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# INDUSTRIAL APPLICATIONS OF WILD AND GENETICALLY-MODIFIED STRAINS OF ACETIC ACID BACTERIA

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*Abstract*: Acetic Acid Bacteria (AAB) have been known for many years, since humans first used them to produce vinegar. AAB serve as biocatalysts in industrial production of, inter alia, acetic acid, dihydroxyacetone, gluconic acid, bacterial cellulose or levan. Apart from the traditional industrial applications of wild strains of AAB, scientists strive to develop novel methods for the production of selected compounds using genetically-modified AAB. The application of such mutants in the industry entails both positive and negative aspects. Modifications of the bacterial genome have a significant effect upon the functioning of the entire cell. This review presents industrial applications of metabolites produced by both wild and genetically-modified strains of AAB.



**PRZEMYSŁOWE ZASTOSOWANIE DZIKICH I GENETYCZNIE ZMODYFIKOWANYCH SZCZEPÓW BAKTERII OCTOWYCH**



*Streszczenie*: Bakterie kwasu octowego znane są od wielu lat, od kiedy ludzie po raz pierwszy wykorzystali je do wytworzenia octu. Bakterie octowe biorą udział w produkcji wielu związków o dużym znaczeniu przemysłowym, m.in. kwasu octowego, dihydroksyacetonu, kwasu glukonowego oraz celulozy mikrobiologicznej. Oprócz tradycyjnych kierunków wykorzystania potencjału biochemicznego dzikich szczepów bakterii octowych, coraz więcej uwagi poświęca się badaniom nad konstruowaniem zmodyfikowanych genetycznie szczepów o zwiększonych możliwościach wytwarzania określonych metabolitów. Zastosowanie mutantów bakteryjnych w przemyśle wzbudza wśród społeczeństwa zarówno pozytywne jak i negatywne odczucia. W artykule przedstawiono zastosowanie dzikich szczepów bakterii octowych oraz opisano możliwości przemysłowej aplikacji genetycznie zmodyfikowanych szczepów tych mikroorganizmów.

**Key words:** AAB, acetic acid, genetic modifications, mutant
**Słowa kluczowe:** bakterie octowe, kwas octowy, modyfikacje genetyczne, mutant

## 1. Application of wild strains of AAB in the industry

Acetic acid bacteria (AAB) are Gram-negative, non-spore-forming, aerobic bacilli [40]. The optimal temperature for their growth ranges from 25 to 35°C, and optimal pH – from 5.4 to 6.3 [11, 28]. AAB are chemoorganotrophs [26]. They may develop in an environment with high osmotic pressure, e.g. in fruits and fruit juices, nectars, bee honeys, ciders or beers [8, 29].

A typical trait of all AAB is its capability of producing acids that are formed as the terminal or transient products of oxidation of alcohols and carbohydrates. The key compounds produced by AAB include: acetic acid, bacterial cellulose, dihydroxyacetone, gluconic acid and levan [30, 33, 35, 38]. These bacteria are also promising starter cultures, used either to better control known food fermentation processes or to produce novel fermented foods and beverages. They play an important role in natural food fermentation processes such as lambic beer, water kefir, kombucha, and cocoa [6].

The most popular product obtained from AAC is vinegar. This compound is mainly used for food preservation, but also for flavour enhancement of dishes. Vinegar is additionally used for the preparation of sauces, mayonnaises and mustards. It improves the sensory attributes of a ready product, and, through its acidifying medium, enables the preservation of food [29].

Recently, there has been a big increase in interest and research regarding the obtaining of bacterial cellulose (BC, also known as microbial cellulose). BC exists as a basic structure of fibril that consist of a β-1,4-glucan chain with the molecular formula $(C_6H_{10}O_5)n$. The glucan chains are held together by inter- and intra-hydrogen bonding. Microfibryls of BC are about 100 times smaller than plant cellulose [9]. Moreover, the three dimensional structure of BC is much more

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organized compared to the structure of plant cellulose [22]. Sheets of dried BC have better mechanical properties compared with traditional paper obtained with the addition of plant cellulose [31].

