2.0	2.5 to 10
Slope	0.0464	0.1056	0.222	0.0409
Intercept	0.033	0.004	0.093	0.0045
Correlation coefficient (r)	0.9991	0.9998	0.9882	0.9998

The accuracy of the proposed method was assessed by determining the recovery of metals from analyzed tea samples using the standard addition method (Table 3). Recovery assays of nickel, copper, zinc and iron demonstrated satisfactory, obtaining mean recoveries of 99.29, 100.31, 99.94 and 99.79 %, respectively.

Table 3. Recovery metals from tea samples

Tea samples	Ni [%]	Cu [%]	Zn [%]	Fe [%]
1	87.02	100	93.27	93.28
2	92.20	97.48	103.64	104.29
3	120.61	103.45	102.46	103.45
Mean recovery	99.29	100.31	99.94	99.79

The results of total contents of the studied trace elements namely nickel(II), copper(II), zinc(II) and iron(III) are presented in Table 4. Determination of transition metals in samples was carried out by FAAS. The values are expressed as mean values (three separate determinations) ± standard deviation of the mean.

The results show the ability of tea as a plant to accumulate transition metals, particularly Fe, to a lower extent Ni, Cu and Zn. Iron as the most abundant metal in granulated tea ranged from 242.33-589.33 mg kg^{-1}. The highest value of Fe was analyzed in the cheapest tea, the smallest in sample No. 2 (the most expensive). The other metals are less abundant than Fe, their values varied from 8.50-8.90 for Ni, 7.80-10.4 for Cu and 8.13-12.6 for Zn mg kg^{-1}.

Table 4. Mean total contents of selected transition metals (Each value is the mean of three samples)

Concentration of metals with standard deviation [mg kg^{-1}] and coefficients of variation (CV) [%]	Tea samples		
	1	2	3
Ni±SD [mg kg^{-1}]	8.50±0.10	8.90±0.53	8.87±0.06
CV [%]	1.2	5.9	0.7
Cu±SD [mg kg^{-1}]	8.47±0.46	7.80±0.95	10.4±1.40
CV [%]	5.4	12.2	13.5
Zn±SD [mg kg^{-1}]	9.43±0.91	8.13±1.02	12.6±0.89
CV [%]	9.6	12.5	7
Fe±SD [mg kg^{-1}]	276.67±6.43	242.33±100.81	589.33±21.59
CV [%]	2.3	41.6	3.6

These metals could be put in a descending order according to their contents in granulated tea as follows:

$$\text{Fe}\ (242.33\text{-}589.33) > \text{Zn}\ (8.13\text{-}12.6) \geq \text{Cu}\ (7.80\text{-}10.4) \geq \text{Ni}\ (8.50\text{-}8.90)$$

The presented order of the total metal content was illustrated graphically in Figure 1. In laboratories precision is expresses as a coefficient of variation, which is nothing more than the standard deviation divided by the mean and expressed as a percentage. In accordance with analytical consideration of World Health Organization the precision determined at each concentration level should not exceed 15% of the coefficient of variation. In this study values of CV for Ni, Cu and Zn do not exceed recommended level. Only in the case of Fe in tea sample No. 2 CV value was significantly higher (41.6%).

Analyzing the contents of four metals in the tested tea samples a variety of results can be concluded (Table 4). Among the tested tea samples, the sample No. 3 as the cheapest one has the highest contents of (Cu, Zn and Fe). The maximum level of Fe (589.33 mg kg^{-1}) in this sample was too high in comparison to teas No. 2 and 3. Such a high iron content in tea the consumers should consider. Generally, Fe showed the highest values in all teas in relation to other metals. The lowest values of Cu and Zn were found in tea sample No. 2 and the highest - in tea sample No. 3. This study shows that the Cu and Zn contents in all the tea samples were less than 13 mg kg^{-1}, which is well below the permissible limit of 20 mg kg^{-1} and 50 mg kg^{-1} respectively, under Polish Regulation of the

Minister of Health and Social Welfare of 27 December, 2000 [23]. According to Falandysz & Kotecka [24] the content of microelements in black tea leaves, including zinc is much higher, compared to green tea. Copper is a component in fungicides applied regularly on the plantations of coffee, tea and cocoa, for protection against the pathogen causing leaf disease.

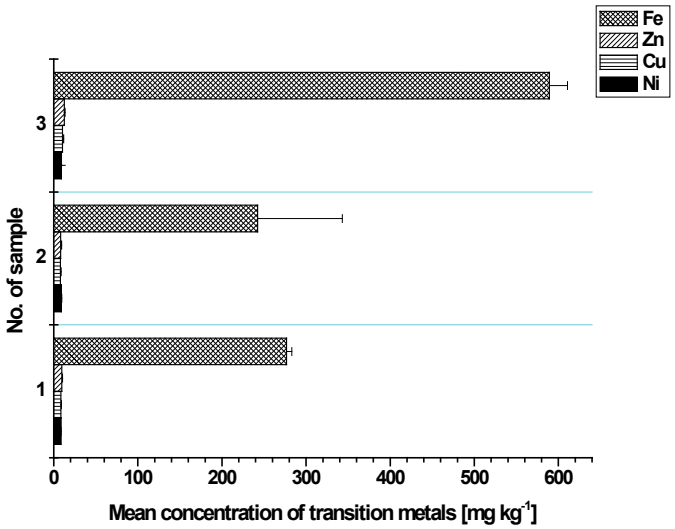

Figure 1. Comparison of data of total contents of metals in black tea samples imported from India

Table 5. The total contents of transition metals in tested tea samples

Tea samples	Used analytical method	Ni [mg kg-1]	Cu [mg kg-1]	Zn [mg kg-1]	Fe [mg kg-1]
Indian teas (own data)	FAAS	8.50-8.90	8.0-10.4	8.13-12.6	242-589
Indian teas [25]	ICP-AES	4.88	23.21	27.26	146.9
Lipton (lose) [26]	ICP	4.8	24.08	31.96	194,.
Lipton® *Yellow label* [27]	ICP-QMS	6,.4	25.3	34.2	344
Indian teas [16]	AAS-GF	24.07	2.53	-	-
Imported teas [24]	FAAS	-	29-34	31-35	230-260

Natural copper content in tea leaves is approximately 28 mg kg^{-1}, and levels above 70 mg kg^{-1} dry weight suggest that copper compounds are regularly used on the plantation [24]. Considering the obtained results, it should be noted that the plant material, used for our analysis, has not been exposed to copper fertilizers. Since Ni and Fe are the toxic elements, not having any tolerance limit in tea, the agro inputs used in tea fields should be analyzed for heavy metal impurities.

Comparing the total content of selected metallic components in analyzed tea samples imported from India with other studies (Table 5) [24-28], values of Cu and Zn from own studies have not exceeded the concentration from literature data. Whereas, the contents of Ni and Fe, and especially the last metal, significantly exceeded the amounts given in the references. Probably, these differences may be due to different working tools, the type of analytical method, measurement error, tea cultivation area or agricultural differences. The granular teas have a lower quality, nevertheless the beverage is popular among the poorer because it is the cheapest. Therefore, monitoring the contents of transition metals in such teas is important and necessary.

The information specifying the rational total contents of transition metals in granular black tea is lacking in literature. The maximum allowed concentration of each metal in granular black tea is needed. Occasional analysis and frequent examination of beverages is advisable to avoid problems that could arise from intake beyond the tolerance limits standards.

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Review article

ZnS Cu-doped quantum dots

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***Abstract**: The paper presents a survey of literature on the structure and optical properties of ZnS and copper ion-doped ZnS quantum dots. The effect of other metal dopants on the spectral properties of ZnS:Cu quantum dots was also considered. The influence of such parameters as dopant concentration, temperature of the synthesis and compounds which form or modify the additional layer on dots on spectral properties of the quantum dots was described. Examples of application of ZnS:Cu quantum dots are also given.*

***Keywords**: quantum dots, photoluminescence, fluorophores, fluorescence, chemosensor, biosensor.*

Introduction

The continuous development of biotechnology, food production and medicine requires new methods of medical diagnostics and food analysis. Considerable attention is paid to analytical methods for fast, easy and selective determination of many chemical substances, such as hydrogen peroxide, carbon monoxide and oxygen [1], or physicochemical parameters, such as pH [2] and temperature [3].

A response to the existing demand may be optical sensors characterized by high sensitivity and selectivity [1, 4, 5]. The main component of a fluorescence optical sensor is fluorophore immobilized on a carrier whose emission varies in the presence of a determined substance or under the influence of physicochemical parameters [6]. Operation of this type of sensor is based on the detection of fluorescence changes which take place under the influence of the agent being determined [2, 7, 8]. The biosensor is formed as a result of applying in the carrier, beside the fluorophore, a biological receptor such as an enzyme, antibody or microorganism which greatly extends the range of determined substances with important biological compounds such as glucose and urea [4, 6, 9, 10]. However, organic fluorophores are often unstable in solution and can exhibit toxicity which prevents their use *in vivo*. They are characterized by a broad emission band making interpretation of results difficult [11]. These drawbacks have contributed to the search for new solutions.

