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Vinegar and other products

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TABLE OF CONTENTS

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Opening Session

FOOD SAFETY, A TOOL FOR SUCCESSFUL INNOVATION

A.M. Troncoso-González[1*]

[1]*Nutrition and Food Science. Faculty of Pharmacy, University de Sevilla, Spain.*

[*]*Corresponding author: amtroncoso@us.es*
Phone: +34 954556759.

The globalisation of the food chain is continually throwing up new challenges and risks to the health and interests of consumers. The central goal of the European Union's food safety policy is to achieve the highest possible levels of protection for human health and consumers' interests in relation to food. This is accomplished by ensuring that food is safe and appropriately labelled - taking into account diversity, including traditional products - while at the same time ensuring the effective functioning of the internal market. General Food Law of 2002 sets out the basic principles and recognises the shared responsibility of all actors in ensuring the safety of our food.
Safety is the essential precursor on which the food industry is built – the absolute bottom line. Without safety, issues such as quality and innovation have no grounding The EU has developed a comprehensive body of food safety legislation, which is being continually monitored and adapted as new developments arise. This legislation is based on risk analysis. The establishment of the European Food Safety Authority (EFSA) was a key step to support the work of the EU institutions in protecting EU consumers in this field, providing independent scientific advice on existing and emerging risks. Safety issues have to be taken into account since the beginning and play an important role for successful innovation, Several examples will be discussed in this presentation.

Keywords: food safety.

SESSION I. Why do we care about Acetic Acid bacteria? History. Traditional vinegar. Food and Health.

S1-KN
PROTECTED GEOGRAPHICAL STATUS: LIMITS AND OPPORTUNITIES FOR VINEGARS

P. Giudici[1*]

[1]*Department of Agricultural and Food Sciences (DipSAA), University of Modena and Reggio Emilia, via Amendola 2 (Padiglione Besta), 42122 Reggio Emilia, Italy.*

[*]*Corresponding author: paolo.giudici@unimore.it*
Phone: +39 0522522034.

Protected Geographical Status (PGS) is a legal structure defined by European Union to protect regional food and specialties. It groups three distinctive systems: Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialty Guaranteed (TSG). The geographical appellation to distinguish food and beverages, especially wine, is very old, but it was applied systematically, starting from 1935 for the French wines with the constitution of the *Institut National des Appellations d'Origine*. Then, legally enforced with the *Appellation d'Origine Controlée*. Nowadays, geographical appellation or PGS is quite spread and many countries has adopted systems that remind the PGS structure of EU, were the number of regional foods with a PGS is huge. E.g. France claims 1578 PGS plus 640 wines with appellation, Italy has 238 PDO/PGI products, plus 467 wines. The other EU countries follow with relatively less products. This huge number of PGS products clearly shows how much is considered this policy in the EU. The reason is due to the broad idea that geographical appellation policy is a key to improve social and economical life of local agricultural area. In particular the PGS has been accredited of five main features: 1) to protect the reputation of regional food; 2) to promote rural and agricultural activity; 3) to help producers obtaining better price; 4) to eliminate unfair competition; 5) to help consumers to discriminate among authentic and non-genuine products.
Now, after few decades since the implementation of the PGS structure, it is time to make a balance cost/benefit of the system. The first question to answer is: does the PGS reach the initial purposes? In particular, does it help and promote rural area? Does it help consumers to obtain better price for authentic products? If we consider that many PDO products have cyclic commercial difficulties and prices paid to farmers are often lower than the production cost, the answer is no. Furthermore, the price of PDO products paid by consumers on the retail centers is higher than the price paid for analogous products, it means that something wrong it happen in the distribution chain.
The vinegars with a PGS are a restricted number: Vinagre de Jerez; *Vinagre del Condado de Huelva; Aceto Balsamico Tradizionale di Modena; Aceto Balsamico Tradizionale di Reggio Emilia*. Then, PDO attribution is in progress for two other vinegars, *Vinagre de Montilla-*

Moriles and the Chinese *Zhenjiang Xiang Cu* (镇江香). The concerns about GPS policy are particularly relevant for PDO/PGI vinegars.

Keywords: protected geographical status, vinegars, PGI, POD.

S1-O-01
THIRD JUBILEE OF THE DISCOVERY OF THE MECHANISM OF THE ACETOUS FERMENTATION BY LOUIS PASTEUR IN 1861

J.F. Bourgeois,[1*] I. García-García[2]

[1]Consultant for H. Frings, D Bonn, 19 route de Chevrier, CH 1244 Choulex, Switzerland; [2]Department of Chemical Engineering, Faculty of Sciences, University of Córdoba, Campus Universitario de Rabanales, Ctra(a) de Madrid km 396. 14071 Córdoba, Spain.

**Corresponding author: jacquesfbourgeois@gmail.com Phone: +41 227501267.*

In July 26th 1861 Pasteur addressed a first memorandum to the "Société Chimique de Paris" regarding his studies about the acetous fermentation. That same year 1861, Pasteur obtained a patent called "For the fabrication of acetic acid", dated July 9th, sent by the Institut Pasteur in Paris. Pasteur wrote also: "I am determined from today to restitute this patent to the public domain". A mark of his generosity. It is interesting that Pasteur used the new nomenclature *Mycoderma aceti* in the patent. The genus *Mycoderma* was described for the first time by Persoon in 1822, the year Pasteur was born. In "Etudes sur le vin" (1873), Pasteur wrote: "Wine and vinegar are very similar with respect to the causes of their susceptibility to diseases". The style of Pasteur is very clear and explicatory. He is full of enthusiasm and modesty when he writes about his own artistic drawings: "The reality is much superior to a drawing... and, I may say, superior to the beauty of these charming little strings".

Pasteur is often remembered for his extraordinary discoveries in Microbiology. For instant, Prof. Pasteur Vallery-Radot, grandson of Louis Pasteur, wrote in the Introduction of Volume III of his book "Oeuvres de Pasteur, Etudes sur le vinaigre et sur le vin", in 1924: "In 1861 Pasteur discovers the role of *Mycoderma*". In a special edition of "Branntweinwirtschaft" in 1961, called "Pasteur Jubilee, 100 years of vinegar bacteriology", the great microbiologist J.L. Shimwell wrote: "It was not until 1861 that Louis Pasteur made the momentous announcement that vinegar was not made by a purely chemical oxidation of ethanol, but by the agency of a minute living "plant"". In the same edition, Prof. Pasteur Vallery-Radot wrote: "Pasteur, immersed in his research on fermentation, studies acetous fermentation. He finds out that the acetification is due to a thin biofilm which covers the alcoholic liquid, on which it is growing. This biofilm consists of a particular *Mycoderma*, with the property of absorbing and condensing large quantities of oxygen, and fixing it on alcohol. This process transforms alcohol into acetic acid". But, in the same publication, Shimwell wrote: "It seems that it was not until the next year (1862) that he definitely named it *Mycoderma aceti*. That would constitute a round figure of exactly 150 years, to celebrate the Jubilee of the naming of the species *aceti*. The occasion of the celebration of the Pasteur Jubilee of his discovery of the mechanism of acetous fermentation occurred in 1961, in Dole (France), the birth place of Pasteur. It coincided with the annual celebration of his birth on December 27th 1822. A local newspaper "Les Dépêches" published on December 27th 1961 an article to commemorate the event making reference to Mr. Bourgeois as "a vinegar brewer, instigator of the Pasteur Jubilee". So, the same "instigator" suggests this Conference to be an excellent occasion for the "Third Jubilee of the discovery of the acetous fermentation".

Keywords: mycoderma aceti, acetous fermentation, acetic acid bacteria, jubilee.

S1-O-02
CHINESE CEREAL VINEGARS IN SOLID-STATE FERMENTATION

F.S. Chen,[1] L. Li,[1] J. Qn,[1] C.X. Chen[1*]

[1]College of Food Science and Technology, Huazhong Agricultural Univerisity, Wuhan, Hubei, 430070 P.R. China.

**Corresponding author: chenfs@mail.hzau.edu.cn Phone: +86 2787282927.*

In China there is a proverb saying that in a daily life seven indispensable substances are firewood, rice, edible oil, salt, sauce, vinegar and tea. From the proverb, we can see the vinegar has a very important position in Chinese life. In the historical literature, we can find that vinegar originated more than three thousands years ago in China. It is reported that the first mention about vinegar was in 1058 B.C. in the book named Zhou Li about rites of the Zhou Dynasty, and the professional workshop for vinegar appeared in Chunqiu Dynasty (770 B.C. ~ 476 B.C.). At that time, vinegar was so costly that only the noble could afford it. Vinegar became popular in folk in Donghan Dynasty (25A.D.-220 A.D.). Up to Northern and Southern Dynasties (420A.D.-581 A.D.), the book named Qi Ming Yao Shu about the essential techniques of farming, written by Sixie Jia, recorded 23 kinds of methods about how to brew vinegars in detail.

Besides seasoning, vinegar is regarded as a herbal medicine in China. In the book of Compendium of Materia Medica written by Shizhen Li in 1857, medicinal functions of vinegar were described as dissipating the blood stasis, treating the diseases of jaundice and yellow sweat, improving appetite and nourishing the liver. In Dictionary of Chinese Medicine, it was also recorded that the vinegar had the medicine's curative effect for acute and chronic hepatitis.

Nowadays, in Chinese markets, there are more than 20 types of homemade cereal vinegars, most of which are brewed with starchy materials such as rice, sorghum, corn, barley and wheat. Among them, the most famous Chinese vinegars are Shanxi aged vinegar, Zhenjiang aromatic vinegar, Sichuan bran vinegar and Fujian Monascus vinegar, respec-

tively using sorghum, sticky rice, wheat bran and red yeast rice as the main raw materials or the starter. These vinegars are also honored as Four China-style Famous Vinegars due to their unique favors, long production histories, massive yields and characteristic fermentation processes.
Besides the raw materials, according to the colors, the special favors and the production processes, Chinese vinegars can be classified into different groups, such as black (brown) vinegar, red vinegar, white vinegar, smoky vinegar, herbal vinegar, solid-state fermentation (SSF) vinegar, liquid-state fermentation (LSF) vinegar and so on.
In this paper, we will take Shanxi aged vinegar as an example to make a description about the history, the general SSF process, the main microorganisms and functional materials of Chinese cereal vinegars.

Keywords: chinese cereal vinegars, solid-state fermentation, microorganisms functional materials.

S1-O-03
SIMPLE PROCEDURE TO DISCRIMINATE AMONG BALSAMIC VINEGARS

F. Lemmetti,[1*] P. Giudici[1]

[1]*Department of Agricultural and Food Sciences (DipSAA), University of Modena and Reggio Emilia, via Amendola 2 (Padiglione Besta), 42122 Reggio Emilia, Italy.*

[*]*Corresponding author: federico.lemmetti@unimore.it*
Phone: +39 0522522034.

A lot of sweet and sour vinegars and dressing, derived from grape must and appealed as "balsamic", are currently available in the market all over the world. We have grouped the different products on the basis of their Protected Geographical Status, ingredients and additives used, as well as the production technology.
The consumer perception of the attribute "balsamic" is associated with a series of common features that clearly discriminates them from other types of vinegars. They are dark, opalescent, shiny, thick and viscous, and the acidic taste is modulated by a variable amount of sugars. The features described are usually achieved in different ways and this make the difference among the balsamic vinegars and sauces. The key point refers to the fact that different methods, used for the same purpose, can have dramatically different costs, which then affect the price and quality of the finished product; i.e. the dark colour can be obtained with addition of caramel (E150d) or by long aging. The differences on production cost is huge and obvious.
The most expensive step in industrial production is aging, and of course this is particularly important for traditional balsamic vinegars. Aging plays a key role in determining many valuable characteristics of balsamic, for example, the dark brown colour belongs from the initial process of must cooking, which triggers a series of non-enzymatic browning reactions that slowly continue over the years. The process of aging in wood barrels increases the specific gravity and viscosity due to the progressive loss of water and the formation of high molecular weight compounds (melanoidins) that modify the rheological properties of the product.
We set up a simple procedure able to discriminate among different balsamic products, with evident convenience for producers and for consumers protection. The procedure is based on few descriptors like the difference from Brix value and reducing sugars, pH, total and fixed acidity. The samples of Traditional Balsamic Vinegar are clearly separated from all the other balsamic vinegars and dressings. This could be explained with the higher concentration of melanoidins and other sugar degradation compounds that are formed during the long aging.

Keywords: traditional balsamic vinegar, aging, melanoidins.

S1-P-01
AGING OF WINE VINEGARS WITH DIFFERENTS WOODS: INFLUENCE IN THE POLIFENOLS AND COLOUR

V. González,[1*] I. López,[1] J.M. León,[1] J. Lasheras,[1] F. Heredia,[2] P. Ramírez[1]

[1]*IFAPA Centro de Cabra. Carretera Cabra-Doña Mencía, km 2.5. 14940 Cabra (Córdoba), Spain.* [2]*Food Colour & Quality Laboratory, Department of Nutrition & Food Science, Facultad de Farmacia, Universidad de Sevilla, 41012 Sevilla, Spain.*

[*]*Corresponding author:*
virginia.gonzalez.c@juntadeandalucia.es
Phone: +34 957596644.