Bacterial cellulose is called "the material of the future" due to its wide application in various branches of industry and medicine [12]. In 1992, BC was acknowledged to be a compound safe to human health and life by the Food and Drug Administration (FDA) [24]. BC does not enter into reactions with substances contained in food products, owing to which it does not change their organoleptic properties. It remains stable in a wide range of pH values and temperatures; hence, it may be applied to produce food packages [24]. Microbiological cellulose may also be used as a fat substitute for the production of low-calorie food products. It is non-toxic and resistant to mechanical damage, whereas its structure is semi-permeable, which allows its use as a stabilizing agent of suspensions and as a filler in food products with greasy consistency [5]. Currently, this biopolymer is produced by wild strains of AAB belonging to the *Komagataeibacter xylinus* (previously *Gluconacetobacter xylinus*) and *Gluconobacter oxydans* species [12, 19]. The synthesis of BC is possible thanks to cellulose synthase complex (CS complex), which is well-structured machinery. The CS complex includes a series of subunits working in a concerted way that synthetize and export the β-glucan chains in the extracellular space [12].

Another interesting substance of industry importance, produced by wild strains of AAB (e.x. *G. oxydans*), is dihydroxyacetone (DHA). DHA is a product of the microbiological oxidation of glycerol, catalyzed by bacterial membrane-bound glycerol dehydrogenase [35]. The most common traits of DHA are the formation of colour compounds as a result of contact with human skin; therefore, it is applied as an active ingredient of tanning cosmetics [7].

Some wild strains of AAB, including *G. oxydans*, are able to oxidize glucose to gluconic acid, which also finds applications in food production. This organic acid can play a significant role in the acidification of food products as well as being a preserving agent. It is also applied to produce artificial honey, cheese, and sausages [25]. Another, less popular but still important application of AAB, is the production of levan (homopolysaccharide of fructan type with β-(1-6) glycosidic linkages). Some strains of AAB ex. *Kozakia baliensis* DSM 14400, *Neoasaia chiangmaiensis* NBRC 101099, can play a significant role in producing buckwheat-sourdough bread [37]. Ua-Arak *et al.* [36] have demonstrated the possibility of using selected strains of AAB in gluten-free sourdough production, which contained levan in situ [18, 37].

Apart from the well-known biochemical potential of AAB strains, scientists are still searching for new methods to improve the industrial application of AAB. New research involves molecular techniques to increase the microbial production of compounds obtained from these bacteria.

## 2. Application of genetically-modified strains of AAB in the industry

Genetic modifications of bacteria allow many problems faced during the application of wild strains in the industry and medicine to be solved. The isolation and characterization of mutants expands the knowledge of the metabolic pathways running in bacterial cells. The industrial application of genetically-modified strains of AAB enables the production of a specified metabolite and, at the same time, minimizing or eliminating troublesome by-products and compounds contaminating the natural environment [17, 20].

Ethanol oxidation is primarly catalyzed by aldehyde dehydrogenase (ALDH). It is expected that the increased level of this enzyme, caused by the dosage effect from cloned ALDH genes, brings about an increase in the ethanol oxidation rate. The ALDH gene was cloned from *A. polyoxogenes* and integrated into a vector that was constructed from a plasmid indigenous to *Acetobacter* and introduced into strain NBI 2099, isolated from commercially available vinegar and identified as *A. aceti*. The mutant showed increased ALDH activity, which resulted in higher acetic acid productivity. The mutant concomitantly produced a higher concentration of acetic acid than the wild strain (causing 2-fold increases in the production rate and in the maximum concentration of acetic acid) [10].

Habe *et al.* [13, 15] facilitated the mutation of AAB from the species *Gluconobacter frateurii*. The wild strain was carrying out biotransformation of glycerol to glyceric acid, while glycerol dehydrogenase (GlyDH, EC 1.1.99.22) was catalyzing the oxidation of substrate to DHA, which was accumulated in bacterial cells [13–15]. In order to facilitate the recovery of pure gluconic acid from the post-reaction mixture, the *sldA* gene encoding GlyDH was removed from the genome of *G. frateurii*. The resultant deletion mutants were characterized by a higher production of glyceric acid (12.5 g/L) compared to the wild strains (9.8 g/L), and the final product was free of DHA, which facilitated its recovery [15].