Quantum dots have become an attractive alternative to organic fluorophores. Due to unique spectral properties they are in the focus of research of many centers in the world [12-14].

Structure and properties of quantum dots

The structure of quantum dots

Quantum dots are semiconductor nanocrystals with a size of 1 to 100 nm, where the quantum confinement effect occurs. Therefore, it is possible to modify their spectral properties by changing the quantum dot size or by introducing into its structure a dopant in the form of metal ion. Due to unique optical properties, quantum dots may find application as fluorophores in sensor systems in such areas as food analysis, biotechnology or biomedicine [14,15]. Quantum dots are made up of elements from groups II – VI and III – V [16]. The most commonly used quantum dots include these composed of CdSe [17], ZnSe [18] and ZnS [19].

A quantum dot consists of a semiconductor core (Figure 1A), with a diameter of 2 nm to 5 nm, responsible for the dot luminescence [19,20]. The most common core-forming compounds include CdTe [21], CdSe [22], ZnSe [23] and ZnS [19]. The luminescence of quantum dots is caused by the quantum confinement effect and defects in the crystalline structure of quantum dots [24], which is particularly important in the case of ZnS dots. However, defects on the surface can also reduce luminescence of the dots because atoms on the quantum dot surface have dangling bonds which trap electrons after absorbing a quantum of energy. Relaxation of the electron-hole system occurs in this case by non-radiative way and as a result the luminescence quantum yield of quantum dots is significantly reduced. For these reasons it is important to control the quality of a quantum dot surface [24]. For this purpose the dot core is covered with the second, semiconductor layer (Figure 1C, D). This modification leads also to strengthening of the quantum confinement effect which enables quantum dots to reach higher emission intensity or shift the maximum in the emission spectrum toward longer wavelengths. The compounds used most commonly as quantum dot coatings are semiconductors such as ZnS, ZnSe and InP, and dot size is reaching 10 nm [20].

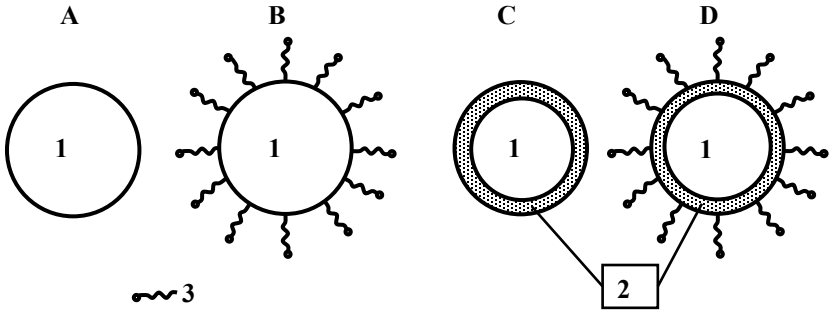

Figure 1. Scheme of quantum dot structure
1 – core, 2 – shell, 3 – organic molecule, A, B, C, D – described in text

To be used in biological systems, i.e. in aqueous media, quantum dots are covered with an additional hydrophilic organic layer (Figure 1B or 1D) whose aim is to give hydrophilic character to the dot surface in order to allow its solubilization in water [24, 25]. The coating can be composed of amino acids, e.g. cysteine, methionine or glycine [26] and compounds containing the –SH group, such as mercaptopropionic acid (MPA) [19, 25], 2-mercaptoethylamine [27] or 2-mercaptoethanol [27]. These compounds are extremely easily bonded to the surface of chalcogens, since the sulfur atom is incorporated into their crystalline structure. In addition, the organic layer which covers the quantum dot surface can also have a protective character, preventing degradation of the dots and their aggregation. Polymers used most commonly for this purpose include sodium polyphosphate [28], polyethylene oxide [29] and polystyrene [29].

Application of organic compounds with functional groups such as –COOH or $–NH_2$ on the dot surface allows for another modification of the surface of quantum dots by binding various ligands to the dot surface (Figure 1C). The ligands are typically biologically active compounds such as proteins [9, 15], antibodies [30, 31], enzymes [5] or amino acids [32]. Their selection is directly related to the use of quantum dots in a particular system. The quantum dot-bonded biological material system becomes a biochemical (nanobiosensor) or chemical nanosensor for the determination of biologically important compounds (glucose, urea) [5, 19], ions such as Na^+, K^+, Cu^{2+}, Mg^{2+} [19, 32], enzymes [15], cancer cells [15, 16] or for measuring the amount of microorganisms [30, 31]. The size of ligand-modified quantum dots can reach even 100 nm [20].

Optical properties of quantum dots

Quantum dots owe their unique properties to the quantum confinement effect which results from limitation of the movement of electrons in a quantum dot through a potential energy barrier. A quantum dot, as said, is essentially a semiconductor crystal with a size in the nanometer range. In the bulk semiconductor energy levels of atoms overlay and form, as a result, conduction band (empty) and valence band occupied by the valence electrons of atoms (Figure 2). They are separated by a bandgap (forbidden gap), where electrons cannot be present. The bandgap energy in semiconductors is relatively low (e.g. for ZnSe about 2.5 eV [33] and 3.7 eV for ZnS [19]), which allows for the passage of an electron from the valence band to the conduction band and is responsible for electrical conductivity of semiconductors. In a quantum dot, which is a nanocrystal built from a finite and small number of atoms, energy bands are again split into different energy levels, and the width of the bandgap increases compared to the bulk semiconductor. This effect is the stronger the smaller the size of the semiconductor crystal. As a result, the movement of electrons is confined to the area determined by the dot edge and quantum confinement occurs.

The optical properties of quantum dots are determined by the transfer of an electron from the valence to conduction band due to absorption of light quanta. Since the bandgap in semiconductors is relatively narrow, they absorb light in the

ultraviolet and visible spectrum. As a result of electron transfer to the conduction band, an 'empty' place (a so-called hole) remains in the valence band. The return of the electron from the conduction to valence band is accompanied by the emission of light quantum with energy close to bandgap energy. The smaller the quantum dot, the wider the energy bandgap. Thus, in this case the size of nanoparticles directly affects the wavelength of the emitted light [19, 34]. This dependence is a big advantage of quantum dots. An additional option allowing us to 'adjust' the energy bandgap and thus the wavelength of the emitted light is the application of dopants in the form of metal ions in the quantum dot. The dopants form additional energy levels within the forbidden gap (Figure 2).

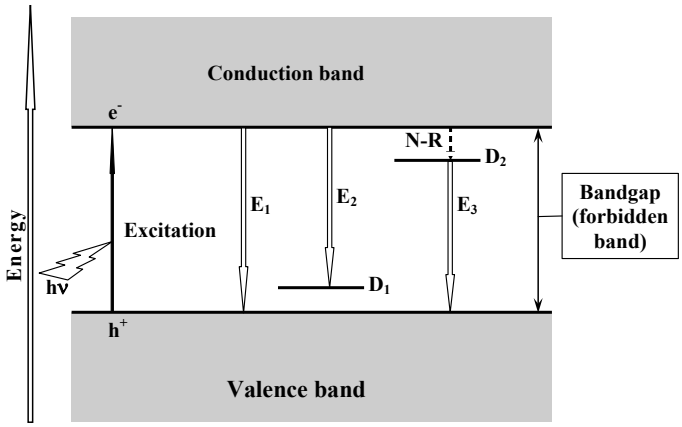

Figure 2. Schematic energy level diagram of quantum dots
D_1, D_2 – dopant levels, E_1, E_2, E_3 – emission of light, N-R – nonradiative process

Other advantages of quantum dots as fluorophores can also include high quantum yield, broad absorption band, narrow emission band with a large Stokes shift, high photostability and resistance to photobleaching [35-37].

The article addresses the synthesis and properties of zinc sulfide quantum dots doped with copper ions (ZnS:Cu) and covers researches carried out in the Chemical Biophysics Team in the Institute of General Food Chemistry.