Colour is an important factor related to the sensorial properties of the products, and it is one of the quality parameters of wine vinegar that is monitored, because of its relevance in sensory analysis and as indicator of processes, such as oxidation and aging.
The aging during 7 months of two kinds of vinegars, with 2 % (v/v) of ethanol (vinegar 1) and with 0.5% (v/v) of ethanol (vinegar 2) have been studied. The barrels were made from two tipes of oak wood, American oak and French oak, and two kinds of barrels new and used.
This study aims to determine how wood barrels affect vinegar quality. Due to the importance that slight changes in phenolic composition have on organoleptic properties analysis of samples included colorimetric parameters and total phenol.
All of the colorimetric parameters evolve constantly in a positive way during the whole period of aging indicating a positive effect. Specifically, the lightness, L*, of the initial must decreased slightly in the vinegar 1 by 2 % and 5%, in the vinegar 2 has remained practically constant. The chroma, C^*_{ab} in the vinegars aged in French oak increased by 28% doubling the values of the American oak vinegar. h_{ab} of the initial must decreased by 1 and 3% in all the vinegars except in the old French vinegar, that decreased of the initial by 7% at 8% in the two tipes of vinegars studied. The total phenol increased by 65% in the vinegar 2 aged on new barrel French oak, it doubling the content respect the American oak vinegar, in the vinegar 1 occurs something similar but with slightly lower values.
Sensorial analysis of wine vinegars were described on the basis of the attributes previously selected. The general

impresion of vinegars aged in new French barrel vinegars were those who scored higher. These results prove that wood used in vinegar aging influences in a positive way in the polyphenol content and color of the final vinegar.

Keywords: quality, american oak, french oak, new barrels, used barrels.

S1-P-02
ANALYSIS OF VARIATION OF MAIN COMPONENTS AND FUNCTIONAL FACTORS DURING AGING PROCESS OF SHANXI AGED VINEGAR

T. Chen,[1] J.J. Shi,[1] F.S. Chen[1*]

[1]College of Food Science and Technology, Huazhong Agricultural Univerisity, Wuhan, Hubei, 430070 P.R. China.

**Corresponding author: chenfs@mail.hzau.edu.cn*
Phone: +86 2787282927.

Shanxi aged vinegar is one of the most famous traditional vinegars in China. It is produced from several kinds of cereal including sorghum as main raw material, a large dosage of starter *Daqu* (about 60% of the raw material and made from barley and pea), wheat bran and millet chaff as supplementary and filling material with traditional solid state fermentation techniques.
Shanxi aged vinegar was found be beneficial to human health, and large numbers of researches focused on nutrition, flavor and functional components of the vinegar. However, there are no reports on the changes of main components and functional factors during aging process in Shanxi aged vinegar.
In this study, the volatile flavor compounds, amino acids, organic acids, trace elements, other conventional ingredients and functional factors in Shanxi aged vinegar were detected by instrument technology. The results showed that most conventional ingredients and functional factors (polyphenols, flavones, GABA, tetramethyl pyrazine and ferulic acid) were increased during aging process. There were abundant nutritional and functional components in 8-year-old vinegar. Total polyphenols and total flavonoids were 12.01 mg/mL and 3.79 mg/mL in 8-year-old vinegar, and tetramethy pyrazine, ferulic acid, GABA were 321.20 μg/mL, 19.64 μg/mL and 54.52 mg/100 mL respectively in the 8-year-old vinegar.

Key words: shanxi aged vinegar, components analysis, common components, volatile components, functional components.

S1-P-03
EVOLUTION OF WINE VINEGAR COMPOSITION DURING AGING WITH OAK BARRELS

V. González,[1*] P. Ramírez,[1] J.M. León,[1] J. Lasheras,[1] I. López[1]

[1]IFAPA Centro de Cabra. Carretera Cabra-Doña Mencía, km 2.5. 14940 Cabra (Córdoba), Spain.

[*]Corresponding author:
virginia.gonzalez.c@juntadeandalucia.es
Phone: +34 957596644.

A crucial step in the production of high-quality wine vinegars is ageing in wood. This study aims to determine how wood barrels affect vinegar quality. For this purpose, two kinds of barrels new and used were evaluated, vinegars were aged for 7 months in barrels made from two tipes of oak wood, american oak and french oak. To test the effect of alcohol content on the extraction from wood, two kinds of vinegars were studied, one of them with 2 % (v/v) of ethanol (vinegar 1) and the other one with 0.5% (v/v) of ethanol (vinegar 2).
Analysis of samples included dry extract, ashes, alcoholic degree, total acidity fixed acidity, volatile acidity, pH and color intensity.
Barrels aging increased the concentration of dry extract specially in the vinegar 1 in french oak (new and used) and in the vinegar 2 of new american oak, total acidity increased in all the vinegars mostly on the vinegar 2 of french oak, fixed acidity increased in all the vinegars specially in the vinegar 2 of french oak and of american old oak, ashes increased a greater proportion in vinegar 2 of new american barrels and color intensity increaded considerably in vinegar 1 french oak barrels.
Sensorial analysis of wine vinegars were described on the basis of the attributes previously selected and from these results their spider charts were drawn. The intensity of ethyl acetate odor is more evident in vinegar 1 in new french barrels and old american barrels. Woody odor in french barrels (new and used) and new american barrel in vinegar 1 were the greatest. Vainilla odor in french barrels new and used in vinegar 1 were the greatest.
The remaining alcoholic degree of vinegars plays an important role in the ethyl acetate and woody aroma components. Overall impression, aroma intensity and quality clearly increase with aging.
These results prove that wood for aging in vinegar determines both the chemical composition and sensorial properties.

Keywords: high-quality, american oak, french oak, new barrels, used barrels.

S1-P-04
ORAL ADMINISTRATION OF LIPOPOLYSACCHARIDE OF ACETIC ACID BACTERIA PROTECTS POLLEN ALLERGY IN MICE MODEL

H. Inagawa,[1,2,3] S. Amano,[2] T. Nagashima,[2,4] C. Kohchi,[1,2,3] G.I. Soma[1,2,3,5]

[1]Faculty of Medicine, Kagawa University, Kagawa, 761-0793; [2]Control of Innate Immunity, Technology Research Association, Kagawa, 761-0301; [3]Macrophi Inc., Kagawa, 761-0301; [4]Toyo Hakko Co., Ltd., Aichi, 474-0046; [5]Institute for Health Sciences, Tokushima Bunri University, Tokushima, 770-8514, Japan.

[*]Corresponding author: pxs07205@nifty.ne.jp
Phone: +81 878677712.

Acetic acid bacteria are one of Gram-negative bacteria produce many fermented foods such as vinegar and Kefir (Caspian yogurt) which contribute to health, but the substances contributing to health in these foods are not clarified yet. Lipopolysaccharide (LPS), a major component of cell wall of Gram-negative bacteria, is known to have strong immune-regulatory activity. We have found and reported the existence of biologically active LPS in acetic acid bacteria. The LPS shows limulus positive activity and activation of macrophage to produce nitric oxide and tumor necrosis factor. In this study, to estimate anti-allergic effect by oral administration of acetic acid bacteria extract containing with LPS were investigated, the cedar pollinosis model was used. Establishment of the protection of pollen allergy with food derived materials are quite important matter in Japan.

In this study, we isolated acetic acid bacteria from various fruits by GYC medium. The bacteria were cultured with modified Nodai medium and the collected cells were suspended with distilled water and heated (120°C, 20 min) to prepare LPS containing extract. The extract contained about 0.001-0.01 mg/mL of Limulus positive substances. The extract of acetic acid bacteria induced higher level of IL-10 and FOXP3 mRNA expression in macrophage (RAW246.7 cell) by DNA microarray analysis.

Then the anti-allergic effect of acetic acid bacteria extracts was investigated. BALB/c mice were immunized with mixture of cedar pollen and alum into peritoneal cavity, and additional immunizations of pollen to nasal cavity. After immunization, the mouse were administered the pollen into nasal cavity for trigger allergy reaction and the number of scratch their nose were counted during 5 minutes. By the oral administration of acetic acid bacteria extract demonstrated significantly less the scratch number than control water group with pollen immunization.

These results showed that LPS in acetic acid bacteria have a possibility to protect of allergic reaction, especially cedar pollenosis. Further analysis should be needed to clarify the mechanism for anti-pollen effect of LPS of acetic acid bacteria.

Keywords: lipopolysaccharide, oral administration, anti-allergy, macrophage.

S1-P-05
THE AGE OF BALSAMIC VINEGAR: RESIDENCE TIME AND CHEMICAL DESCRIPTORS

F. Lemmetti,[1*] P. Giudici[1]

[1]*Department of Agricultural and Food Sciences (DipSAA), University of Modena and Reggio Emilia, via Amendola 2 (Padiglione Besta), 42122 Reggio Emilia, Italy.*

**Corresponding author: federico.lemmetti@unimore.it Phone: +39 0522522034.*

Traditional balsamic vinegar (TBV) production involves some distinct steps: the cooking of the must is followed by its alcoholic and acetic fermentation; then, during the aging in a barrel set, the volume loss due to evaporation is restored according to the technique of "rincalzo" (topping up) that remind the Solera system. This procedure leads to a set of barrel containing a mixture of vinegars of different ages, and the TBV age is defined as "residence time" (RT), that is the weighted average among each aliquots of vinegar mixed through the years. From an algebraic point of view, the problem has been solved and the result is a mathematical model in which the TBV age is easily estimated from the volumes involved in the maintenance of the barrel set (Giudici and Rinaldi, 2007). While it is intuitive and commonly accepted that losses affect the yield of the barrel set, it is more interesting to clarify the relationship between yield, evaporation rate and age of TBV. The lower is the yield, due to losses by evaporation and other causes, the lower is the RT and thus the effective aging. The intensity of aging of TBV is indeed inversely proportional to the flow of vinegar through the barrel set.

This simulation system has been modified by excluding the water and the calculation is based on the actual dry matter that undergoes aging, i.e. the solutes dissolved in the vinegar. To this end, the solute losses, due to chemical and physical phenomena and biological activities, have been quantified for every stages of TBV production. Based on the mass balance performed, a rigorous calculations can be set, in order to simulate the trend of the RT and concentration profile for the product. Yields calculated in this manner are consistent with data collected from different barrel sets during a few years and it is clear that the yield significantly influences the RT of TBV: older TBVs correspond to high yields. The calculation of the RT on the basis of dry mass, besides being more correct because water is excluded from the calculation, provides higher values than those calculated by the volume, and these differences are greater for the most aged barrels of vinegar. In short, increasing the yield during the aging process correspond to the increase of TBV quality.

From a chemical point of view, melanoidins are potential indicator of TBV age and quality, since these compounds vary in terms of quality and quantity during the residence time of TBV in the barrels.

Keywords: traditional balsamic vinegar, aging, residence time, yield.

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SESSION II. What are they? Taxonomy and Phylogenesis. Ecological aspects (natural occurrence of AAB; Biodiversity).

S2-KN-01
TAXONOMY OF ACETIC ACID BACTERIA AND MOLECULAR TECHNIQUES SUITABLE FOR THEIR IDENTIFICATION AT THE SPECIES LEVEL

I. Cleenwerck,[1*] P. De Vos[1,2]

[1]BCCM/LMG Bacteria Collection, Laboratory of Microbiology, Faculty of Sciences, Ghent University, Belgium; [2]LM-UGent, Laboratory of Microbiology, Faculty of Sciences, Ghent University, Belgium.

[*]Corresponding author: ilse.cleenwerck@ugent.be Phone: +32 92645105.

The taxonomy of acetic acid bacteria (AAB) has undergone many changes during the last 30 years in accordance with the development and application of new methodologies. The genera *Acetobacter* and *Gluconobacter* were introduced in 1898 and 1935, respectively, and the early classifications systems of AAB were based mainly on morphological and biochemical characteristics. Gradually, molecular and chemotaxonomic techniques were introduced, and the family *Acetobacteraceae* was proposed by Gillis & De Ley in 1980 on the basis of DNA-rRNA hybridization data supported by phenotypic data. In the same period, Gosselé and co-workers examined a wide variety of *Acetobacter* and *Gluconobacter* strains by numerical analyses of 177 phenotypic features, since it was expected that objective comparison of large sets of characteristics for a large number of strains would yield superior classifications. Based on their work, several taxonomic changes were proposed. Mid eighties, the first DNA-DNA relatedness studies with AAB strains were published by Yamada *et al.* (1984) and Micales et al. (1985). Both authors studied independently the genus *Gluconobacter* by DNA-DNA hybridizations and revealed the existence of at least three genospecies. At the end of the eighties, Gillis and co-workers were the first to describe a novel species of the family *Acetobacteraceae* based on a polyphasic approach, *A. diazotrophicus*. Shortly thereafter, the first phylogenetic studies of AAB based on 16S rRNA gene sequences were published. In 1997, Yamada and co-workers introduced a major change in the classification of AAB by proposing the genus *Gluconacetobacter* for the Q-10 containing *Acetobacter* species *(A. xylinus, A. liquefaciens, A. hansenii, A. diazotrophicus* and *A. europaeus*) on the basis of partial 16S rRNA sequences and chemotaxonomic comparisons of ubiquinone systems. Finally, since 1998, polyphasic taxonomic studies of AAB from novel niches and from culture collections, led to reclassifications in the family *Acetobacteraceae*, as well as to the introduction of new taxa at the generic and species levels.