Another species of AAB subjected to genetic modification in order to inhibit the synthesis of an undesirable compound was *Gluconacetobacter europaeus*. Bacteria of these species are commonly applied for the production of rice vinegar [1]. The organoleptic properties of the finished product are affected by the raw material subjected to the fermentation process and by multiple chemical substances synthesized during its microbiological production. One of them is acetoin:

a volatile compound with a strong buttery-creamy aroma, that is undesirable in Japanese rice vinegar and sake [1]. Various strains of *G. europaeus* bacteria have been modified to ensure a high quality of these products. Genome modifications consisted mainly of the impairment of the activity of enzymes that catalyzed respective biochemical transformations in the acetic acid synthesis pathway. For instance, the *aldC* gene that encoded α-acetolactose decarboxylase (EC. 4.1.1.5) – an enzyme catalyzing the transformation of α-acetolactate to acetone – was removed from the genome of *G. europaeus* KGMA4004 bacteria. The resultant deletion mutant of *G. europaeus* KGMA4004 was characterized by acetoin production lower by ca. 30%, compared with the non-mutated strain [1].

In some cases, the application of genetically-modified AAB in the industry allows a reduction of costs usually incurred for the production of specified chemical compounds with traditional microbiological methods. Many mutants are capable of utilizing components of cheap culture media in the form of, for example, post-production wastes for their growth. One of the means to minimize the costs of bacterial cellulose production may be the application of a cheap and easily available substrate, e.g. lactose [3]. This saccharide occurs in cheese whey, which is a by-product of the cheese making process. The global production of whey is estimated to exceed $145 \times 10^6$ t annually, and only half of this is converted into usable products [32, 39]. Whey is characterized by a high biological oxygen demand (BOD above 30 kg/m$^3$) and a high demand for chemical oxygen (above 60 kg/m$^3$), [3] owing to which it poses a serious problem to the natural environment. Whey is managed through the microbiological fermentation of lactose, and products of its transformation include, i.a., lactic acid, gellan, and ethanol. *E. coli* bacteria display the activity of β-galactosidase (EC. 3.2.1.108), which catalyzes the hydrolysis of lactose to glucose and galactose. β-galactosidase is encoded by the *lacZ* gene [3]. It was demonstrated that when the *lacZ* gene from *E. coli* was transferred to *Acetobacter xylinus*, this mutant was capable of growing on whey, by consuming 85% of its lactose (for 4 days of culture), and simultaneously produced 0.78 g/L of pure cellulose [3]. The use of genetically-modified cells of *A. xylinus* on an industrial scale could greatly facilitate whey management.

A positive aspect of the industrial application of genetically-modified acetic acid bacteria is better utilization of culture medium components and enhancement of the activity of some enzymes produced by these microorganisms. To achieve this, cells of *G. frateurii* THD32 strain bacteria were subjected to mutation [34]. The modification consisted of the removal of the *sboR* gene responsible for the regulation of *sldSLC* gene transcription (encoding the flavin adenine dinucleotic dependant on D-sorbitol dehydrogenase FAD-SLDH). Wild strains of the genus *Gluconobacter* are characterized by their capability to oxidize different saccharides and alcohols and to accumulate appropriate products of these reactions, and by a low effectiveness of biomass production [34]. The deletion mutant of *G. frateurii* THD32Δ*sboR* exhibited better growth on D-sorbitol and L-sorbose compared with the parental organisms. It was also characterized by an almost twofold higher activity of NADPH-dependent L-sorbose reductase (EC. 1.1.1.289), responsible for the assimilation of L-sorbose being a significant link during ascorbic acid production [34].

Genetic modifications have allowed the improving *G. oxydans* strain the capability to produce dihydroxyacetone [2, 13, 23]. The main problem encountered during DHA production is the inhibition of the metabolic activity of bacteria caused by the improper choice of substrate concentration and an increasing concentration of the product [2]. *G. oxydans* produce DHA and glyceric acid at the same time. These reactions are catalyzed by glycerol dehydrogenase (EC. 1.1.1.6) and alcoholic dehydrogenase (EC. 1.1.1.1), respectively. It was demonstrated that the *G. oxydans* mutant, deprived of the gene encoding the alcoholic dehydrogenase (Δ*adhA*), became capable of growing in the medium with a high glycerol concentration (150 g/L) and, simultaneously, of producing DHA with the concentration of 125 g/L [13].