ZnS quantum dots, synthesis and optical properties

Quantum dots used most frequently, *inter alia*, in optoelectronic devices include CdSe [22,38], CdS [33] or CdTe dots [22]. They are characterized by high quantum yield and fluorescence [39, 40]. However, these dots contain cadmium which is highly toxic to living organisms, so it is not advisable to use them in sensor systems for clinical diagnostics [15, 41]. Therefore, ZnS quantum dots with low toxicity [19] are of interest to many research centers. Zinc sulfide is one of the earliest discovered semiconductor materials and has long been used in optoelectronic devices [19]. ZnS has two possible structures: cubic and hexagonal, with the bandgap energy amounting to 3.68 eV for the regular form

and 3.8 eV for the hexagonal one [42]. Depending on such factors as pH of the environment or temperature, ZnS quantum dots exhibit absorbance in the range from 250 to 375 nm, and photoluminescence with a maximum of about 360 – 545 nm [19, 43, 44]. Bindu *et al.* [42] showed that pure ZnS is characterized by a strong quantum confinement effect. They recorded two bands in the excitation spectrum at 246 nm and 375 nm and corresponding photoluminescence bands at 362 nm (λ_{exc} 245 nm) and 455 nm (λ_{exc} 362 nm) for ZnS quantum dots in dry form. The first band was broad and irregular, and the second one narrow and symmetrical.

For ZnS quantum dots coated with mercaptoethanol Li *et al.* [45] found that with the increase of dots aging time at temperature 80°C their radius grows which changes the shape of absorption spectrum and causes a shift of its maximum from 290 nm toward longer wavelengths. On the other hand, in the photoluminescence emission spectrum only an increase of intensity was observed with a maximum persisting at 423 nm. Presumably, the increase of photoluminescence intensity was caused by the increased crystallinity of dots and disappearance of defects on their surface as a result of aging. Li *et al.* [46] synthesized ZnS quantum dots coated with 3-mercaptopropionic acid (MPA), which was then partly exchanged to (3-mercaptopropyl) trimethoxysilane (MPS). This modification led to an increase of both the intensity of photoluminescence emission and the quantum yield. Senthilkumar *et al.* [47] immobilized ZnS quantum dots on a polyvinyl alcohol substrate. In the photoluminescence spectrum which they recorded there were two overlapping emission bands with peaks at 415 nm and 440 nm, which the authors explained by the presence of the sulphur and zinc ions vacancies. In general, it is observed that for pure ZnS quantum dots its size has no significant effect on the position of the photoluminescence emission peak, and defects of crystalline lattice (sulphur and zinc ions vacancies) are responsible for light emissions (Figure 3).

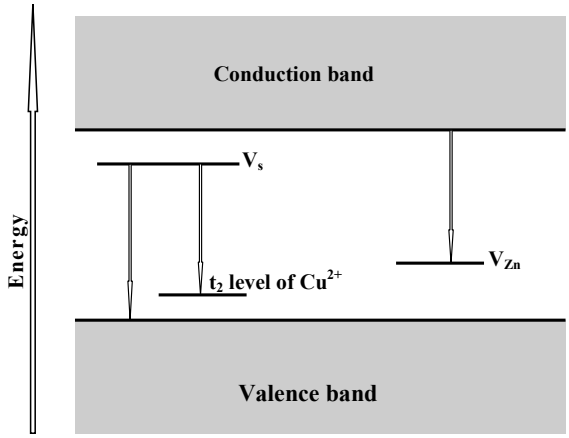

Figure 3. Schematic energy level diagram of ZnS and ZnS:Cu quantum dots according to [55]
V_s – sulphur vacancy, V_{Zn} – zinc vacancy

The addition of transition metal ions to the ZnS quantum dots often causes quenching of the photoluminescence of ZnS matrix by itself and at the same time the emission properties of dots. It was found that doping the ZnS quantum dots with metal ions, e.g. Mn^{2+} [48, 49], Co^{2+} [50], Fe^{3+} [51], Cu^{2+} [52] caused modification of their optical properties, in particular shifted the emission maximum toward longer wavelengths, and could also increase the quantum yield of photoluminescence and emission intensity. Best known are the dots doped with Mn^{2+} ions which are characterized by a very narrow emission band with maximum at about 595 nm almost independent of the dot size. In this case, the $^4T_1 \rightarrow {}^6A_1$ transition in Mn^{2+} ions is responsible for light emission and the observed photoemission has the character of phosphorescence [48, 49].

ZnS:Cu quantum dots

In the case of Cu-doped ZnS quantum dots there are still questions about their properties to which the current researches give only a partial response. The basic problems are:

- The oxidation state of copper ions in doped nanocrystal (Cu^+ or Cu^{2+}),
- The exact location of dopant levels in the forbidden gap,
- The nature of photoemission from doping centers (fluorescence or phosphorescence),
- Selective adsorption of copper ions on the surface of nanocrystals.

Synthesis of ZnS:Cu^{2+} quantum dots

There are two basic types of quantum dot synthesis: a high-temperature synthesis in organic medium and low-temperature synthesis in aqueous medium. In the case of ZnS:Cu dots the latter is mainly used and it will be discussed here in detail.

ZnS is known to be a hardly soluble salt which is formed by mixing the salts which contain Zn^{2+} and S^{2-} ions in a solution. In the case of its doping the solution must contain dopant ions, i.e. Cu^{2+}. By choosing appropriate conditions of synthesis such as reagent concentration, pH and temperature, one can obtain ZnS in the form of nanocrystals and not as a precipitate. As a zinc source water-soluble salts $Zn(CH_3COO)_2$ [28, 53-56], $ZnCl_2$ [57-60] or $ZnSO_4$ [61] are used. Copper ions are doped using $Cu(CH_3COO)_2$ [28, 53, 55, 56], $CuCl_2$ [57-59] or $CuSO_4$ [54, 61]. A source of sulfide ions is usually Na_2S [28, 54-59] and organic compounds, hydrolysing in water forming S^{2-} ions, such as thiourea [53, 64] or thioacetoamide [67, 76].

The applied conditions of synthesis such as pH, heating time and temperature of the solution, differ depending on source cited. Thus, the pH of the synthesis medium may range from 7 [53] to 11.5 [62, 63], with high pH leading to the formation of dots exhibiting high photoemission [71]. When using Na_2S the process of synthesis is usually carried out at room temperature [28], while in the case of thiourea high temperature is applied, reaching even 180°C [64] when the synthesis is performed in the autoclave. Lee *et al.* [65] examined the effect

of temperature on quantum dot synthesis with the use of thiourea. In all cases the nanoparticles which they obtained have a crystalline structure and their photoluminescence intensity increases as a function of synthesis temperature from 70 to 85°C, and then decreases. In the photoluminescence emission spectrum two bands were observed: first, weak with a maximum at 420 nm from zinc sulfide, and a second, strong with a maximum at 520 nm for which copper ions are responsible. The decrease of emission intensity, when the temperature of synthesis was higher than 85ºC the authors explained by the formation of CuO which created centers of non-radiative recombination of electrons and holes.

The applied time of synthesis and aging of ZnS:Cu quantum dots varied from 30 minutes [54] to several hours [52, 59, 62, 64]. In some cases the synthesis was carried out under anaerobic conditions by purging the solutions with nitrogen [59, 62].

All these parameters of synthesis affect optical properties of quantum dots, as will be discussed in more detail below.

The effect of dopant concentration on spectral properties of Cu^{2+}-doped quantum dots

The concentration of dopant metal ions in the starting solution during synthesis is one of the most important factors affecting the optical properties of quantum dots. Jayanthi *et al.* [53] examined the effect of copper addition in the range 0.001% M-0.1% M on the emission of quantum dots in the form of dried films. In the emission spectrum three bands were distinguished, the first two with maxima at about 390-420 nm and 480 nm for which defects in the ZnS structure are responsible, and the third one at about 520 nm caused by the presence of Cu^{2+} ions. Intensity of the fluorescence emission increased at Cu^{2+} concentration from 0.001% M to 0.01% M, and then it decreased to 0.1% M. According to the authors, the reason of reduction of the fluorescence intensity in this range was the formation of copper sulfide resulting in a decrease in the amount of Cu^{2+} ions which may serve as active luminescence centers in ZnS. Peng *et al.* [55] studied ZnS:Cu quantum dots with 0.5% to 2% addition of copper (molar ratio of copper to zinc ions). In the emission spectrum of dry powders they recorded one band for which the maximum was shifting toward longer wavelengths (from about 411 nm to 500 nm) with an increasing content of copper ions, and the highest emission intensity corresponded to quantum dots containing 1% of copper ions. Peng [55], like Jayanthi *et al.* [53], attributed photoluminescence quenching in the dots which contained more than 1% of copper ions Cu^{2+} to the formation of CuS. Kole *et al.* [56] studied optical properties of ZnS:Cu quantum dots with addition of copper ions in the range from 0.25 to 1.00% in methanol solution. They found that the absorbance spectrum was the same regardless of the concentration of copper added with a maximum at 305 nm and band edge at 334 nm. This indicated that the resulting dots were characterized by a low degree of polydispersity and their sizes did not depend on the amount of copper added. Photoluminescence emission spectra of the dots can be separated into three bands

with maxima at 407 nm, 460 nm and the third one at about 495-518 nm depending on the concentration of copper ions added. The first two bands appear also in the dots of pure ZnS. The last band is related to the emission during the transition of an electron from the conduction band to dopant level within the band gap formed as a result of doping with Cu^{2+} ions. It was also found that there was an optimal concentration of the dopant (in this case 0.75%) over which the photoluminescence of quantum dots decreased. Like Jayanthi *et al*. [53], the authors attributed this phenomenon to the formation of copper sulfide.