Although the classification scheme for AAB became more robust, accurate identification of AAB at the species level remained time-consuming. During the last decade several molecular techniques, such as sequence analysis of 16S-23S rDNA spacer regions, MLSA of the housekeeping genes *dnaK, groEL* and *rpoB*, and DNA fingerprinting techniques such as rep-PCR and AFLP™ have been proven useful for rapid and more reliable identification of AAB.

Keywords: *taxonomy, fast and accurate identification of AAB.*

S2-KN-02
ECOLOGY OF ACETIC ACID BACTERIA AND THEIR ROLE AS PLANT GROWTH-PROMOTERS

R.O. Pedraza[1*]

[1]National University of Tucumán. Faculty of Agronomy and Zootechnics. Av. Kirchner 1900. (4000) Tucumán, Argentina.

[*]Corresponding author: rpedraza@herrera.unt.edu.ar Phone: +54 3814390003.

Aerobic bacteria able to oxidize ethanol to acetic acid in neutral or acid media are candidates of belonging to the family *Acetobacteraceae* that includes several genera. They are widespread in nature and most species have been isolated from vinegar, wine, fruits and flowers. However, some species were found also associated to different plants, colonizing inner tissues and roots. Several of these species are capable of promoting plant growth through different mechanisms such as the biological nitrogen fixing process, phytohormones production, phosphate solubilization and siderophores production, among others. Some of the species showing these features belong to the genera *Gluconacetobacter, Swaminathania* and *Acetobacter*.

The first N_2-fixing acetic acid bacterium (AAB) (*Gluconacetobacter diazotrophicus*) was described in Brazil, colonizing inner tissues of sugarcane plants. Then, two new species associated to coffee plants were described in Mexico: *G. johannae* and *G. azotocaptans*. In 2004, a salt-tolerant bacterium named *Swaminathania salitolerans* was found associated to wild rice plants. And later, N_2-fixing *Acetobacter peroxydans* and *Acetobacter nitrogenifigens*, associated with rice plants and Kombucha tea, respectively, were described in India.

Gluconacetobacter diazotrophicus, the most studied AAB as plant growth-promoter, is an endophytic bacterium, able to colonize several plant species (e.g., sugarcane, pineapple, coffee, sweet potato, *Pennisetum pupureum*, etc.). Besides being able to fix nitrogen it has shown to possess some other characteristics potentially valuable in the area of agriculture such as the production of substances responsible for plant growth-promotion (phytohormones), action against pathogens and mineral nutrient solubilization.

In the last two decades it was observed that plant growth-promoting bacteria are valuable for agriculture as a tool for improving crop performance and environmental conditions, as they may reduce and avoid the use of chemical fertilizers and pesticides. Hence, at present N_2-fixing acetic acid bacteria are also considered to be used as biofertilizers in different crops to exploit their benefits derived

from an efficient association, mainly related to the biotechnological potential for a sustainable agriculture.

Keywords: Gluconacetobacter diazotrophicus, nitrogen-fixing AAB, plant growth-promoting bacteria.

S2-O-01
MALDI-TOF MASS SPECTROMETRY FINGERPRINTING TOOL FOR RAPID IDENTIFICATION AND CLASSIFICATION OF ACETIC ACID BACTERIA

C. Andrés-Barrao,[1] M.L. Chappuis,[1] C. Benagli,[2] M. Tonolla,[2,3] F. Barja[1*]

[1]Microbiology Unit, Botany and Plant Biology Dept., University of Geneva, Ch. Des Embrouchis 10, CH-1254 Jussy, Geneva, Switzerland; [2]Cantonal Institute of Microbiology, Via Mirasole 22A, CH-6500 Bellinzona, Switzerland; [3]Microbiology Unit, Botany and Plant Biology Dept., University of Geneva, Quai Ernest-Anserment 30, CH-1211 Geneva, Switzerland.

[*]Corresponding author: françois.barja@unige.ch Phone: +41 223793750.

Acetic acid bacteria (AAB) are involved in multiple natural processes leading to high valued food and beverages products, such as vinegar. The taxonomy of these microorganisms has always been very tedious, overall when using classical phenotypic traits. Molecular techniques have been shown to overcome in some extent this constraint, but the unequivocal identification of several AAB species remains unresolved. The most extensively used technique for the identification of microorganisms is the analysis of the 16S rRNA gene sequence, but the analysis of this solely gene fails to discriminate between bacterial species that are very closely related. Additionally, the necessity of culturing to isolate bacteria, and all subsequent manipulations until the obtaining of the 16S rRNA gene sequence, makes this approach still time-consuming.

The analysis of whole cells by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been revealed as a methodology of great interest for the identification and typing of bacteria and other microorganisms due to the really short time that is required for the complete analysis.

The aim of this study was to evaluate the suitability of MALDI-TOF MS for the identification of cultured AAB that are related with the industrial production of vinegar. Reference and isolated strains belonging to the genera *Acetobacter, Gluconobacter* and *Gluconacetobacter* were used. The analysis of the reference strains was consistent with their phylogenetic classification. Most isolates were clustered in accordance with their molecular identification. Some isolates that were not clustered within a group in the AAB MALDI-TOF MS dendogram were revealed as non AAB or mixed cultures. Our results showed that MALDI-TOF MS analysis is a fast and reliable method for the clustering and identification of AAB species.

Keywords: *acetic acid bacteria, phylogeny, MALDI-TOF MS, whole cell identification.*

S2-O-02
CULTURE-DEPENDENT AND CULTURE-INDEPENDENT COMMUNITY DYNAMICS ANALYSIS OF ACETIC ACID BACTERIA DURING COCOA BEAN FERMENTATION

Z. Papalexandratou,[1] K. Illeghems,[1] L. De Vuyst[1*]

[1]Research Group of Industrial Microbiology and Food Biotechnology, Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium.

[*]Corresponding author: ldvuyst@vub.ac.be Phone: +32 26293245.

Acetic acid bacteria (AAB) play an important role during cocoa bean fermentation, as they oxidize ethanol produced by yeasts and lactic acid produced by lactobacilli into acetic acid and further oxidize acetic acid and lactic acid into carbon dioxide and water, thereby generating heat that, together with volatile acetic acid, kills the seed embryo. Alternatively, AAB are strictly aerobic microorganisms that oxidize their substrates (alcohols, sugar alcohols, organic acids) under certain temperature and pH conditions. Therefore, isolation of AAB from and monitoring AAB community dynamics during fermentation processes is not straightforward. The present study aimed at developing culture-dependent and culture-independent techniques to follow up the identity and size of AAB communities during cocoa bean fermentation. Selective isolation and enumeration of AAB colonies was performed on different agar media (DMS, BME, AAM, GY) at 37°C aerobically. Colony identification was performed through $(GTG)_5$-PCR fingerprinting making use of an extended reference database. Culture-independent AAB community dynamics were monitored by denaturing gradient gel electrophoresis (DGGE) of PCR amplicons targeting certain regions of 16S rRNA genes, making use of group-specific (WBAC primers targeting both LAB and AAB) and bacterial universal primers. The results indicated that selective isolation of AAB species depends on the agar media used. For instance, DMS/GY and AAM agar selected for *A. pasteurianus* and *Acetobacter* spp., respectively. BME agar was not selectively enough for AAB. In addition, enterobacterial and lactic acid bacterial species could easily grow on BME and AAM agar, respectively, while DMS/AAM agars allowed the isolation of pimaricin-resistant yeasts. Whereas *Glucon(acet)obacter* could be detected culture-independently and isolated from AAM agar only occasionally, several new *Acetobacter* species were found among the isolates from fermenting cocoa pulp-bean mass. Further, the culture-dependent community dynamic results indicated that AAB survived during the initial anaerobic phase of a cocoa bean fermentation process, followed by slow growth up to high densities in the mid-phase of the fermentation. PCR-DGGE analysis with universal primers allowed the detection of AAB at genus level, while WBAC primers could reveal some of the *Acetobacter* species as well. However, the development of a new primer set targeting AAB solely was necessary.

Keywords: acetic acid bacteria, isolation, (GTG)5-PCR, PCR-DGGE.

S2-O-03

DIVERSITY OF ACETIC ACID BACTERIA PRESENT IN HEALTHY AND MOULDY-INFECTED GRAPES FROM AUSTRALIA VINEYARDS

E. Mateo,[1*] M.J. Torija,[1] A. Mas,[1] E. Bartowsky[2]

[1]Departamento de Bioquímica y Biotecnología. Facultad de Enología. Universitat Rovira i Virgili. C/ Marcel•lí Domingo s/n. E- 43007 Tarragona, Spain; [2]Autralian Wine Research Institute, Urrbrae, SA, Australia.

[*]Corresponding author: estibaliz.mateo@urv.cat Phone: +34 977558688.

Grapes have a complex microbiota ecology which plays central roles in the quality of this fruit and wine. It is well known that if oxygen conditions are appropriate, Acetic acid bacteria (AAB) are the main family of bacteria that can proliferate and cause wine spoilage.

The aim of this study was to analyse the diversity of AAB population isolated from grape bunches of vineyards in the Adelaide Hills region of Australia. Grapes were classified as healthy, mouldy-infected and dry.

Up to 628 AAB colonies were directly recovered from grapes in glucose medium supplemented with piramicin (200 mg/L) to suppress fungal and yeast growth. Molecular techniques were used to analyze AAB isolates. The typing by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR and $(GTG)_5$-PCR techniques showed a high AAB diversity, identifying more than 50 different profiles. The species identification by 16S-23S rRNA gene internal transcribed spacer (ITS) region phylogenetic analysis grouped these genotypes inside the *Gluconobacter*, *Acetobacter* or *Asaia* genera.

Some of the species detected have been described in previous studies of grapes and wine as *Gluconobacter oxydans, Acetobacter malorum* and *Asaia siamensis*. However, other species never before described either in wine or on grapes as *Gluconobacter cerinus*, *Gluconobacter frateurii, Asaia lannensis* or *Asaia bogorensis* have also been recovered and identified in this study. The first two are very closely related to *Gluconobacter oxydans*, widely described on grapes and wine.

As some recently described species have also been reported in ecological studies of wine and grapes, it is necessary to conduct an in-depth revision of AAB diversity using appropriate molecular tools in order to determine the real AAB microbiota present on grapes.

Keywords: wine, spoilage, vinegar, Asaia, Gluconobacter.

S2-O-04

NO-CULTURE STRATEGY FOR TRACKING AAB AND YEASTS IN LOW-ACIDIC AND HIGH POLYPHENOLIC NICHES

D. Mamlouk,[1*] L. Solieri,[1] M. Gullo[1]

[1]Department of Agricultural and Food Sciences (DipSAA), University of Modena and Reggio Emilia, via Amendola 2 (Padiglione Besta), 42122 Reggio Emilia, Italy.

[*]Corresponding author: dhouhamamlouk@yahoo.fr Phone: +39 3467373449.

Community of acetic acid bacteria (AAB) and yeasts inhabits low acidic and high polyphenolic niches such as fermented beverages. In this work, we selected *kombucha* as model to optimize a no-culture strategy for tracking the dynamics of AAB and yeasts. The strategy consisted of: i) Genomic DNA extraction optimization, ii) PCR/DGGE setup and iii) Validation of the assay. For gDNA extraction optimization, we evaluated the suitability of the following gDNA preparations for a standard PCR-DGGE assay: 1) CTAB/AAB; 2) CTAB/vinegar; 3) Freeze/bead/CTAB1; 4) Freeze/bead/CTAB2 methods. Different extraction methods affected not only the quantity and purity of DNA but also the complexity of PCR/DGGE profiling. Method 4 allowed us to obtain suitable DNA for PCR assays, as well as the highest level of complexity in PCR/DGGE band patterns both for AAB and yeasts. Moreover changes from exopolysaccharidic to liquid phases did not affect the suitability of this method. PCR/DGGE setup was performed considering the following variables: DNA template concentration, PCR conditions and specificity, effect of GC-clamp and nested PCR. For AAB, primers pair WBAC1/WBAC2 enabled to study AAB dynamics as well other bacterial groups, whereas 341f/518r permitted the detection of members of the *Gluconacetobacter* genus. For yeasts, primers pair U1/U2 was more efficient then NL1/LS2 in community profiling of the majority of *kombucha* samples. GC-clamp added to forward primers negatively affected gDNA suitability and a nested re-PCR for yeasts and AAB was required. Finally, validation of the assay was carried out during *kombucha* fermentation following AAB and yeasts dynamics from the inoculum to 12 days of incubation. Within AAB species, *Gluconacetobacter xylinus* responsible for the exopolysaccharidic network matrix, was mainly detected, whereas *Zygosaccharomyces spp, Dekkera spp, Pichia spp* and *Schizosaccharomyces pombe* in the case of yeasts.