Apart from beneficial effects, the application of genetically-modified microorganisms may have some negative outcomes. It sometimes happens that the intended modifications of the genome initiate the uncontrolled mutations. Discontinuity of a specified gene may, for example, cause enhanced expression of another gene. The literature reports on some cases of mutations that failed to bring the expected outcomes. An example may be mutants of *G. oxydans*, which, despite achieving novel, desirable traits, were losing the primary ones that had earlier been exploited in the pharmaceutical industry [21]. Genetic modification consisted of removing genes responsible for the synthesis of enzymes: self-dependent glucose dehydrogenease associated with the cytoplasmic membrane (*mgdH*) and cytoplasmic glucose dehydrogenase (*mgdH sgdH*) inactivated with it. Recombinants of *G. oxydans* N44-1 Δ*mgdH* and *G. oxydans* N44-1 Δ*mgdH sgdH* produced a higher (compared to the wild strain) biomass yield in the culture medium with glucose; however, they lost their capability to convert 2,5-diketogluconate and 2-ketogluconate that are substrates in vitamin C production [21].

Genetic modifications consisting of the removal of a specified fragment of a bacterial genome may inhibit the action of a gene encoding typical traits of the organ-

ism. Such a change in the metabolism was observed in one of the strains of AAB: namely in the *G. frateurii* mutant [34]. These bacteria were deprived of the *sboR* gene, which encoded the enzyme being responsible for the regulation of transcription of another gene (*sld-SLC*) indispensable for the production of L-sorbose from D-sorbitol. Compared with the wild strain, the mutant of *G. frateurii* was characterized by enhanced activity of the NADPH-SR enzyme responsible for the assimilation of L-sorbose and for decreased dynamics of action of NAD-SLDH catalyzing D-sorbitol oxidation to D-fructose [34].

An example of the undesirable (from the perspective of the food industry) effect of genetic modifications on the production of selected chemical compounds is also the mutation of *G. oxydans* IFO 12528 [14]. This strain is capable of producing glyceric acid from glycerol in the presence of alcoholic dehydrogenase (mADH), which is, in turn, encoded by the *adhA* gene. The action of mADH is based on ethanol oxidation to acetic aldehyde, which is next converted into acetic acid under the influence of aldehyde dehydrogenase (mALDH). The genetic modification of *G. oxydans* 12528, consisting of the cutting out of the *adhA* gene, caused the halt of acetic acid production from ethyl alcohol and lowered by half the production of glyceric acid, which was still being produced despite the absence of mADH. Study results suggest that another enzyme could be present in the mutant's cells, that could take part in the production of glyceric acid [14].

## 3. Opinion on GMOs used in industry

Genetically modified organisms still have a rather bad reputation in Europe. The anti-GMO lobby accuses proponents of this technology of pushing the introduction of GMOs into agriculture without adequately considering health and environmental risks. The pro-GMO camp charges its opponents with blowing potential risks out of proportion in order to manipulate public opinion against this new technology. During this mutual finger pointing, both sides have taken to blaming the public for a lack of understanding [27]. Consumers worldwide are displaying limited understanding, misconceptions, and even unfamiliarity with GMO food products. In The United States, GMO labelling is obligatory (similar to current European standards); however, consumer awareness of current GMO labelling is still low [41]. The 48% of respondents said that they knew very little about GMOs, whereas 16% felt they knew nothing at all, compared with 30% knowing a fair amount and just 5% knowing a great deal about GMOs [16]. It seems that the coming years may see exponential increases in GMO product development, as researchers gain increasing access to genomic resources that are applicable to organisms beyond the scope of individual projects. It is generally thought that genetic engineering is the inevitable wave of the future, and that we cannot afford to ignore a technology that has such enormous potential benefits [4].

## 4. Summary

The wild strains of AAB are commonly applied in various branches of industry. The same genetically-unmodified strains have been used for the production of vinegar for years. The industrial application of chemical compounds, that were produced with microbiological methods exploiting wild strains of AAB, does not generate any negative emotions among consumers.

Thus far, achievements of genetic engineering in studies addressing acetic acid bacteria have enabled better recognition of their metabolic pathways. In addition, they have contributed to imparting novel traits to these microorganisms, thereby making them producers of many valuable compounds (e.g. dihydroxyacetone, bacterial cellulose).

To apply recombinant DNA technology to improving AAB to an even greater extent, further research concerning gene expression, protein engineering, and other fundamental studies are required. Progress in process development is also indispensable for exhibiting the potential of recombinant bacteria.

Scientific research indicates that the mutated and characterized strains of acetic acid bacteria may be successfully applied to produce important compounds in processes that are more effective, economically profitable and safer to the environment compared with traditional ones. However, for many scientific experts and ordinary people the question: "what are the risks of tampering with mother nature?" will remain open for the next few years.

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