All authors agree to the fact that with an increasing concentration of copper ions in the quantum dots, the photoluminescence emission maximum shifts systematically toward longer wavelengths and the intensity of photoluminescence increases to a certain concentration limit. Too high amount of copper dopant causes a decrease of emission which is caused probably by copper sulfide formation during the synthesis. In the results obtained by various authors one may observe some differences between the shape of emission spectra, position of the recorded maxima of each band and copper ions concentration optimal for a maximum emission. This is due to differences in the methods of dots synthesis and measurement methods used to study them, in particular whether the dots were tested in a dry form or in solution [53, 55, 56].

The degree of oxidation of copper in dots – Cu^{2+} or Cu^{+} ?

The degree of oxidation of a dopant in the form of Cu^{+} or Cu^{2+} ions has an influence on the optical properties of quantum dots [62, 66-71]. In order to receive quantum dots doped with Cu^{+} ions, the reducing agent is used during synthesis and the most commonly used ones are sodium sulfite Na_2SO_3 [66, 67] and sodium borohydride $NaBH_4$ [70]. The role of a reducing agent may play also sulfide ions S^{2-}. Here, the ESR measurements can be helpful because Cu^{+} ions are paramagnetic, while Cu^{2+} are not. Labiadh *et al.* [62] basing on X-ray photoelectrons measurements and ESR found that in the quantum dots which they obtained copper was present in the form of Cu^{+} ions and sulfide ions were a reducing agent. Similar conclusions were reached by Corrado *et al.* [71].

Since Cu^{+} ions in an aqueous medium are unstable, to prevent their oxidation they may be complexed with appropriate ligands [60, 67]. In the synthesis of ZnS:Cu^{+} dots Yang *et al.* [67] used stable complexes with SCN^{-} ions. On the other hand, Sun *et al.* [60] used as ligands complexing Cu^{+} ions thiourea and thiosulfate ions. Comparing the fluorescence spectra of quantum dots doped with Cu^{2+} and Cu^{+} ions, Yang *et al.* [67] observed significant differences between them which demonstrates different mechanisms of fluorescence. They proposed the following mechanism of fluorescence of Cu^{+}-doped ZnS dots. Once photons are absorbed by ZnS, electrons from the valence band, transferred to the conduction band, are trapped by defects in the crystalline lattice of the quantum dot. Then, recombination occurs between the defects and energy level t_2 introduced by Cu^{+} ions and light is emitted. Manzoor *et al.* [66] examining the effect of the amount of Cu^{+} ions on photoluminescence emission spectra, found

two acceptor centers. The first is formed as a result of exchanging Zn^{2+} by Cu^{+} ion in the crystalline lattice of ZnS and is responsible for the emission with a maximum at 470 nm. The second type of centers is formed with an increasing amount of copper ions. This results in a close binding of copper ions which replace Zn^{2+} ions with Cu^{+} ions in the interstitial position. The centers exhibit a maximum emission at 500 nm. The mechanism of fluorescence emission for Cu^{2+} is explained as the transfer of an electron from the conduction band to the energy level t_2 of Cu^{2+} ion (Figure 3) [55, 56, 72].

Modifications in the structure of ZnS:Cu quantum dots

During the synthesis of ZnS:Cu quantum dots, to water solutions of inorganic salts containing Zn^{2+} [28, 53-56, 60], Cu^{2+} [28, 53-56] or Cu^{+} [60, 67] and S^{2-} ions [28, 53-55, 60] compounds are added to modify properties of the dots. Acids such as α-methacrylic [55] or mercaptopropionic (MPA) [62, 63] are used to increase solubilization of quantum dots [62, 73] and their stability in a solution [55]. For ZnS:Cu quantum dots coated with MPA Labiadh *et al*. [62] observed increased photoluminescence emission during irradiation with UV light. They attributed this effect to MPA photooxidation on the dot surface. This led to the release of sulfide ions binding with Zn^{2+} ions and forming an additional ZnS coating around the existing ZnS:Cu quantum dot. In the synthesis of ZnS:Cu quantum dots Wang *et al.* [58] used glycine as a ligand to reduce the difference in solubility of ZnS and CuS, which allowed them to incorporate a larger amount of copper into ZnS:Cu quantum dots. Sun *et al.* [60] used thiourea or sodium thiosulfate as ligands in the synthesis of ZnS nanocrystals doped with Cu^{+}. Both of these ligands were used to reduce the difference between the solubility of Cu_2S and ZnS, and also by forming complexes with Cu^{+} ions to stabilize them in the aqueous medium. The type of ligand applied had an effect on the size of quantum dots obtained. When using thiourea the dot diameter was 3.4 nm, and in the case of thiosulfate it was 3.8 nm. The photoluminescence emission spectra of the dots were similar for both applied ligands.

As film-forming compounds covering the core of quantum dots which were intended to protect the quantum dot against degradation and aggregation, also such polymers as polymethacrylic acid [54], polyvinylpyrrolidone [73] as well as inorganic compounds such as sodium polyphosphate [28] were used.

Immobilization of ZnS:Cu quantum dots

For repeated use of ZnS:Cu quantum dots or their application in optoelectronics, the dots must be immobilized. For this purpose research regarding their immobilization on various media are conducted. The dots can be simply applied as thin layers on a substrate like glass or quartz plate [53], but in this case they can be used only in dry form. Bodo et al. [61] immobilized ZnS and ZnS:Cu quantum dots in polyvinyl alcohol in the form of thin films on glass plates. On the other hand, Huang et al. [72] immobilized ZnS:Cu quantum dots in sulfonated polystyrene. Quantum dots in this case were characterized by a very narrow photoluminescence

emission spectrum with a maximum at 415 nm. Thin films obtained from a polymer containing quantum dots were used as an emitter in the light-emitting diode (LED) whose electroluminescence showed a maximum at about 440 nm [72]. For the same purpose Nath et al. [59] immobilized dots in the zeolite.

ZnS:Cu quantum dots doped by other ions

ZnS:Cu, X (X-F^-, Br^-, Cl^-) quantum dots

Halogens are among the most popular elements used as co-dopants for ZnS:Cu quantum dots. Quantum dots doped with halogens are characterized by a higher photoluminescence intensity as compared to ZnS:Cu dots and a shift of the emission spectrum generated by zinc sulfide toward longer wavelengths [66]. Manzoor *et al.* [66] studied the effect of addition of halide anions on ZnS and ZnS:Cu dots containing Cu^+ ions. In the case of pure ZnS the addition of halide anions did not change optical properties of the dots. However, in the case of Cu^+ doped dots it caused an increase of photoluminescence intensity and a shift of the maximum toward longer wavelengths, with the best activator being the F^- ions. The authors attributed this effect to the increase Cu^+ ion solubility in the ZnS lattice under the influence of halides. By appropriate selection of the amount of added Cu^+ and F^- ions one can obtain quantum dots with maximum emission ranging from 434 to 514 nm.

Corrado *et al.* [69] described the high-temperature synthesis of ZnS:Cu quantum dots in an organic medium with the use of Br^- as a co-dopant. The ZnS:Cu dots doped with Br^- obtained in the optimized conditions showed approximately 5 times greater emission than the dots without this co-dopant. They attributed the strengthening of emission to the increase of solubility of the copper ions in the ZnS structure in the presence of Br^- ions and to the donor-acceptor transition between the electron trapped on the dopant level formed by Br atoms and the hole on the dopant level formed by the Cu atoms.

While studying the synthesis of ZnS:Cu quantum dots in the aqueous medium with the use of $CuCl_2$ as a source of copper ions, Corrado *et al.* [71] found that at the same time chloride ions were incorporated into the structure of synthesized dots. This caused an increase of photoluminescnce emission intensity as a result of formation of additional donor levels.

The authors of the above studies agreed that the mechanism of donor-acceptor transition was most probably responsible for the photoluminescence emission of quantum dots doped additionally with halogens, with a donor being the halide ions and acceptor the copper ions [66, 69, 71].

ZnS:Cu quantum dots with a metallic co-dopant

Addition of the second metal ion to ZnS:Cu dots makes it possible to change their optical properties. Begun *et al.* [70] synthesized ZnS quantum dots co-doped with Cu^{2+} and Mn^{2+} ions stabilized with chitosan. In the emission spectrum of these dots two separated bands were observed, a weak one with a maximum at 460 nm connected with a Cu dopant and the other at 592 nm related to the presence of manganese ions. Addition of a reducing agent ($NaBH_4$) resulted in

a decay of the band with a maximum at 460 nm and formation of a new strong band at about 520 nm. The authors attributed this to the reduction of Cu^{2+} ions to Cu^{+}. Position of the band originating from Mn^{2+} ions as a result of the reduction was virtually unchanged, only an increase of emission was observed.