Keywords: AAB, yeasts, no-culture method, kombucha, dynamics, PCR/DGGE.

S2-O-05

INFLUENCE OF EXOGENOUS PHYTOHORMONES ON THE GROWTH AND PELLICLE FORMATION OF GLUCONACETOBACTER XYLINUS

J.L. Strap,[1*] O. Qureshi,[1] H. Sohail,[1] A.J. Latos[2]

[1]Faculty of Science, University of Ontario Institute of Technology, Oshawa, Ontario, L1H 7K4, Canada; [2]Faculty of Dentistry, University of Toronto, Toronto, Ontario, M5G 1G6, Canada.

[*]Corresponding author: janice.strap@uoit.ca Phone: +1 9057218668 ext 2935.

Gluconacetobacter xylinus forms a pellicle at the liquid-air interface of statically grown broth cultures. A major structural component of this interfacial biofilm is cellulose which serves to protect the organism from unfavorable environmental fluctuations. In addition, cellulose formation enhances surface colonization to provide a competitive advantage for resource acquisition.

Exogenously supplied phytohormones are known to affect plant growth and development. Some microorganisms can also synthesize phytohormones and many have been investigated for their potential as plant growth promoters; however, there have been few studies investigating the effect of phytohormones on bacteria. The aim of this study was to investigate the effect of the phytohormones indole-3-acetic acid (IAA), gibberellic acid (GA), abscisic acid (AA) and zeatin on growth and pellicle formation in *G. xylinus*.
The growth, cellulose yield and pellicle properties of *G. xylinus* were significantly affected by exogenous phytohormones. Each of the phytohormones tested stimulated the growth rate of *G. xylinus* between 1.1 to 1.7 times that observed for untreated controls. The addition of phytohormone caused a significant reduction in pellicle thickness for most concentrations tested. Exogenous AA caused *G. xylinus* to produce pellicles with greater wet weight than the other three hormones tested. While pellicles produced by control cultures were thicker, the yield of bacterial cellulose was greater in the presence of AA and zeatin suggesting that these pellicles were denser. Bacterial cellulose yield decreased in the presence of IAA. GA increased the cellulose yield at higher concentrations with the greatest effect seen at 10 μM. The crystallinity index (CI(IR)) of cellulose produced in each treatment was determined by Fourier transform infrared spectroscopy. The observed effect on cellulose crystallinity was concentration and hormone dependent. GA affected the cellulose crystallinity the most with the highest CI(IR) of 1.01 observed at 5 μM. Zeatin treated cultures produced the least crystalline cellulose of any treatment with CI(IR) of 0.56 at 0.5 μM.
In conclusion, these results underscore the importance of studying the broader ecological effect of agricultural practices in which exogenous growth promoters are applied. In addition, phytohormones can be used as a tool to manipulate the properties of bacterially produced cellulose for industrial application.

Keywords: cellulose, phytohormones, crystallinity, Gluconacetobacter xylinus.

S2-P-01
ACETIC ACID BACTERIA CULTURE COLLECTION: SIGNIFICANCE AND MANAGEMENT

L. De Vero[1*]

[1]Department of Agricultural and Food Sciences (DipSAA), University of Modena and Reggio Emilia, via Amendola 2 (Padiglione Besta), 42122 Reggio Emilia, Italy.

*[*]Corresponding author: luciana.devero@unimore.it
Phone: +39 0522522057.*

Acetic acid bacteria (AAB) occur in very specialized habitats such as plants, fruits and flowers, as well as several kinds of sugary or alcoholic substrates. In the last decades, some AAB species have been extensively investigated not only for foods and beverages but also others industrial biotechnology applications as the development of processes for high-value products or biosensors. The potential exploitation of AAB strain diversity requires the existence of culture collections which represent important biological resources for selection and genetic improvement of microbial cultures useful for biotechnological applications. The greatest hurdles to acetic acid bacteria study are related to their difficult isolation, thus several media and incubation conditions are necessary for successful cultivation and preservation of pure cultures. Furthermore culture collections have to evaluate microbial changes over time as well as guarantee appropriate tools in preserving ''authentic'' strains.
These are also the tasks of the Food and Industrial Microbial Collection (FIMC) of the Department of Agricultural and Food Sciences (DipSAA) that, currently, holds about 300 AAB strains, above yeast and lactic acid bacteria strains, isolated from several matrices. Specifically, the AAB strains maintained in the collection have been isolated from musts, wines, various vinegars, solid-state fermentation matrix and *kombucha tea*. Different preservation techniques are used, ranging from continuous growth methods, for short-term maintenance, to cryopreservation methods at -80°C, for long-term maintenance and for avoiding genetic drift or instability. Full understanding of metabolic potential of the AAB strains collected is achieved by the integration of sequence data with transcriptional and functional studies. A "Strain Database" (www.renewx.org) and a Bioinformatics Software (GelCompare II - Applied Math) support all the information related to the strains starting from isolating source until the full characterization, as well as the collection management.

Keywords: acetic acid bacteria, culture collections, preservation methods.

S2-P-02
CHARACTERISATION OF ACETIC ACID BACTERIA PRODUCING HIGH-ACID VINEGAR

C. Andrés-Barrao,[1] D. Bravo,[2] M.L. Chappuis,[1] R. Ortega Pérez,[1] M. Ojha,[3] P. Junier,[2] F. Barja[1*]

[1]Microbiology Unit, Department of Botany and Plant Biology, University of Geneva, Chemin des Embrouchis 10, CH-1254 Jussy-Geneva, Switzerland; [2]Laboratory of Microbiology, Institute of Biology, University of Neuchâtel, Emile-Argand 11, PO Box 158, CH-2009 Neuchâtel, Switzerland; [3]Departement de Biochimie, University of Geneva, 30, Quai Ernest-Ansermet, CH-1211 Genève 4, Switzerland.

*[*]Corresponding author: françois.barja@unige.ch
Phone: +41 223793750.*

The modern submerged fermentation for the production of vinegar is currently the main method used in industrial vinegar factories. Rapid acetification rate as well as high concentration of acetic acid (up to 20%) can be obtained. Several strains of the genus *Gluconacetobacter* are involved in the production of high-acid vinegars produced by this methodology. These strains are characterised by their higher ADH stability and activity, and also by a higher acetic acid resistance, than other acetic acid bacteria.
In this study, two spirit vinegar fermentations were

performed by using the submerged method. Both processes reached a maximum acidity of 14%. The molecular characterisation was performed using culture-dependent: restriction fragment length polymorphism (RFLP) and multilocus sequence alignment (MLSA) and culture-independent: denaturant gradient gel electrophoresis (DGGE) methodologies. The characterisation showed a major abundance of one strain in both processes, confirmed the monoculture nature of industrial processes. The phylogenetic analysis identified the species present in each fermentation as *Ga. oboediens* and a species related to *Ga. hansenii/entanii respectively*.
In the other hand, scanning and transmission electron microscope observations showed that high-acid vinegar bacteria lacked the characteristic polysaccharide layer surrounding the outer membrane, which has been previously observed in low-acid Acetobacter strains.

Keywords: submerged high-acid vinegar, Gluconacetobacter, direct identification, RFLP, MLSA, DGGE, polysaccharides.

S2-P-03
DIVERSITY AND DYNAMICS OF CULTIVABLE POPULATION OF ACETIC ACID BACTERIA AND YEASTS IN KOMBUCHA

L. Solieri,[1] D. Mamlouk,[1] L. Kallel,[2] M.Gullo[1*]

[1]Dept. of Agricultural and Food Science, University of Modena and Reggio Emilia, Italy; [2]National Institute of Applied Sciences and Technology, LETMI, Tunisia.

*[*]Corresponding author: maria.gullo@unimore.it
Phone: +39 0522522057/63.*

In recent years functional foods promoted with health claims have attracted increasing attention on the market. Among them *kombucha* is a fermented beverage widely consumed in Eastern Asian countries, but little is known about its constituent microbial communities. In this study two 12 days benchmark *kombucha* fermentations from green and black tea were carried out. A culture-dependent approach was applied both on exopolysaccharidic and liquid phases to monitor dynamics and diversity of acetic acid bacteria (AAB) and yeasts community. Among AAB one main profile was observed (86% of strains); remaining strains were grouped in 4 profiles by 16S/RFLP-based analysis. Whereas by (GTG)5/PCR typing sixteen clusters were obtained. 16S rRNA gene sequencing confirmed the occurrence of *Gluconacetobacter xylinus* as predominant both in green and black samples at 0, 6 and 12 days of fermentation. Mainly on ACB medium minor bacterial groups often colonizating tea leaves (*Paenibacillus spp.*), plants (*Plantibacter spp.*) and moisturing environments (*Williamsia spp.*), were detected starting from 6th fermentation day. Yeast population consisted of a restricted number of dominant species: *Dekkera sp., Schizosaccharomyces sp., Zygosaccharomyces sp., Dekkera sp.* and P*ichia sp. D. anomala* was prevailing in both phases though all black and green *kombucha* fermentation times. *Sc. pombe* was detected only within 6 days of both green and black tea and it was not isolated after 9 days, when the high ethanol-producing species *D. bruxellensis* was detected. *Z. bailii* was isolated from exopolysaccharidic of black tea from 6 to 12 days. Finally *P. membranifaciens* was detected occasionally at the end of the fermentative process. For each species the degree of diversity was determined by combined M13 and OPA20-based RAPD method. Cluster analysis showed that one to two prevailing biotypes occurred through all the process.

Keywords: AAB, yeasts, kombucha population, culture-dependent approach.

S2-P-04
ISOLATION, CLASSIFICATION AND IDENTIFICATION OF YEAST FROM SHANXI AGED VINEGAR

J.J. Wu,[1] X.Y. Zhang,[1] F.S. Chen[1*]

[1]College of Food Science and Technology, Huazhong Agricultural Univerisity, Wuhan, Hubei, 430070 P.R. China.

*[*]Corresponding author: chenfs@mail.hzau.edu.cn
Phone: +86 2787282927.*

Shanxi aged vinegar（SAV） is one of famous traditional Chinese vinegars made from several kinds of cereal by spontaneous solid-state fermentation techniques, in which *Daqu* (Chinese pronunciation) is used as the initial starter. Because *Daqu* is prepared in an/a "open" or "half-open" workplace, many kinds of microorganisms from the raw materials and production environments are enriched in it. Yeast, as one of the main microbial populations in *Daqu*, plays important role in alcoholic fermentation and flavor formation of SAV. Here, the yeast were isolated from different fermentation period and identified by phenotypic methods combined with molecular tools including ITS-5.8S rDNA RFLP, inter-delta/PCR and 26S rRNA D1/D2/PCR. 47 yeast strains were isolated during SAV alcoholic fermentation. Among them, 29, 9 and 9 strains were isolated from earlier stage (the1st~3rd day of aerobic fermentation), middle stage (the 9th day of anaerobic fermentation) and later stage (the18th day of anaerobic fermentation), respectively. According to their morphological characters, 36 strains were classified as *S. cerevisiae* and 2 strains were classified as *P. anomala*. The rest 9 stains had the same morphological characters with *S. cerevisiae*, but they had pseudohyphae on the culture medium containing corn powder and couldn't form ascospore on MacConkey medium. According to the physiological and biochemical characteristics, the 47 yeast strains were also classified as the same 3 groups. The results of ITS-5.8S rDNA RFLP (restriction fragment length polymorphisms) analysis with HeaIII and Hif I indicated that 45 strains had the same profile with *S. cerevisiae* CBS1171^{T}, while the other 2 strains had the same profile as *P. anomala* CBS5759^{T}. And 45 presumptive *S. cerevisiae* strains identified by ITS-5.8S rDNA RFLP could be grouped into 4 clusters according to the results of inter-delta PCR analysis, the first cluster included 20 strains and had 5 amplified fragments (1000bp, 680 bp, 580 bp, 500 bp and 350 bp), the second cluster included 19 strains and had 2 amplified

fragments (750 bp and 460 bp),the third cluster included 4 strains and had 2 amplified fragments (750 bp and 550 bp),the rest 2 strains had 4 amplified fragments (1700bp,1100bp,760bp and 400 bp) and had the same results with *S. cerevisiae* CBS1171^T. The results indicated that the isolated *S. cerevisiae* had different genotype. Finally, 4 *S. cerevisiae* strains from 4 different clusters and 1 *P. anomala* strain were identified by 26S rDNA D1/D2 region sequencing. The results indicated that the amplified fragments were 650bp. The sequence similarity of the *S. cerevisiae* strains from 4 different clusters were 100% with the sequence of *S. cerevisiae* in genbank, and the sequence similarity of isolated *P. anomala* were 100% with the sequence of *P. anomala* in genbank. All the results showed that most of isolated yeasts were *S. cerevisiae*, but different *S. cerevisiae* strains may had different phenotype and genotype. Studies on yeast involved in Shanxi aged vinegar fermentation are helpful for starter selection, regulation process parameter, and provide reference for other strain isolation and identification.