Similar results were recorded by Ummartyotin *et al.* [68] who synthesized powdered, ceramic ZnS quantum dots doped with Mn^{2+} and Cu^{+} ions. For ZnS quantum dots they observed the maximum of emission at 452 nm, for ZnS:Cu quantum dots at 517 nm, and for ZnS:Mn dots at 595 nm. For zinc sulfide quantum dots doped with copper and manganese together they obtained two emission bands: one at 457 nm, which corresponding to the shifted ZnS band, and another one at 595 nm, for which manganese ions were responsible. The band emitted by copper was not recorded for these dots. The authors presumed that due to a higher degree of oxidation, the Mn^{2+} ions replaced the Zn^{2+} ions in ZnS, easier than Cu^{+} ions. However this conversion was not complete, as evidenced by the presence of emission band at 457 nm corresponding to the emission of pure zinc sulfide.

In the case of other co-dopants their mutual interaction in a nanocrystal is observed. One of such co-dopants is lead. Lead ions exhibit higher activity than copper ions, so they react with zinc sulfide more easily than Cu^{2+} ions. In zinc sulfide quantum dots doped with Cu^{2+} and Pb^{2+} ions the so called composite luminescence centers are formed for which the energy gap is smaller than in the case of quantum dots doped only with copper ions or lead ions. The quantum yield of emission also increases, since more electron – hole pairs can be created [74, 75]. Yang *et al.* [75] synthesized crystalline ZnS:Cu:Pb nanoparticles of a size from 2 to 4 nm, with a broad emission band with the maximum ranging from 500 nm to 550 nm. The spectrum differs from the spectra of quantum dots doped only with Cu or only with Pb, which indicates that distinct composite centers are formed. By appropriate selection of the Cu:Pb ratio and concentration of these elements in the dots they reached nearly eightfold increase of the emission as compared to pure ZnS, while the separate addition of either Cu or Pb resulted in reduced emissions.

Ehlert *et al.* [74] obtained ZnS:Cu:Pb quantum dots by the high-temperature method in an organic medium. They tested the effect of an additional shell of pure ZnS on the emission properties of nanocrystals obtained in dry form. They found that the emission spectrum of ZnS:Cu:Pb nanocrystals was a combination of emission spectra derived from the same ZnS matrix and both dopants. In the case of dots of the core/shell type with the ZnS shell they observed a decay of emission component at high wavelengths (about 700 nm) associated with the presence of Pb^{2+} ions.

Another co-dopant may be Co^{2+} ions. Addition of the cobalt ions alone to ZnS usually results in reduction of the fluorescence intensity of quantum dots. Yang *et al.* [76] were the first to describe the properties of zinc sulfide nanocrystals doped with Co^{2+} and Cu^{2+} ions. Co-doped quantum dots obtained in this way

exhibited strong fluorescence, much higher than that of pure ZnS with a maximum in the range from 515 nm to 560 nm with both peak position and emission increase dependent on the Cu:Pb ratio and the concentration of these elements in the ZnS matrix. The authors attributed strengthening of the emission by co-doping with Cu^{2+} and Co^{2+} ions to the formation of composite centers like in the case of co-doping with Pb^{2+} ions [75]. Similar results were obtained by this research group for co-doping with In^{3+} ions [78]. For copper and cobalt-doped quantum dots Iqubal and Iftekhar [77] observed a non-symmetric emission spectrum with a maximum at 480 nm much different from the results obtained by Yang et al. [76]. This was caused by a different synthesis method, in this case in an anhydrous medium (acetonitrile).

Applications of ZnS:Cu quantum dots

So far Cu ion-doped ZnS quantum dots have found a few applications. Most research on the synthesis of such dots and their optical properties is conducted with respect to their application in optoelectronics in the construction of light-emitting diodes (LED) [59, 72], therefore their properties are tested in the solid state, when the dots are applied in the form of powder on the medium or immobilized in the polymer [53, 61]. As is known, semiconductors of the ZnS type are used as photocatalysts. In an aqueous medium, after absorbing a quantum of light, the generated electron-hole pair initiates further reactions leading to the formation of free radicals, and as a result to the oxidation of organic compounds dissolved in water. This process can be widely used in environmental protection in water and sewage treatment. Due to high extension of surface area, nanocrystals (quantum dots) are very effective photocatalysts. The ZnS:Cu dots were used as photocatalysts in model reactions of dye oxidation in water solutions [62, 79]. The ability of quantum dots to emit visible light under the UV illumination makes it possible to use them as markers applied on various media (paper, cotton fabric) by the screen-printing method [80] in order to protect documents or form smart packages or to detect fingermarks in blood on non-porous surfaces [81]. Begum et al. [52] used ZnS:Cu dots coated with chitosan to label tissues and cells.

Although in general quantum dots are widely used as chemosensors, in the case of specific ZnS:Cu dots only a chemosensor for folic acid determination was described [82]. Quenching of quantum dot photoluminescence by this analyte made it possible to determine folic acid at the least level of 11 μM. From studies performed by our research team it follows that the photoluminescnce of ZnS:Cu dots is quenched by oxygen [83], which may suggest that they can be used in the construction of a biosensor for glucose determination with the use of glucose oxidase by measuring oxygen consumption.

Summary

From the literature on copper-doped zinc sulfide quantum dots it follows that they can have interesting properties when used as fluorophores to form chemical nanosensors or nanobiosensors. On the other hand, as results from the literature data, in the case of these particular ZnS:Cu dots there is little opportunity to change the position of emission maximum by manipulating the composition and size of the dots which can limit their potential applicability as markers in cells and tissues. Most of the above presented studies on the effect of formation conditions of quantum dots on the emission properties refer to measurements conducted for objects in the form of dry powders. A small part concerns ZnS:Cu dots solubilized in water solutions. The influence of solubilization medium parameters (e.g. pH) on the stability of dots in solutions is practically unrecognized. There are also very few results of time-resolved measurements of photoluminescence of these dots which would allow us to better recognize the mechanism of emission. The influence of oxygen on emission properties of these dots offers a prospect of using them in the construction of nanobiosensors to determine glucose with the use of glucose oxidase, or other oxidase substrates dependent on oxygen. As can be seen, in the case of ZnS:Cu dots there is still ample room for further research, which we plan to continue with our team.

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Review article

Lactate biosensors for food industry

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Abstract: *Lactic acid and lactate fermentation play important role in food and beverages production, control and quality. Analysis of lactate by standard methods is time, work consuming, and cannot be implemented at the production site. The alternative is the use of biosensors for lactate. The article reviews the biosensors for D- and L-lactic assay, their types, construction and application in food analysis area. The special emphasis is given to the commercial biosensor for lactate. The market survey indicates that there is a lack of lactate biosensors addressed to food industry except wine production. QUALI_JUICE project was the practical attempt to use some of the commercial biosensors in production and quality control in fruit juice industry. The results of the project indicates that commercial biosensors can be used after some minor functional adaptation in fruit juice production control. The application of these biosensors can be broadened to the other sectors of food and beverage industry.*

Keywords: *lactate assay; lactate biosensor, fermented food; food spoilage; process control.*

Introduction

Lactic acid is the end product of glycolysis in anaerobic conditions. There are two optically active stereoisomers of lactic acid: L(+)-lactic acid and D(-)-lactic acid. L-lactic acid is produced in muscles during anaerobic glicolysis and by most of lactic acid bacteria [1]. D-lactic acid is produced by some bacteria, algae and plants [2]. Many lactic acid bacteria are producing racemate mixture of lactic acids [3, 4]. Lactic acid and lactate assay is very important in many areas. Among them are:

- Medicine – indicator of ischemia for critical care, indicator for some inflammations,
- Sport medicine – testing and monitoring of training intensity and recovery,
- Food quality analysis – fermented food, marker for bacteria spoilage of food and beverages,
- Oenology – monitoring malolactic fermentation in wine,
- Agriculture – indicator of bacteria mammary infection (mastitis),
- Veterinary – blood lactate control in animal diseases and training (racing horses or dogs),
- Process control in biotechnology – production of lactic acid.

Lactic acid fermentation is one of the key processes in the food industry. The production of fermented food is one of the oldest food processing technologies used by mankind. Fermentation of milk, meat and vegetables by lactic acid bacteria is known from 6000 BC [3]. The main goal of the use of this fermentation in food processing is a preservation effect. This process helps also to provide products with right taste, aroma and texture, improves the nutritional value of food, can eliminate some toxins [5-7] and prevent growth of undesired microorganisms like Gram-negative bacteria or moulds [8, 9]. The lactic acid fermentation contributes also to some probiotic properties of final product [10].

L-lactic acid (E270) is used in food industry to decontaminate meat or poultry and prevent bacteria growth on them [11-13], to inhibit spoilage yeast grow [14] and also as antibrowning agent [15]. About 50% of lactic acid is used as emulsifying agent in bakery production [16]. It is used also as acidulant, pH buffering agent and flavouring in variety of foods and beverages like candy, jams, jellies, soft drinks and many others [17]. L-lactic acid can be produced either by chemical methods or by lactic acid fermentation [17]. Lactic acid is also used in the chemical industry to produce biodegradable polymers [18], in cosmetics, pharmaceutical, leather and textile industries [17].

On other hand sometimes lactic acid fermentation is undesired in food and is leading to its spoilage. The increased level of L-lactate in egg is an indicator of spoilage by contamination or incubation [19]. Also in canned fruits and vegetables, UHT milk the increase of L-lactate is the marker of spoilage [20]. Contamination of fruit juices with lactic acid bacteria during production and storage often remained unnoticed for a longer time. Lactic acid fermentation of juices is leading to alteration of organoleptic properties of them, not acceptable for human consumption and economic loses for the producer and environmental problems [19, 21]. The Code of Practice of the Association of the Industries of Juice and Nectars from Fruits and Vegetables of the European Economics Community (AIJN) [22] established the maximum permissible concentration of sum of both isomers of lactic acid in fruit juices as 0.5 g L^{-1}. Lactic acid bacteria are also often causing spoilage of beer [23].

D-lactic acid is produced by some bacteria (*Staphylococcus sp.*, *Enterobacter sp.*) and its increased level is observed in urine and blood in some bacteria infections [24]. The presence of D-lactic acid is an indicator of food spoilage especially raw meat [25], tomato and products from them [26]. D-lactic acid is not utilized in humans so its consumption must be controlled. WHO recommend a daily intake of this isomer in man to be less than 100 mg kg^{-1} body weight [27] and do not recommend its consumption by infants and young children [28].

In wine and cider production very important is malolactic fermentation carried out by lactic acid bacteria (mainly *Oenococus oeni*) after main alcoholic fermentation. L-malic acid is converted to L-lactic acid reducing the acidity of wine and leading to improvement of taste and flavour [4, 29]. Some strains of lactic acid bacteria can carry out fermentation producing D-lactic acid causing „piqûre

lactique" taste [30], which is undesired and lowers wine quality. The presence of D-lactic acid in wine and cider is used as an indicator of their spoilage [4].

The concentration of lactic acid (sum of both isomers) is typically measured using HPLC with UV/VIS or conductometric detection [21, 31, 32] or capillary zone electrophoresis [33]. Attempts were made for chiral resolution of D- and L-lactic acid by capillary electrophoresis to assay them in food products [34]. The lactic acid stereoisomers can be assayed independently by enzyme kits with stereospecific NAD^+ dependent dehydrogenases [2, 35]. Such kits are produced for example by Boehringer, (Germany) or Megazyme, (Ireland). Other possibility of lactic acid assay (sum of both isomers) is the use of colorimetric test strips and Reflectoquant® supplied by Merck (Germany). Chromatographic methods and enzyme ones are expensive, time consuming and need well trained laboratory stuff. They need also intensive sample pre-treatment and cannot be used in food industry at line or on line. The alternative for these methods is the use of biosensors for lactic acid assay. Biosensors provide rapid, simple and direct measurement, with no need of sample preparation [36, 37].

What is a Biosensor ?

According to definition given by IUPAC, a biosensor is an integrated receptor-transducer device, which is capable of providing selective quantitative or semi-quantitative analytical information using a biological recognition element in direct spatial contact with transducer element [38]. Biosensors can be classified according to the transducer element into [39]:

- Electrochemical (amperometric, potentiometric, coulometric, conductometric),
- Optical (absorbance, fluorescence, chemiluminescence, surface plasmon resonance),
- Piezoelectric (quartz crystals, surface acoustic wave),
- Calorimetric,
- Semiconductors (field effect transistors, light addressable potentiometric sensors),
- Others.

The first biosensor for lactic acid (L-lactate) was reported in 1970 by Wiliams et al. [40]. It was amperometric electrode with cytochrome b_2 as biological sensing element with $Fe(CN)_6^{3-}$ as redox mediator.

Biological Recognition Elements in Lactate Biosensors

As the most important part of every biosensor is the biological recognition element, the review of possible biological receptors for lactate (both isomers) is given below.

The most common biological receptor for L-lactic acid is L-lactate oxidase (LOD) which catalyzes the reaction:

$$L\text{-}lactate + O_2 \xrightarrow{L-lactate\ \ oxidase} pyruvate + H_2O_2 \qquad (1)$$

L-lactate content can be assayed by measuring consumption of oxygen by means of oxygen electrode [41, 42] or electrooxidation of H_2O_2 produced in reaction (1)

on platinum electrode. In this case, the current produced by electrochemical reaction is directly proportional to the lactate concentration [42-44]. Because ascorbate, uric acid and paracetamol react at the electrode at nearly the same potential as H_2O_2, their presence in the samples can interfere with lactate assay. To eliminate interferences the additional layers can be added covering the immobilized LOD. Typically Nafion® is used as the anti-interference layer [45], aromatic polyamines like polyaniline or polytyramine [46] or overoxidized polypyrrole [47]. The elimination of interferences influence could be achieved also by lowering the potential of H_2O_2 oxidation by modification of electrode surface by phtalocyanine [48] or Prussian blue [49].

LOD is the enzyme that accepts artificial redox mediators instead oxygen:

$$L\text{-}lactate + Med_{ox} \xrightarrow{L-lactate\ \ oxidase} pyruvate + Med_{red} \tag{2}$$

The reduced mediator is oxidized at electrode surface generating current which is proportional to the lactate concentration.

$$Med_{red} - z\bar{e} \rightarrow Med_{ox} \tag{3}$$

Typical redox mediators used in lactate biosensors are transition metal complexes like $Fe(CN)_6^{3-}$ [50], ferrocene and its derivatives [51-53], Prussian blue [54] or organic dyes like Meldoda blue [55]. Application of osmium complex conjugated with polymer leads to "wiring" of the mediator to LOD and the direct exchange of electrons between active centre of the enzyme and electrode could be achieved giving the electrode response at the absence of oxygen [56].

L-lactate oxidase was used also in construction of electrochemilumenescent biosensors, in which oxidation of luminol by H_2O_2 produced in reaction (1) is followed by measuring either current or intensity of produced light [57]. Oxidation of luminol by H_2O_2 produced in reaction (1), catalysed by peroxidase is accompanied by chemiluminescence. This phenomenon is the basis of chemiluminescent optical biosensor for lactate [58, 59].

The other biological receptors for L-lactate are:

- Lactate monooxygenase (specific for L-lactate) which was used in optical sensor [60]

$$L-lactate + O_2 \xrightarrow{lactate\ \ monooxygenase} CH_3COOH + CO_2 + H_2O \tag{4}$$

- Cytochrome b_2 from yeast catalyzing reaction [40]

$$L-lactate + Med_{ox} \xrightarrow{cytochrome\ \ b_2} pyruvate + Med_{red} \tag{5}$$

- PQQ (pyrroloquinoline quinone) dependent L-lactate dehydrogenase [61],
- Microorganisms like *Saccharomyces cerevisiae* [62], *Escherichia coli* [63], *Shewanella putrefaciens* [64], *Acetobacter pasteurianus* [65],
- Porcine kidney tissue used in chemiluminescent biosensor [66].

Unless cytochrome b_2 the other biological receptors mentioned above are rarely used and their practical importance is negligible.

As it was mentioned earlier, there are two stereoisomers of lactic acid. All biological receptors described before are specific for L-lactic acid but sometimes there is a need to assay D-lactic acid alone or sum of both isomers. There are two stereospecific NAD^+ dependent lactate dehydrogenases (L-LDH and D-LDH):

$$L-lactate + NAD^+ \xrightarrow{L-lactate\ \ dehydrogenase} pyruvate + NADH + H^+ \quad (6)$$

$$D-lactate + NAD^+ \xrightarrow{D-lactate\ \ dehydrogenase} pyruvate + NADH + H^+ \quad (7)$$

The lactate content can be in this case measured amperometricaly by electrooxidation of NADH [67-69] or optically by absorbance [70] or fluorescence of NADH [71]. The application of lactate dehydrogenases in biosensor construction is connected with many problems:

- The electrooxidation of NADH is not fully reversible and is leading partially to a dimer production which is enzymatically inactive,
- The electrooxidation of NADH needs high overpotential,
- The reactions (6) and (7) are reversible. Because of that L-LDH is used sometimes to assay pyruvate in reverse reaction at low pH,
- Reaction rate of reactions (6) and (7) is strongly pH-dependent and is high enough at pH greater than 7.