Keywords: vinegar, yeast, isolation, identification.

S2-P-05
ISOLATION OF ACETIC ACID BACTERIA SPECIES IN GRAPE MUSTS: "NEW" AND "OLD" SPECIES

D. Navarro,[1] E. Mateo,[1] A. Mas,[1*] M.J. Torija[1]

[1]Departamento de Bioquímica y Biotecnología. Facultad de Enología. Universitat Rovira i Virgili. C/ Marcel•lí Domingo s/n. E- 43007 Tarragona, Spain.

**Corresponding author: albert.mas@urv.cat*
Phone: +34 977558688.

Acetic Acid Bacteria (AAB) were routinely isolated and identified in grape musts from the Experimental Cellar of the Oenology Faculty in Tarragona (Spain). Different serial dilutions were plated onto glucose solid media and incubated at 28°C. Each bacterial colony was then replicated in glucose with carbonate calcium medium and those that produced a clear halo were considered putative AAB and were typed by $(GTG)_5$-fingerprinting and identified by 16S rRNA gene RFLP-PCR and gene sequencing. Further identification by 16S-23S rRNA ITS sequencing was performed. Consensus sequences from different genotypes were compared with those available in Genbank database.
Out of 43 isolates that produced a clear halo around the colonies, only 37 yielded a clear electrophoretic profile and those could be grouped into 21 genotypes. Most of them were identified as *Gluconobacter oxydans* by RFLP-PCR of 16S rRNA gene. However, sequencing (16S rRNA gene or 16S-23S rRNA ITS) analysis of these genotypes revealed that only one of them was *G. oxydans*, whereas the other were identified as the close related species *G. japonicus, G. albidus, G. cerinus* and *G. thailandicus*. More surprisingly, two of these genotypes were identified as a member of another genus, *Kozakia baliensis*, which had never been reported in grape must. The other genotypes were identified as *Acetobacter malorum, A. cerevisiae, A. pasteurianus* and *Gluconacetobacter europaeus*, and all of them, needed a further identification by 16S-23S rRNA ITS sequencing. Previous analysis of the AAB presence in grape must provided a main presence of *G. oxydans* and *A. aceti*. However, it is most likely that closely related species that have been described lately are also present in relevant numbers in grapes and wines.

Keywords: sequencing, wine, fingerprinting.

S2-P-06
KOMBUCHA TEA: A BIORESOURCE FOR ACETIC ACID BACTERIA

L. Kallel,[1*] M. Gullo,[2] M. Hamdi[1]

[1]Laboratory of Microbial Ecology and Technology, Department of Biological and Chemical Engineering, National Institute of Applied Sciences and Technology (INSAT), Centre Urbain Nord, 2 Boulevard de la Terre, B.P. 676, 1080 Tunis, Tunisia; [2]Department of Agricultural and Food Sciences, University of Modena and Reggio Emilia, Via G. Amendola 2, Pad. Besta, 42100 Reggio Emilia, Italy.

**Corresponding author: daddalina@yahoo.fr*
Phone: +21 624330001.

Kombucha or Tea fungus is a traditional fermented beverage obtained during sweetened tea fermentation conducted by acetic acid bacteria (AAB) and yeasts. Historically, *Kombucha* appeared first in Asia and thereafter its uses and consumption spread from Russia, central Europe and then over the world. It is composed of a cellulose floating pellicle and a sour slightly and sparkling broth. However, in spite of vinegar, *kombucha* remained not well explored mainly as a niche of AAB strains exploitable in biotechnological applications. The aim of this study was to overview the occurrence of AAB in *kombucha* samples from liquid and pellicle phases. *Kombucha* from black and green tea was obtained by inoculating a previously grown culture into a freshly prepared tea infusion containing 10% of sucrose, 3% (w/v) of cellulose pellicle and 10% (v/v) of broth and led to fermentation up to 15 days in a batch system under aerobic condition. Titrable acidity reached a maximum of 12 g/L at the end of fermentation in black *kombucha* and 6 g/L in green one. pH dropped from approximately 3.7 until 2.75 for both samples as a result of acids formation. Maximum ethanol accumulated at the day 15 did not exceed 0.5% (v/v) for both samples. Cellulose layer become thicker during fermentation, starting from 2-3 mm until 10 mm. AAB strains were isolated from pellicle and liquid phases by sampling each 3 days using GYC and ACBS media. A total of 93 strains were collecting during 15 days of fermentation of which 50 are cellulose producing. The majority of strains were tolerant to 5% (v/v) of ethanol and to 30% (w/v) of glucose. This work opens an issue to explore the diversity of AAB from *Kombucha* focusing their ability to produce cellulose and to grow in low-acidic and sugary environment.

Keywords: kombucha, fermentation, acetic acid bacteria, cellulose.

S2-P-07

PHYLOGENETIC AFFILIATION OF TWO NOVEL GLUCONACETOBACTER STRAINS PRODUCING EXTRACELLULAR POLYSACCHARIDES

N. Slapšak,[1] I. Cleenwerck,[2] K. Matsushita,[3] J. Tr ek[1*]

[1]Dep. of Biology, Faculty of Natural Sciences and Mathematics, University of Maribor, Slovenia; [2]BCCM/LMG Culture Collection, Faculty of Sciences, Ghent University, Belgium; [3]Dep. of Biological Chemistry, University of Agriculture, Yamaguchi University, Spain.

**Corresponding author: jtrcek03@yahoo.com*

The genus *Gluconacetobacter* contains several acetic acid bacteria that are capable of producing extracellular polysaccharides, such as bacterial cellulose and acetan. Bacterial cellulose has potential applications in the biomedical field (e.g. artificial skin and vessels, controlled-drug carriers), but is at present mainly applied in food products (e.g. nata, kombucha), paper products, acoustics (e.g. diaphragm for headphones and loudspeakers), electronics and medical devices (e.g. temporary wound coverage). *Gluconacetobacter* species that are known to contain cellulose-producing strains are *G. xylinus, G. oboediens, G. intermedius, G. hansenii, G. swingsii* and *G. rhaeticus*, a set of phylogenetic closely related species. Identification of such acetic acid bacteria through phenotypic approaches is not very reliable, partly because these acetic acid bacteria are exposed to a high frequency of spontaneous mutations, possibly due to the presence of many insertion elements present in the genome of these bacteria. Therefore, molecular-biological methods, based on the identification/characterization of specific DNA regions, are regarded to be more reliable for the identification of these bacteria. In this study, two strains of *Gluconacetobacter* have been investigated using different molecular-biological methods. Comparative analysis of nearly complete 16S rRNA gene sequences, and of partial *adhA, dnaK, groEL* and *rpoB* gene sequences, restriction analysis of the 16S-23S rDNA spacer region, AFLP™ DNA fingerprinting and DNA-DNA hybridizations revealed that these strains belong to a novel species in this genus.

Keywords: Gluconacetobacter, novel species, extracellular polysaccharides.

S2-P-08

SEMI INDUSTRIAL VINEGAR PRODUCTION FROM HARVESTED FRUIT LEFTOVER

N. Calo,[1] C. Andrés-Barrao,[1] M. Chappuis,[1] C. Revillod,[2] R. Eklu-Natey,[3] F. Barja[1*]

[1]Department of Botany and Plant Biology, Microbiology Unit, University of Geneva, Chemin des Embrouchis 10, CH-1254 Jussy-Geneva, Switzerland; [2]Vinagrerie artisanale de Cottens, Chemin du Sau 2, CH-1116 Cottens, Switzerland.

**Corresponding author: françois.barja@unige.ch Phone: +41 223793750.*

Tropical, Subtropical and Mediterranean countries produce large amounts of fruits such as pineapples, mangos, persimmons, raspberries, strawberries, kiwis, figs or apricots. These fruits are particularly biodegradable and highly perishable due to the high content of sugar and water. Because of this, 10-40 %; up to more than 60% in some tropical areas, of the harvested fruits deteriorates quickly and become unsuitable for consumption, implying a loss of earning for farmers. Acetic acid bacteria (AAB), which are naturally present in fruits and flowers, participate to this economic spoilage by natural fermentation, transforming sugars and alcohols into their corresponding acids. However, they are also used in industrial processes leading to high valued food and beverage products, such as vinegar, cocoa and kombucha. Therefore, such bacteria can be used in order to valorise these leftovers, in addition to allow the effective utilization of fruits that fail to meet the quality standards required for their direct commercialization. Currently, vinegar producers are looking for new original resources suitable for vinegar production, and various raw materials like sugarcane, rice or honey have already been used for this purpose. Fruits like pineapple constitute attractive raw materials for such production as they have high amounts of sugars, vitamins, minerals and nutrients, but also unique organoleptic properties.

In the vinegar production industry, only few genera of AAB are used among the large number of bacteria that are able to produce acetic acid, the most predominant being *Acetobacter* and *Gluconacetobacter*.

In this study we have focused in setting up the physical and chemical parameters of effective submerged vinegar production by two-step fermentation: alcoholic and acetic, starting from various fruit juices (mango, pineapple, raspberry, kiwi and fig). A heterogeneous population was observed using scanning and transmission electron microscopy during the pineapple vinegar acetification. Additionally, the bacterial population during this process shifted from *Acetobacter cerevisiae* to *Gluconacetobacter europaeus*.

Keywords: submerged vinegar production, acetic acid bacteria, fruits, morphology, identification.

SESSION III. How do they work?

SESSION III.A. Genetics and Molecular Biology.

S3A-KN
MOLECULAR BIOLOGY APPROACHES TO ACETIC ACID BACTERIA POPULATION STUDIES

A. Mas,[1*] J.M. Guillamón,[1] E. Mateo,[1] M.J. Torija[1]

[1]Departamento de Bioquímica y Biotecnología. Facultad de Enología. Universitat Rovira i Virgili. C/ Marcel•lí Domingo s/n. E- 43007 Tarragona, Spain.

[*]Corresponding author: albert.mas@urv.cat Phone: +34 977558688.

Acetic Acid Bacteria (AAB) are considered one of the most common wine spoilage microorganisms and a threat for the oenologists. Their ability to transform most of the sugars and alcohols into organic acids produces easily the transformation of glucose into gluconic acid on damaged grapes and ethanol into acetic acid in wines. Traditional methods have identified *Gluconobacter oxydans* as the main AAB species on healthy grapes and *Acetobacter aceti* and *Acetobacter pasteurianus* in wines as main spoilers. However, new molecular methods have expanded the number of genera and species. As a consequence new species are now considered present in relevant numbers in grapes or wines. For instance, *Acetobacter oeni* in Portugal, *Acetobacter cerevisiae* in Chile or *Acetobacter malorum* and *Acetobacter tropicalis* in Canary Islands (Spain) may also have a significant role on grape, wine ecology or spoilage.
Another main characteristic of AAB is their consideration as "fastidious microorganisms" or its possible entry into a Viable But not Culturable status (VBNC), especially when they are present in extreme media such as wine. Thus, new culture-independent methods may derive new insights into the presence of AAB in wine making. Methods such as Real-Time PCR allow the detection of AAB, both at species or genus level without the need of previous plating on solid medium. The effectiveness of yeast inoculation or sulphur dioxide upon AAB population is challenged when AAB wine ecology is analysed by culture independent methods. Thus, although most of the previous knowledge on AAB wine ecology done by traditional microbiological techniques is still valid and useful, the use of molecular biology approaches and especially combined with culture-independent methods can challenge, at least partially, some of these well established concepts.

Keywords: sequence analysis, typing, real-time PCR, grape, wine.

S3A-O-01
GENOME-WIDE ANALYSES FOR HYPER GLUCOSE TOLERANCE OF ACETIC ACID BACTERIA

H. Hadano,[1] K. Omura,[1] J. Tomatsu,[1] H. Hirakawa,[2] S. Takebe,[1] K. Matsushita,[3] Y. Azuma[1*]

[1]BOST, Kinki University, Japan; [2]Kazusa DNA Research Institute, Japan; [3]Yamaguchi University, Japan.

[*]Corresponding author: azuma@waka.kindai.ac.jp Phone: +81 736770345 ext.5201.

Acetic acid bacteria (AAB) inhabit niches where sugars are accumulated, such as fruits and flowers. The sugar concentration and thus osmotic status in such environments might be modified in natrue drastically by rain and dehydration and modestly by ripeness of fruits. To achieve a model system to comprehend environmental responses of AAB and to acquire new enzymes and metabolic systems practicable in high concentration of sugars, we have started genome-basis analyses using two AAB species, *Tanticharoenia sakaeratensis* and *Asaia bogorensis*, which are relatively phylogenetically close to each other and share an interesting features, i.e. the ability of multiplication in media containing glucose up to 30%. First, we have determined draft genome DNA sequences of *T. sakaeratensis* and *A. bogorensis* using a next generation sequencer, Illumina HiSeq 2000, and it was shown that their genomes encode 3,155 and 2,787 genes, respectively. Paralog analysis among more than 20 AAB genomic DNA sequences including unpublished draft sequences implicated that 41 paralog groups were specific to only *T. sakaeratensis* and *A. bogorensis*, such as monosaccharide-transporting ATPase and oxidoreductase molybdopterin-binding protein. But functions for approximately half of the genes were unknown. For narrowing down the candidates genes involving in the hyper glucose tolerance, we carried out comparative physiological and omics analyses in both *T. sakaeratensis* and *A. bogorensis* grown. Under 30% glucose conditions, *T. sakaeratensis* and *A. bogorensis* could utilize glucose and produce acids, however both strains multipicated more slowly than under lower glucose concentration conditions. *A. bogorensis*, but not *T. sakaeratensis*, showed drastic morphological changes under high glucose conditions. The proteome analyses were carried out using proteins fractionated into soluble, membrane-bound and SDS-mediated extractants. Comparative analyses illustrated that several proteins were accumulated and decreased in only high glucose condition, for instance, proteins similar to pyruvate decarboxylase and *A. pasteurianus* OmpA, respectively. In this meeting, we will present current omics data of *T. sakaeratensis* and *A. bogorensis*.

Keywords: hyper glucose tolerance, genome, proteome, Tanticharoenia sakaeratensis, Asaia bogorensis.

S3A-O-02
STUDIES OF AN FNR-LIKE TRANSCRIPTIONAL REGULATOR IN *GLUCONOBACTER OXYDANS* 621H

S. Schweikert,[1*] S. Bringer,[1] M. Bott[1]

[1]Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, D-52425 Jülich, Germany.

[*]Corresponding author: s.schweikert@fz-juelich.de Phone: +49 2461613975.

The strictly aerobic α-proteobacterium *Gluconobacter oxydans* is used for a wide variety of industrial applications such as vitamin C synthesis. A special characteristic is the incomplete oxidation of substrates like sugars or sugar alcohols in the periplasm. Despite its industrial importance, knowledge of the metabolism of *G. oxydans* and its regulation, especially concerning the sugar metabolism, is still very scarce (Hanke et al. 2012). Our *in silico* analysis of the genome sequence of strain 621H identified 117 putative transcriptional regulators (TRs) belonging to 38 different regulator families. None of these has been characterised yet. Based on a number of criteria we preselected a number of TRs which might be of particular interest. Of these, an FNR-like regulator was chosen for detailed studies. FNR (fumarate nitrate reductase regulator) of *Escherichia coli* is an oxygen sensor which controls the switch from aerobic to anaerobic respiration. As *G. oxydans* is considered to be strictly aerobic and no pathways for anaerobic respiration or fermentation are known, the role of the *G. oxydans* FNR homolog, in particular its target genes, was enigmatic. Therefore its function was studied by chromosomal deletion of the corresponding gene and analysis of the mutant strain with respect to growth properties and global gene expression (using DNA microarrays). In addition, preliminary studies on the biochemical properties of the FNR homolog of *G. oxydans* were performed.
The results of these studies will be presented.

Keywords: Gluconobacter oxydans, transcriptional regulation, Fnr, GOX0974.

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Hanke, T., Richhardt, J., Polen, T., Sahm, H., Bringer, S., Bott, M. (2012): Influence of oxygen limitation, absence of the cytochrome bc(1) complex and low pH on global gene expression in *Gluconobacter oxydans* 621H using DNA microarray technology. *J Biotechnol, accepted manuscript*.

S3A-O-03
HIGHLIGHTS ON *ACETOBACTER PASTEURIANUS* PROTEOME MODIFICATIONS

C. Andrés-Barrao,[1] M.M. Saad,[2] M. Chappuis,[1] R. Ortega Pérez,[1] M. Boffa,[3] X. Perret,[2] F. Barja[1*]

[1]Department of Botany and Plant Biology, Microbiology Unit, University of Geneva, Chemin des Embrouchis 10, CH-1254 Jussy, Switzerland; [2]Department of Botany and Plant Biology, Microbiology Unit, University of Geneva, Quai Ernest-Anserment 30, CH-1211, Geneva, Switzerland; [3]Political Economy, University of Geneva, Bd du Pont d'Arve 40, CH-1211, Geneva, Switzerland.

[*]Corresponding author: françois.barja@unige.ch
Phone: +41 223793750.

Acetic acid bacteria (AAB) show a unique resistance to ethanol and acetic acid. Several members of the *Acetobacter* and *Gluconacetobacter* genera, which are capable to transform ethanol to acetic acid via the action of alcohol dehydrogenase and aldehyde dehydrogenase, are used for the industrial production of vinegar.
Known resistance mechanisms of AAB to the moderate to high acetic acid concentrations present in vinegar include (i) the assimilation of acetate through the TCA cycle, (ii) the secretion of acetate by efflux systems, and (iii) modifications in the lipid and polysaccharide composition of the bacterial cell wall. Yet, except for a few acetate-specific proteins identified, little is known about the global responses of AAB to acetic acid at the proteome level.
This prompted us to follow the changes that occur in the proteome of *Acetobacter pasteurianus* during acetic acid fermentation. Using two-dimensional differential in-gel electrophoresis (2D-DIGE), the proteome of the strain LMG1262^T was examined at different stages of the fermentation process, starting with 4% ethanol until its complete depletion. Using an ANOVA factor <0.05 and 1.5-fold as the minimal level of differential expression as thresholds, at least 325 proteins were shown to be either up- or down-regulated.
In addition, scanning electron micrographs confirmed that the median length of AAB cells was reduced by about 30% in length as a consequence of the increasing acidity. A modification in the nature of membrane polysaccharides was also revealed by PATAg specific staining.

Keywords: Acetobacter pasteurianus, 2D-DIGE, morphology, acetic acid fermentation.

S3A-O-04
PROMOTERS OF MEMBRANE-BOUND DEHYDROGENASES FROM GLUCONOBACTER OXYDANS 621H AND THEIR ABILITY IN A NEW EXPRESSION SYSTEM

M. Mientus,[1*] B. Peters,[1] D. Kostner,[1] W. Liebl,[1] A. Ehrenreich[1]

[1]Lehrstuhl für Mikrobiologie Technische Universität München, Emil-Ramann-Str. 4, D-85354 Freising, Germany.

[*]Corresponding author: mmientus@tum.de
Phone: +49 8161715468.

Acetic acid bacteria are used in biotechnology due to their ability to incompletely oxidize a great variety of carbohydrates, alcohols and related compounds in a regio- and stereo-selective manner. Most of these reactions are catalyzed by membrane-bound dehydrogenases with a broad substrate range.
As currently very little is known about the promoters of membrane-bound dehydrogenases we investigated the regulation and transcription starting points of different dehydrogenases including one regulated promoter. Furthermore the promoter strength was quantified by a reporter. These promoters are now available tools for the expression of (novel) membrane-bound dehydrogenases using *G. oxydans* 621H as host.
A multi-deletion strain of *G. oxydans* 621H which is lacking its main membrane-bound dehydrogenases was constructed with a clean deletion method (Δ*upp*) by our group. In order to complement the deletions of membra-

ne-bound dehydrogenases we constructed a shuttle vector system for their functional expression. This system was successfully used for the complementation of the deleted membrane-bound dehydrogenases in *G. oxydans* 621H and could also be used in other *G. oxydans* strains. Furthermore this vector system is available for the expression and characterization of membrane-bound dehydrogenases from a vinegar metagenome.

Keywords: promoters, expression, membrane-bound dehydrogenases, Gluconobcater oxydans.

S3A-O-05
TALES OF TWO ENZYMES: MEMBRANE-BOUND ALCOHOL DEHYDROGENASE AND ALDEHYDE DEHYDROGENASE OF ACETIC ACID BACTERIA

T. Yakushi,[1*] H. Mukai,[1] T. Kodama,[1] M. Matsutani,[1] G. Theeragool,[2] K. Matsushita[1]

[1]Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan; [2]Faculty of Science, Kasetsart University, Bangkok, Thailand.

[*]Corresponding author: juji@yamaguchi-u.ac.jp Phone: +81 839335857.

Acetic acid fermentation, in which ethanol is oxidized to acetic acid by acetic acid bacteria, is the most characteristic process in vinegar production. The process is catalyzed by two membrane-bound enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH).
ADH has ethanol:ubiquinone oxidoreductase activity to work as a primary dehydrogenase in the ethanol-oxidizing respiratory chain. ADHs purified from *Acetobacter* and *Gluconobacter* consist of three subunits, dehydrogenase (sub I encoded by *adhA*), ubiquinone reduction cytochrome c (sub II, *adhB*), and chaperone (sub III, *adhS*) subunits. On the other hand, ADHs purified from *Gluconacetobacter* spp. do not contain sub III. Recently, we determined the genome sequence of *Gluconacetobacter xylinus* IFO3288 and found a homologous gene for sub III. We constructed Δ*adhB* (Δ*GLX_26600*) and Δ*adhS* (Δ*GLX_16410*) strains of IFO3288. The Δ*adhB* cells dramatically lost ethanol oxidase and ethanol dehydrogenase activities. Since a sub I-like band could be detected in heme-catalyzed peroxidase staining of SDS-PAGE analysis, sub I having heme C with very low activity may be produced in the absence of sub II. Ethanol oxidase and ethanol dehydrogenase activities at the wild-type level could be observed in the cell-free extract of the Δ*adhS* strain. This gene homologous to *adhS* is not required for ADH activity.
ALDH catalyzes oxidation of acetaldehyde produced by the action of ADH to produce acetic acid. Although the genes for ALDH of *Gluconacetobacter europaeus* are cloned and sequenced, their physiological relevance to acetic acid fermentation is not clear yet. Here, we disrupted the aldH gene encoding dehydrogenase subunit of ALDH in *Acetobacter pasteurianus* SKU1108, a thermotolerant strain, of which the draft genome sequence is available. The Δ*aldH* strain failed to do acetic acid fermentation in the medium with 4% ethanol, suggesting that the *aldH* gene is responsible for acetic acid fermentation. However, the Δ*aldH* strain still showed ALDH activity in the membrane fraction at approx. 30% of the wild-type strain. This remaining ALDH activity is not derived from the ADH molecules, because it was not affected by heat inactivation of ADH. We found another set of genes homologous to *aldFGH* in the genome, which may account for the remaining in vitro ALDH activity.

Keywords: Gluconacetobacter, alcohol dehydrogenase, PQQ, cytochrome, molecular assembly.

S3A-O-06
A NEW CLEAN DELETION SYSTEM FOR DIFFERENT GLUCONOBACTER OXYDANS STRAINS

D. Kostner,[1*] B. Peters,[1] M. Mientus,[1] W. Liebl,[1] A. Ehrenreich[1]

[1]Lehrstuhl für Mikrobiologie Technische Universität München, Emil-Ramann-Str. 4, D-85354 Freising, Germany.

[*]Corresponding author: david.kostner@tum.de Phone: +49 8161715468.

The genus *Gluconobacter* from the family of Acetobacteriaceae is well known for its ability to perform rapid incomplete oxidation of a great variety of carbohydrates, alcohols and related compounds. In a multitude of biotechnological processes for example *G. oxydans* is used because of its regio- and stereo-selective oxidative capabilities. The incomplete oxidation of substrates is catalyzed by various dehydrogenases. For the detailed molecular investigation of *Gluconobacter*, a well-established and easily applicable clean deletion system is essential.
A method for markerless clean deletion in *G. oxydans* strain 621H is already available from our group. This method is based on the use of uracilphosphoribosyl transferase (UPRTase) as a counter-selectable marker in the presence of the toxic pyrimidin analogue 5-fluorouracil (5-FU). The bottleneck of the method is the requirement for previously generated mutants of the UPRTase gene (Δ*upp*). The construction of such Δ*upp* mutants is difficult in some strains with effective restriction systems or they might be inconvenient for biotechnological purposes.
To allow usage of wild-type strains instead of Δ*upp*-mutants, we developed an improved clean deletion system based on a cytosine-deaminase as the counter-selectable marker in the presence of toxic 5-fluorocytosine (5-FC). This principle is applicable for several other genera of Acetobacteria to establish clean deletion systems.

Keywords: Gluconobacter oxydans, clean deletion system, fluorocytosin.