To overcome the problems connected with electrooxidation of NADH the surface of the electrode was modified by redox mediators like Meldoda blue [30, 72, 73] or polyaniline [74].

The other possibility is co-immobilization of diaphorase – the enzyme catalysing reaction:

$$NADH + Med_{ox} \xrightarrow{diaphorase} NAD^+ + Med_{red} \quad (8)$$

The reduced form of synthetic redox mediator is then oxidised on the electrode according to reaction (3). Typical redox mediator used in such approach is $Fe(CN)_6^{3-}$ [75, 76].

NADH can be also oxidised by specific oxidase:

$$NADH + O_2 + H^+ \xrightarrow{NADH\ \ oxidase} NAD^+ + H_2O_2 \quad (9)$$

When lactate dehydrogenase and NADH oxidase are coimmobilized on electrode surface, lactate concentration can be evaluated either by oxygen consumption or H_2O_2 production or with the use of redox mediator $Fe(CN)_6^{3-}$ [77, 78].

L-LDH and LOD can be immobilized together yielding enzyme electrode with much greater sensitivity as compared with electrode with each enzyme alone due to the substrate recycling [79, 80]. This combination of enzymes with additional catalase was used in enthalpy meter for lactate [81] in which L-lactate was assayed by measuring temperature increase. By coimmobilzation of D-LDH, LOD and horseradish peroxidase on oxygen electrode the biosensor for total lactate was constructed [82].

The equilibrium of reactions (6) and (7) can be shifted to the products by consumption of pyruvate, as it is done in enzyme kits, by application of additional enzyme L-alanine aminotransferase (glutamic-pyruvic transaminase) catalysing reaction:

$$pyruvate + glutamate \xrightarrow{L\text{-}alanine\ \ aminotransferase} L-alanine + 2-oxoglutarate \quad (10)$$

Such approach was used in some constructions of lactate electrodes [74]. The same sequence of enzyme reactions connected with *Vibrio sp.* luciferase was used in bioluminescent biosensor for L- and D-lactate [83].

Specific D-lactate cytochrome c oxidoreductase (D-LCR) isolated from *Saccharomyces cerevisiae* catalysing reaction:

$$D-lactate + 2\ ferricytochrome\ c \xrightarrow{D\text{-}LCR} pyruvate + 2\ ferrocytochrome\ c + 2\ H^{+} \quad (11)$$

was used with phenazine methosulphate as the mediator in amperometric screen-printed electrode for D-lactate [84].

Key Factors in Lactate Biosensor Aplication

Practically only L-lactate oxidase and both lactate dehydrogenases have real importance in lactate biosensors construction. Most of the lactate biosensors described in literature are amperometric ones and are reviewed up to 2007 by Nikolaus & Strehlitz [19] and only they found application in commercial biosensors for lactic acid. The main trends in construction of amperometric electrodes are: miniaturization to obtain implantable probes, incorporation in flow system, production by screen-printing technique allowing for cheap and mass production of biosensors.

The key factor in developing of a biosensor is the immobilization of the enzyme at the transducer surface. The performance factors (lifetime, sensitivity, selectivity, stability, response time and linear range) depend strongly on the method used to immobilize the enzyme [19]. Typical methods of immobilization are physical adsorption, entrapment in polymer matrix or inorganic gel, crosslinking by bi-functional reagents, covalent binding and enclosing by semi-permeable membrane [38]. Immobilization method influences the enzyme properties like thermal stability, pH optimum, Michaelis constant [52, 85, 86]. Very promising strategy in production of enzyme electrodes is screen-printing using inks composed of powdered carbon or graphite, enzyme, mediator and polymer as binder to obtain working electrode [55, 71, 84, 86, 87], Such approach enables miniaturisation of the biosensor, which can be used as disposable one.

The important parameters of biosensor operation are sensitivity, linear range and lower detection limit. These parameters are connected together and depend on the biological receptor, method of immobilization and construction of the biosensor [19]. For most of the lactate electrodes with LOD or LDH the upper limit of linear range is between 1 and 2 $mM \cdot L^{-1}$, when the biosensor is used in batch mode. The linear range can be extended considerably by adding covering

membrane limiting the substrate diffusion to the biosensor surface [43]. The response time of lactate biosensors is typically less then 5 minutes reaching in some cases even 10 seconds [50].

One of the important problems in practical application is the shelf and operational lifetime of the biosensor determined greatly by the stability of the enzyme but also by the immobilization method. The activity of the enzyme immobilized on the biosensor is decreasing in time, what causes the necessity of recalibration during prolonged use of the biosensor. This problem can be omitted by the use of disposable biosensors if the shelf life is long enough. Such approach is used in some commercial amperometric biosensors for lactate. The data in literature about operational stability of lactate biosensors are hardly comparable, because this parameter was examined in too many different ways [19]. Generally, the described operational lifetimes are not longer than 2 weeks. The shelf life (storage stability) of lactate biosensors is much longer reaching 6 months for biosensor kept in refrigerator [88].

The electrochemical lactate biosensor can work in batch mode or in flow system. The incorporation of the biosensor into flow system allows automatic measurements, less work and time, with extended linear range [47, 89].

Application of Lactate Biosensors in Food Analysis

Most of the applications of lactate biosensors described in literature are in the area of clinical analysis and sport medicine – L-lactate assay in blood and serum but also in saliva and tears [90]. Apart typical medical applications lactate biosensors were used to assay lactate content in food and beverages. Most of biosensors proposed in literature to assay lactate in different food samples are amperometric enzyme electrodes with L-lactate oxidase or with L- or D- NAD^+ dependent dehydrogenases [19].

Although other types of sensor (potentiometric, field effect transistors, (electro)chemiluminescent) are used to construct lactate biosensor [90], only few of them were applied to assay lactate in real food or beverage samples. Nguyen-Bosse et al. described conductometric biosensor with L-lactate oxidase and horseradish peroxidase immobilized in bovine serum albumin gel [91]. They claimed that LOD lost after immobilization its stereospecifity and applied with success proposed biosensor to assay both stereoisomers of lactic acid in yoghurt samples. Fluorimetric biosensor with L-LDH was used in flow injection system to assay L-lactate in milk drink through increase of NADH fluorescence [71]. Girotti et al. described a flow system with D- or L-LHD, luciferase from *Virio sp.*, (NADH:FMN) oxidoreductase and L-alanine aminotransferase immobilized on the tip of optical fibre measuring D- or L-lactate in beer samples by bioluminescence [83]. Luminol oxidation by H_2O_2 formed in reaction (1) catalysed by peroxidase accompanied with chemiluminescence was used to assay L-lactate in milk [66] and yoghurt [59]. Electrochemiluminescence accompanying luminol electrochemical oxidation coupled with reaction (1) was used in biosensor for assay of L-lactate in whey [57].

Amperometric lactate biosensors were used to assay L-lactate in milk and milk products like yogurts [53, 92-95], cream or cheese [96, 97] without special pre-treatment of samples only with appropriate dilution. The concentrations of L-lactate measured by the biosensors were in good agreement with the results obtained by standard enzyme spectrophotometric methods. The other area of lactate biosensor application in dairy industry is following the fermentation of milk [43, 46, 98] or mozzarella cheese production and ripening [99]. There were attempts to use lactate biosensor to assay traces of antibiotics in milk through inhibition of LOD [100]. L-lactate biosensor was also applied to detect bacterial contaminations in UHT milk leading to its spoilage [20]. Other important area of lactate biosensors application is wine production. Wine contains L-lactate as the result of malo-lactic fermentation but can contain also D-lactate in case of spoilage [76]. Because of that, for wine quality control, both types of biosensor for L- and D-isomers were developed [30, 72, 73, 75, 85, 101]. Mazzei et al. proposed a multienzyme biosensor with LOD and D-LDH for assay the sum of D-and L-lactic acid in wine [82]. L-lactate biosensors were used with success to follow the malo-lactic fermentation in wine[29, 102]. The polyphenolic compounds present in wine, especially in red ones, give some matrix effect to the biosensor response [53] but this problem can be solved by dilution of samples. Lactate biosensors were used also for quantification of L-lactate in other beverages like cider [74], beer [83, 103] or kvass [104]. There are only few examples of lactate biosensor application in other food sectors. Lactic acid was assayed by biosensors in tomatoes and products from them [26, 47] or in meat extracts [105]. Lactate biosensor was also used to assay L-lactate in fermented food like olive and cabbage brine [95], kimchi [106], sport drinks [107].

Assay of L-lactate concentration by lactate biosensor can be used to monitor fermentation processes [31, 79]. L-lactate biosensors can be helpful in veterinary and agriculture for example to detect cattle mastitis [108] because L-lactic acid is present in milk during cow milking in case of udder inflammation. Additionally lactate biosensors can be used in veterinary in similar cases as in human (sport) medicine.