S3A-P-01
GENOME SEQUENCE OF *GLUCONACETOBACTER EUROPAEUS*

C. Andrés-Barrao,[1] L. Falquet,[2] S.P. Calderon-Copete,[2] M. Docquier,[3] P. Descombes,[3] R. Ortega Pérez,[1] F. Barja[1*]

[1]*Department of Botany and Plant Biology, Microbiology Unit, University of Geneva, Chemin des Embrouchis 10, CH-1254 Jussy-Geneva, Switzerland;* [2]*Vital-IT, Swiss Institute of Bioinformatics, Quartier Sorge-Bâtiment Genopode, CH-1015 Lausanne, Switzerland;* [3]*Genomics Platform, CMU, University of Geneva, Rue Michel-Servet 1, CH-1211 Geneva 4, Switzerland.*

[*]*Corresponding author: françois.barja@unige.ch Phone: +41 223793750.*

The main acetic acid bacteria (AAB) strains involved in the production of vinegars with high acetic acid concentration belong to the genus *Gluconacetobacter*, and from them, *Gluconacetobacter europaeus* is one of the most prominent species that have been isolated from industrial submerged vinegar fermentors. *Gluconacetobacter* strains are characterised by an extremely high resistance to acetic acid (up to 18-20%).
The genome sequences of other industrially important AAB have been published, such as *Gluconobacter oxydans, Acetobacter pasteurianus, Gluconacetobacter hansenii* or *Gluconacetobacter xylinus*. We describe here the genome sequence of three strains of *Ga. europaeus* (LMG 18890^T, LMG 18494, 5P3), as well as one strain of *Ga. oboediens* (174Bp2). Strains 5P3 and 174Bp2 were isolated from red wine and spirit vinegar running submerged fermentations that were carried in the laboratory.
Sequences were obtained by using Illumina paired-end (PE insert 500bp, 76bp read length) and mate-paired (MP insert 3Kbp, 80bp read length) libraries. The assembly was carried out with SOAPdenovo, combining PE and MP data. The scaffolds were built with Abacas using *Ga. xylinus* as a reference and the gaps were verified by PCR and sequenced. After remapping of the reads onto the scaffolds, the potential connections between scaffolds were assessed with IGV and a utility for displaying connections graphically in Cytoscape. The final sequence was obtained after treatment with PrInSeS to correct for gaps or misassemblies.
We describe here the main common and different features among the four chromosomes.

Keywords: genome sequence, Gluconacetobacter europaeus, high-throughput sequencing.

SESSION III.B. Biochemistry and Physiology.

S3B-KN
GLUCONOBACTER OXYDANS: BIOCHEMISTRY MEETS BIOTRANSFORMATION

U. Deppenmeier[1*]

[1]*Institute of Microbiology and Biotechnology, University of Bonn, Meckenheimer Allee 168, 53115 Bonn, Germany.*

[*]*Corresponding author: udeppen@uni-bonn.de Phone: +49 228735590.*

Gluconobacter oxydans is unsurpassed by other organisms in its ability to incompletely oxidize a great variety of carbohydrates, alcohols and related compounds. Furthermore, the organism is used for several biotechnological processes, such as vitamin C and Miglitol synthesis. For industrial bioconversions, the membrane-bound dehydrogenases of this organism are of major importance since these enzymes are responsible for the stereoselective oxidation of precursors for chemical synthesis of the aforementioned products. Therefore, it would be desirable to improve specific activities by overproduction of the corresponding proteins. Consequently, genetic manipulation and functional gene expression are of increased demand, especially for strain improvement. For this reason, two expression vectors were constructed for protein overproduction in *G. oxydans* and analyzed with respect to their promoter strength. Promoter-reporter fusions and RT-qPCR revealed that the constructs displayed strong and moderate promoter strengths in *G. oxydans*, respectively. To test the system the *G. oxydans* arabitol dehydrogenase gene gox2181, was homologously expressed and protein production was compared to *E. coli* production to investigate the usefulness of these vectors as a protein production platform in *G. oxydans*. Further proof of concept was obtained when the arabitol dehydrogenase (Tm0297) from the hyperthermophilic bacterium *Thermotoga maritima* was also heterologously produced in *G. oxydans* and purified in an active state using the strep-tag system. Finally, The membrane-bound glucose dehydrogenase, encoded by gox0265, was expressed in G. oxydans in an attempt to improve enzyme activities and to streamline the process of integral membrane protein purification. Membranes of the overexpression strain had a specific activity of 15 U/mg with glucose, which was seven-fold higher in comparison to the control strain. The rate of oxygen consumption of these membranes was very high (1100 nmol ½ O_2 min^{-1} mg^{-1}) and about three-times higher in comparison to the control. Glucose dehydrogenase was successfully purified from the membranes by solubilisation with detergent and subsequent StrepTactin affinity chromatography. These results demonstrate that biotransformation rates for industrial applications can be improved. Furthermore, the purification of active membrane proteins from *G. oxydans* by StrepTactin affinity chromatography is possible and can be used for the characterization of novel dehydrogenases.

Keywords: industrial application, strain development, genetic tools, glucose dehydrogenase, protein production.

S3B-O-01
SIMULATED COCOA PULP FERMENTATIONS OF COCOA-SPECIFIC ACETIC ACID BACTERIA REVEAL THEIR FUNCTIONAL ROLE AND INTERACTIONS DURING COCOA BEAN FERMENTATIONS

F. Moens,[1*] T. Lefeber,[1] L. De Vuyst[1]

[1]Research Group of Industrial Microbiology and Food Biotechnology, Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium.

[*]Corresponding author: ldvuyst@vub.ac.be
Phone: +32 26293245.

Cocoa bean fermentation is the first step in chocolate manufacturing. During this fermentation process a microbial succession occurs in which the initial growth and metabolism of yeast and lactic acid bacteria (LAB) is followed by the growth of acetic acid bacteria (AAB). The present study aimed at investigating the kinetics of substrate consumption and metabolite production by cocoa-specific AAB strains to unravel their physiological adaptations to the cocoa pulp ecosystem and their interactions with LAB and yeast to obtain an in-depth view of the functional role of AAB during cocoa bean fermentation. Therefore, four cocoa-specific AAB strains (*Acetobacter pasteurianus* 386B, *A. ghanensis* LMG 23848^T, *A. fabarum* LMG 24244^T, and *A. senegalensis* 108B) were analyzed kinetically and metabolically during monoculture fermentations in a cocoa pulp simulation medium (CPSM) for AAB at pH 4.5 under aerobic conditions. CPSM containing ethanol plus lactic acid and/or mannitol was tested. To get a more detailed view of ethanol oxidation by the AAB strains, ethanol was added at a constant flow rate to the medium to compensate for evaporation losses. *Acetobacter pasteurianus* 386B and *A. ghanensis* LMG 23848^T were analysed during co-culture fermentations with *Lactobacillus fermentum* 222 in CPSM for LAB, containing glucose, fructose and citric acid, and simulating ethanol, pH, and aeration dynamics of a spontaneous cocoa bean fermentation process. A tri-culture fermentation under similar conditions with *Saccharomyces cerevisiae* H5S5K23, *L. fermentum* 222, and *A. pasteurianus* 386B was also performed. Monoculture fermentations with AAB showed that only A. pasteurianus 386B and *A. ghanensis* LMG 23848^T oxidised mannitol to fructose. With respect to oxidation of ethanol and lactic acid to acetic acid, two groups could be distinguished: *A. pasteurianus* 386B and *A. senegalensis* 108B oxidized lactic acid and ethanol consecutively and fast, whereas *A. ghanensis* LMG 23848^T and *A. fabarum* LMG 24244^T did it simultaneously and slow, underlining greater competitiveness of the former species. During co-culture fermentations, *A. pasteurianus* 386B and *A. ghanensis* LMG 23848^T survived the initial suboptimal pH and non-aeration conditions and oxidized lactic acid, produced by *L. fermentum* 222, into acetic acid, indicating their further growth when ethanol becomes limiting. During the tri-culture fermentation experiments, ethanol and lactic acid initially produced by *S. cerevisiae* H5S5K23 and *L. fermentum* 222, respectively, were oxidized to acetic acid by *A. pasteurianus* 386B, which accurately reflected the actual cocoa bean fermentation process.

Keywords: acetic acid bacteria, fermentation, oxidation, cocoa pulp.

S3B-O-02
PROTEOMIC STUDY OF ACETOBACTER PASTEURIANUS NBRC3283 AND ANALYSIS OF FACTORS POSSIBLY RELATED TO ACETIC ACID FERMENTATION

A. Okamoto-Kainuma,[1*] M. Ishikawa,[1] K. Ito,[2] Y. Koizumi[1]

[1]Department of Fermentation Science, Tokyo University of Agriculture, Japan; [2]Central Research Institute, Mizkan Group Corporation, Japan.

[*]Corresponding author: okamoto@nodai.ac.jp
Phone: +81 354772384.

Acetic acid bacteria possess various properties that enable acetic acid fermentation. These bacteria are characteristic in acquiring ATPs by oxidative fermentation, although they can also produce ATPs by normal procedures, which is commonly observed in many aerobic microbes. In addition to this energy-generation property, these bacteria possess resistance ability against various stressors that inevitably exist during fermentation. The combination of these properties enables acetic acid bacteria to perform acetic acid fermentation, which is applied to vinegar production. Hence, elucidation of the expression mechanism or combination manner of these functions is important to develop more sophisticated fermentation techniques. Recently, genomic sequences have been determined for several strains of acetic acid bacteria, which have made it possible to investigate the physiology of these bacteria more comprehensively. In this study, we performed proteomic analysis of *Acetobacter pasteurianus* NBRC3283 and further investigated several factors that have been suggested to be associated with acetic acid fermentation ability.

Agarose-2D electrophoresis was performed on the cell-free extracts of *A. pasteurianus* NBRC3283, and 97% of the CBB-stained protein spots on the gel were identified by ESI-IT MS/MS analysis. According to the results, we created a protein expression map on a 2D-gel profile. Based on this map, the following proteins showed interesting behavior, possibly reflecting characteristic properties of acetic acid bacteria; 1) enolase and pyruvate phosphate dikinase, enzymes belonging to the glycolytic pathway, 2) AarC, an enzyme that compensates for the lack of succinyl-CoA synthetase in the TCA cycle, and 3) GroEL, DnaK, and ClpB, representative molecular chaperones.

Concerning the above factors, we analyzed the detailed behaviors at the transcription level, and the results suggest that the glycolytic pathway is involved in acetic acid fermentation. In addition, we speculate that AarC possibly has some function other than overoxidation of acetic acid. Furthermore, we constructed an *rpoH* disruptant, which does not express the molecular chaperones, and

confirmed its importance for resistance to stressors that exist during acetic acid fermentation.

Keywords: Acetobacter pasteurianus, proteomic analysis, glycolytic pathway, AarC, molecular chaperone.

S3B-O-03
ELUCIDATING THE METABOLIC PROPERTIES OF *GLUCONOBACTER OXYDANS 621H* BY ^{13}C-METABOLIC FLUX ANALYSIS

S. Ostermann,[1*] W. Wiechert,[1] M. Oldiges[1]

[1]*Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, D-52425 Jülich, Germany.*

[*]*Corresponding author: s.ostermann@fz-juelich.de Phone: +49 02461613374.*

The acetic acid bacteria are a very interesting group of organisms, especially for industrial processes. Although they have a great production potential, a lot of their biochemical properties are still unknown. Due to scientific progress in the last years, genomic sequences of first strains are now available. With deeper metabolic insight it will be possible to broaden the range of applications and increase the yield of product by metabolic and bioprocess engineering.
Intracellular metabolic flux rates, the so called Fluxome, can be measured by ^{13}C-metabolic flux analysis (^{13}C-MFA). A labeled ^{13}C-substrate mixture is fed and the resulting labeling pattern is measured via mass spectrometry. In addition all carbon fluxes over the system borders have to be quantified (i.e. substrate uptake rates and product formation rates), and the intracellular isotopic labeling pattern of the metabolites have to be determined. As a result the central carbon metabolism can be visualized in a flux map including absolute values of each reaction with their related standard error. This information about the *in vivo* situation leads to valuable insight into metabolic network operations and could detect so called bottlenecks in production processes.
As a prerequisite for ^{13}C-MFA, a well-controlled continuous cultivation process is necessary. This mode was established in *G. oxydans* 621H with 5 g/L yeast extract and 5g/L glucose as substrates. All relevant carbon uptake rates (glucose) and product formation rates (gluconate, 2-keto-gluconate, acetate, biomass, CO_2) were successfully quantified. In three biological replicates with μ=0,32± 0,02 h^{-1} a carbon recovery between 90,4±5,76% and 110,4±10,13% could be obtained. To get reliable intracellular flux rates a precise and closed carbon balance is essential. Yeast extract is still used in the cultivation media to gain enough biomass. The addition of this complex component significantly interferes with ^{13}C labeling experiments due to error prone labeling data if ^{12}C moieties from yeast extract are incorporated in addition to ^{13}C glucose. It is possible to detect such incorporation of components or precursors from the yeast extract by finding ^{12}C-contamination in the intracellular metabolites. Thus cultivations with uniformly labeled ^{13}C glucose were performed. It could be shown that 100% of the formed CO_2 is ^{13}C-labeled. These data and the closed carbon balance are a hint that no significant amounts of yeast extract are metabolized to CO_2. Once the complete workflow of ^{13}C-MFA is validated for the wildtype *G. oxydans* 621H it will be very easy to adapt it to different production strains and Fluxome data will provide an additional basis for metabolic and bioprocess engineering of acetic acid bacteria.