Commercial biosensors for lactate

Most of the commercial biosensors for lactate available on the market utilize L-lactate oxidase as biosensing element and are devoted to assay L-lactate in blood and serum in emergency rooms in hospitals, in clinical laboratories or for personal use for athletes, as the blood lactate level during exercise and after it is the indicators of training status and recovery. Depending on application, the apparatus measuring L-lactate with biosensor could be a laboratory stand, portable or hand held device. Biosensors used in L-lactate analysers could be reusable or disposable. The last approach is typical for hand held devices in sport medicine for personal use. Some devices present on the market can be applied for bioprocess control and food analysis. The commercial biosensor for lactate are summarised in Table 1.

Table 1. Commercial models of lactate biosensors, their producers and application

Producer	Model	Purpose
YSI Inc (Ohio, USA)	YSI 2300 STAT™ Plus Glucose & Lactate Analyzer	Clinical analysis
	YSI 2700 SELECT™ Biochemistry Analyzer	Bioprocess control, analysis of food and beverages
	YSI 7100 Multiparameter Bioanalytical System	Bioanalysis
	YSI 1500 SPORT™ Lactate Analyzer	Sport medicine
Nova Biomedical (Massachusetts, USA)	Stat Profile® Critical Care Xpress Stat Profile® pHOx	Clinical analysis
	BioProfile® Analalyzers	Bioprocess control
	Lactate Plus	Personal use (sport medicine)
Abbot Laboratories (Illinois, USA)	i-STAT®	Clinical analysis (point of care and emergency room)
EKF Diagnostic GmbH (Germany)	Biosen C_Line Biosen S_Line	Clinical analysis
Sens Lab (Germany)	Lactate SCOUT	Personal use (sport medicine)
ARKRAY (Japan)	Lactate Pro LT-1710	Personal use (sport medicine)
Roche Diagnostic (Germany)	Accutrend® Plus	Clinical analysis (point of care)
	Accutred® Lactate	Personal use (sport medicine)
DiaSys GmbH (Germany)	SensoStar	Clinical analysis
Med-Tronik GmbH (Germany)	Powerlact®	Personal use (sport medicine)
ApexBio (Taiwan)	The Edge™	Personal use (sport medicine)
Bio Sensor Technology GmbH (Germany)	LABTREND	Clinical analysis
	LAC^{PRO}	Clinical analysis (portable)
	LactatProfi 3000	Clinical analysis (portable)
Sensolytics GmbH (Germany)	OLGA (On Line General Analyser)	Bioprocess control
Trace GmbH	Labo TRACE	Clinical analysis
	TRACE C2 Process TRACE Multi TRACE	Bioprocess control
BioFutura s.r.l. (Italy)	Wine checker “Per Bacco”	Wine analysis
Tectronik s.r.l. (Italy)	Senzytec 1	Wine analysis

Only YSI Inc. offers the biosensor for food and beverages analysis purpose. In Italy, which is a great wine producer, two little companies (BioFutura and Tectronik) developed amperometric biosensors for D- and L-lactic acid assay, while concentrations of both of them are important wine quality parameters. These two devices use disposable biochips with immobilized lactate dehydrogenases. Application of both of them can be broadened to other analytes like glucose, ethanol, L-malate and others using appropriate electrodes [75].

Very near to the concept of biosensor is SIRE® Biosensor 101e produced by Chemel AB (Sweden). It consists of small reaction chamber with electrodes. For measurement, a small aliquot of L-lactate oxidase is injected to this chamber and the electric current accompanying reduction of H_2O_2 produced in reaction (1), which is proportional to L-lactate concentration, is measured. SIRE technology was used to measure L-lactate in tomato paste and baby food [109].

QUALI_JUICE project

The aim of QUALI_JUICE project "Quality assurance and development of an early warning system for microbial spoilage for the European fruit juice industry", contract number COLL-CT-2005-012461 was the application of lactate biosensor at juice producing plants (especially from apples) to control the production process and quality of the final product. Small and medium fruit juice producers face the problem of contamination with lactic acid bacteria, provoking undesired fermentation processes leading to juice spoilage and causing financial losses for the affected companies and environmental problems. To reach the goals of the project it was divided into few work packages:

- Definition of the problem assumption (risk of contamination by the lactic acid bacteria, producers requirements, state of art in area of lactate biosensors),
- Laboratory validation of chosen biosensors (stability, sensitivity, limits of detection, accuracy, precision, reproducibility, interferences),
- Functional adaptation of chosen biosensors to measure lactate in fruit juices (preparation of samples, elimination of interferants, changes in measurement protocols),
- Practical application (lactate assay in different juice samples and comparison of the results with other methods – enzyme kits and chromatography, measurements of lactate level in fruit juices at the production plant by the producer personnel, evaluation of the biosensors by juice producers, installation of early warning system with the biosensor on-line),
- Training of the juice producers in the use of the biosensors,
- Dissemination (information campaign in press, at exhibitions and conferences and by internet).

The consortium involved in the project decided to use commercial biosensors as the control instruments. The chosen ones were: Biosen C_Line sport (EKF, Germany), LactatProfi 3000 (ABT, Germany), OLGA (Sensolytics, Germany) and Senzytec 1 (Tectronik, Italy). The first two devices are devoted to assay

L-lactate in blood and serum samples. While the chemical composition and pH of fruit juice is completely different from blood both biosensors needed detailed validation [110]. The obtained results indicated that they can be used to assay L-lactate in juice samples after some modifications in operational protocols. The reliable results were obtained when the samples were purified by polyamide 6 instead polyvinylopirrolidone. This result was adopted by Tectronik in the new version of Senzytec device. In case of LactatProfi 3000 crucial point is the calibration of the device. When L-lactate is measured in fruit juices the calibration must be done before every measurement not every 8 hours as it is stated in the producer instruction. The biosensors were used to assay of D-lactate (only Senzytec 1) and L-lactate in samples of apple pulp, apple must and concentrates from the production site [110, 111] , commercial apple juices [110], other fruit juices and traditional fermented food. The results were compared with those obtained by enzyme kits. The results obtained with three tested biosensors and standard enzyme kit method were in good agreement. The biosensors were used to follow the laboratory fermentations of apple juice and apple pulp by lactic acid bacteria and the results were comparable with those obtained by chromatographic method [31, 32]. The use of the biosensors to assay L-lactate in connection with bacteriological analysis allowed to find the critical point at the production plant where the secondary microbiological contamination of pasteurised juice could happened [111]. Biosen C_Line sport, LactatProfi 3000 and Senzytec 1 were tested by some juice producing companies. The evaluation by the end users indicates that from their point of view the best choice would be Biosen C_line sport because of its accuracy, user's friendly interface and simplicity of measurement. The limiting factor of its use is the economical impact (the price of the device is too high for small and medium enterprises).

Because the increasing level of L-lactate in juice is the indicator of the begging of lactic acid bacteria fermentation [111] lactate biosensor can be a part of early warning system on-line. For this purpose OLGA device was chosen while it is working in stopped flow mode. It was integrated with alarming device and connected with a sampling system at the critical point at the production plant. The functional test of the whole system lasted 4 weeks and showed that continuous control of L-lactate concentration at the production line is possible.

The results of QUALI_JUICE project indicates that commercial biosensors for L-lactate can be used in juice production industry to control the production process and quality of final product. The final choice of the device (biosensor) by the future user (juice producing company) would be defined by its particular demands (simplicity of the measurements, possibility of usage at line) and economical impact (price of the device and consumables).

Conclusions

After more than forty years from the first described lactate electrode the application of lactate biosensors in areas different from medicine (including sport medicine) is still very limited. In food and beverage production the assay of lactic

acid is very important to control lactic acid fermentation processes (also undesired leading to the spoilage) and product quality. The use of the biosensors for this purpose could be the alternative for standard methods like enzyme kits and chromatography. The main advantages of the lactate biosensor use are as follows:

- Shortening the time of analysis,
- The assay can be made by unskilled personnel after short training,
- The measurements can be done at the production site,
- The cost of analysis is lower as compared with standard methods.

The disadvantages of the biosensor use in food and beverage industry are: the fact that the measurement with biosensors is not included in standards and legislation and the lack of commercial biosensors devoted for lactate assay in food except YSI 2700 SELECT™ Biochemistry Analyzer (YSI Inc., USA). Only for the wine industry, the market offers specific biosensors for complex analysis of wine production and quality.

Application of lactate biosensors in food and beverage production described in literature is still limited mainly to dairy products and wine. The results of the QUALI_JUICE project proved that commercial biosensors dedicated for assay of L-lactate in blood could be used without any changes in construction for analysis of fruit juices and other products. There is only a need of some simple sample preparation (absorption of interferants by polyamide 6) and some changes in operation protocols (frequency of calibration). These results open the opportunity for much broader use of lactate biosensors in food and beverage industry.

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