Keywords: metabolic flux analysis, carbon metabolism, defined media.

S3B-O-04
HOW DIFFERENT CARBON SOURCES AND CONDITIONS MAKE AAB "WORKING" STRAINS: *ACETOBACTER PASTEURIANUS* STRAIN AB0220 IN SUPERFICIAL ACETIFICATION SYSTEM AS CASE STUDY

M. Gullo[1*]

[1]*Department of Agricultural and Food Sciences, University of Modena and Reggio Emilia, Via G. Amendola 2, Pad. Besta, 42100 Reggio Emilia, Italy.*

[*]*Corresponding author: maria.gullo@unimore.it Phone: +39 0522522057/63.*

Metabolic potential of acetic acid bacteria (AAB) is of great interest for several fields of the bio-industry. *Acetobacter pasteurianus* species accounts strains relevant for the production of both conventional and innovative fermented beverages. Strain AB0220 was isolated in 2002 during a large spectrum of isolation work aimed to build up an AAB collection from superficial vinegar acetification systems. It was preserved for 9 years by short and long time methods. Ethanol oxidation to acetic acid was stable and confirmed, as well as acetate assimilation during preservation. The strain do not produced cellulose. Cultivability checks showed persistence of phenotypic traits over the extended preservation time. Stability of subcultures related to the culture age and subcultures frequency confirmed the suitability of preservation at least over a period of 9 years. Strain performance during superficial acetification, both in laboratory and industrial scale, was assayed. To this aim, the acetification ability was tested on different carbon sources and conditions miming the basic unit operation of superficial acetification technology. The performance of AB0220 during processes was evaluated implementing a molecular and analytical control system. Under the experimental conditions, acetic acid, ethanol and pH were the main parameters dictating the conduction of scaling-up procedure. When fixing ethanol content between 1 and 3% as upper and lower limits and 3% as the lower limit for acetic acid, suitable acidity (6-7%) was reached. The persistence of AB0220 as starter over the time was evaluated after biofilm-enrichment cultures on GYC plates. The biofilm, totally recovered from plates, was processed for genomic DNA extraction. PCR/DGGE and ERIC/PCR were successfully used to assess species and strain persistence respectively, during 178 days of acetification.

Keywords: Acetobacter pasteurianus, phenotype, AAB selected starter.

S3B-O-05
ROLE OF THE PENTOSE PHOSPHATE PATHWAY AND THE ENTNER-DOUDOROFF PATHWAY FOR CYTOPLASMIC FRUCTOSE CATABOLISM IN *GLUCONOBACTER OXYDANS* 621H

J. Richhardt,[1*] S. Bringer,[1] M. Bott[1]

[1]*Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, D-52425 Jülich, Germany.*

[*]*Corresponding author: j.richhardt@fz-juelich.de Phone: +49 2461613975.*

The obligatory aerobic acetic acid bacterium *Gluconobacter oxydans* 621H oxidizes sugars and sugar alcohols primarily in the periplasm, whereas only a small fraction is metabolized in the cytoplasm. The latter can occur either via the Entner-Doudoroff pathway (EDP) or via the pentose phosphate pathway (PPP). The Embden-Meyerhof pathway (EMP) is non-functional due to the lack of a gene for phosphofructokinase and a cyclic operation of the tricarboxylic acid (TCA) cycle is precluded by absence of the genes for succinyl-CoA synthetase and succinate dehydrogenase. In this study, we tested the relevance of the EDP and the PPP for the cytoplasmic catabolism of the carbon source mannitol. This sugar alcohol is rapidly oxidized to fructose, both in the periplasm and in the cytoplasm. Two defined mutants of *G. oxydans* were constructed, one defective in the EDP by deletion of the genes *edd* (6-phosphogluconate dehydratase) and *eda* (2-keto-3-desoxy-6-phosphogluconate aldolase) and one devoid of the PPP by deletion of the *gnd* gene (6-phosphogluconate dehydrogenase). Based on the growth properties of the two mutants under controlled culture conditions, transcriptome analyses and enzyme activity measurements it was concluded that the PPP is the main route for cytoplasmic fructose degradation in *G. oxydans*, whereas the EDP is dispensable or even unfavorable, as the Δ*edd-eda* mutant showed a significantly increased cell mass compared to the parental strain. In contrast, deletion of the *gnd* gene inhibited growth on mannitol and caused a strong selection pressure for secondary mutations in the *zwf* gene that inactivate glucose 6-phosphate dehydrogenase activity and therefore also prevent fructose metabolism via the EDP. This Δ*gnd zwf** mutant was almost totally disabled in fructose catabolism, but still produced about 16% of the carbon dioxide formed by the parental strain, presumably by catabolizing substrates from the yeast extract.

Keywords: Gluconobacter oxydans, gnd, edd-eda, zwf, mannitol.

S3B-O-06
DIVERSITY OF THE RESPIRATORY CHAIN OF AAB, ESPECIALLY *ACETOBACTER PASTEURIANUS, GLUCONACETOBACTER DIAZOTROPHICUS* AND *GLUCONOBACTER OXYDANS*

H. Miura,[1] S. Nishiyama,[1] M. Arimitsu,[1] Y. Anami,[1] M. Matsutani,[1] T. Yakushi,[1] K. Matsushita[1*]

[1]*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan.*

[*]*Corresponding author: kazunobu@yamaguchi-u.ac.jp Phone: + 81 839335858.*

Acetic acid bacteria (AAB) are well known for their ability to oxidize various sugars and sugar alcohols into their corresponding acids. The oxidation reactions are carried out by membrane-bound enzymes linked to the aerobic respiratory chain, which consists of the primary dehydrogenases and terminal oxidases via ubiquinone. However, the respiratory components, especially NADH dehydrogenases (NDH) and terminal ubiquinol oxidases (Q-oxidase), are varied depending on the genus of AAB. NDH has been classified as NDH-I and NDH-II, both the genes of which are present in all AAB genomes except for NDH-I in *Gluconobacter oxydans*. Whereas, Q-oxidase is classified as cytochrome ba_3, cytochrome bo_3, cytochrome *bd*, and cyanide-insensitive oxidase (CIO). *G. oxydans* genome has genes for cytochrome bo_3 and CIO, *Gluconacetobacter diazotrophicus* has those for cytochrome ba_3 and CIO, while *Acetobacter pasteurianus* has three genes corresponding to cytochrome ba_3, cytochrome *bd*, and CIO.

As for Q-oxidases, cytochrome bo_3 has been isolated long before from *G. oxydans* and *A. aceti (A. pasteurianus)*, and cytochrome ba_3 from *A. pasteurianus* and *Ga. diazotrophicus*. Recently, CIO was purified from *G. oxydans*, and also partially done from *A. pasterurianus* and *Ga. diazotrophicus*. Thus, we will present some of the results obtained from the purified CIOs which exhibit rather unique properties such as CN-insensitivity, low O_2 affinity, or rather high quinol oxidase activity and its kinetics, compared with cytochrome *bd*.

Furthermore, based on microarray or RT-qPCR analyses in *A. pasteurianus*, NDH-I was shown to be highly expressed in the over-oxidation phase but not in the ethanol-oxidation phase of acetic acid fermentation. NDH-II expression was rather low compared with NDH-I in any phases. Whereas, cytochrome ba_3 was highly expressed in all phases of the fermentation, but both cytochrome *bd* and CIO were weakly expressed but the most in the ethanol-oxidation phase. We further isolated NDH-I from the membranes of the over-oxidation phase, and found almost all the subunits of NDH-I complex. Thus, NDH-I-dependent NADH oxidase respiratory chain seems to work mainly in the over-oxidation phase, but not in the ethanol-oxidation phase, together with cytochrome ba_3 oxidase.

Keywords: respiratory chain, cyanide-insensitive oxidase, cytochrome ba_3, cytochrome bo_3, NADH dehydrogenase.

S3B-O-07

THE QUEST FOR NEW OXIDATIVE CATALYSTS: EXPRESSION OF METAGENOMIC MEMBRANE-BOUND DEHYDROGENASES FROM ACETIC ACID BACTERIA IN *GLUCONOBACTER OXYDANS*

B. Peters,[1*] M. Mientus,[1] D. Kostner,[1] W. Liebl,[1] A. Ehrenreich[1]

[1]*Lehrstuhl für Mikrobiologie Technische Universität München, Emil-Ramann-Str. 4, D-85354 Freising, Germany.*

[*]*Corresponding author: bjoern.opeters@wzw.tum.de Phone: +49 8161715468.*

Due to their ability to incompletely oxidize a great variety of carbohydrates, alcohols and related compounds, acetic acid bacteria are widely used in biotechnology. Many of these oxidations are unachievable using organic chemistry. Because these reactions are mostly catalyzed by membrane-bound dehydrogenases in a rapid, regio- and stereo-selective manner, the substrates do not have to be transported into the cytoplasm. We use a metagenomic approach to indentify new membrane-bound dehydrogenases of potential value for biotechnology from a mother of vinegar, because many acetic acid bacteria can not be cultivated in the laboratory.
The membrane-bound dehydrogenases are screened by sequence similarity from the metagenomic sequences and are functionally expressed in specially designed *Gluconobacter oxydans* strains. In these strains all membrane-bound dehydrogenases were deleted using a clean deletion system developed by our group to avoid overlapping enzymatic specificities. To ensure functional integration in the membrane physiology of these organisms we use specifically designed expression vectors with native promoters.
In order to characterize the activity of specific membrane-bound dehydrogenases we set up a high throughput assay, using whole cell catalysis in microtiter-plates. The advantage of this system is a minimized cell preparation together with the ability to compare many stains or substrates in one experiment. We used this approach to determine the *in vivo* substrate spectrum of several specific membrane-bound dehydrogenases from acetic acid bacteria for the first time.

Keywords: metagenome, membrane-bound dehydrogenases, Gluconobacter oxydans.

S3B-O-08

THE ACTIVE AND INACTIVE FORMS OF THE MEMBRANE-BOUND ALCOHOL DEHYDROGENASE FROM GLUCONACETOBACTER DIAZOTROPHICUS

J.E. Escamilla,[1*] S. Gómez-Manzo[2]

[1]*Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria 04510, México;* [2]*Laboratorio de Bioquímica-Genética. Torre de Investigación. Instituto Nacional de Pediatría, Mexico.*

[*]*Corresponding author: eescami@ifc.unam.mx Phone: +52 5556225627.*

The membrane-bound alcohol dehydrogenase of Gluconacetobacter diazotrophicus contain, one pyrroloquinoline quinone moiety (PQQ), one [2Fe-2S] cluster and four *c*-type cytochromes. Here, we describe a novel and inactive alcohol dehydrogenase (ADHi). ADHi, similarly to ADHa is a heterodimer of 72 and 44 kDa subunits and contains the expected prosthetic groups. However, ADHa showed a threefold molecular mass as compared to ADHi. Noteworthy, the PQQ, the [2Fe-2S] and most of the cytochromes in purified ADHi is in the oxidized form, contrasting with ADHa where the PQQ-semiquinone is detected and the [2Fe-2S] cluster as well as the cytochromes *c* remained fully reduced after purification. Reduction kinetics of the ferricyanide-oxidized enzymes showed that, while ADHa was brought back by ethanol to its full reduction state, in ADHi only one quarter of the total heme *c* was reduced. The dithionite-reduced ADHi was largely oxidized by ubiquinone-2, thus indicating that intramolecular electron transfer is not impaired in ADHi. The acidic pH of the medium might be deleterious for the membrane-bound ADH by causing conformational changes leading to changes in the relative orientation of heme groups and shift of corresponding redox potential to higher values. This would hamper electron transfer resulting in the low activity observed in ADHi.
Acknowledgements: CONACyT 152779 and PAPIIT-UNAM IN218710-2

Keywords: alcohol dehydrogenase, Gluconacetobacter diazotrophicus, aerobic fermentation, inactive ADH.

S3B-O-09

STRUCTURES AND FUNCTIONS OF *ACETOBACTER ACETI* COA-TRANSFERASES

T.J. Kappock,[1*] E.A. Mullins[1,2]

[1]*Department of Biochemistry, Purdue University, West Lafayette, Indiana, USA;* [2]*Department of Chemistry, Washington University, Saint Louis, Missouri, USA.*

[*]*Corresponding author: kappock@purdue.edu Phone: +01 7654948383.*

Genome sequencing revealed five acyl-CoA transferase genes in the *A. aceti* strain 1023 genome. One is *aarC*, an acetic acid resistance factor, and the others were designated *uctA* - *uctD*. We previously reported that AarC is succinyl-CoA:acetate CoA-transferase (SCACT) and that its resistance function can be attributed to its role as part of a variant citric acid cycle (vCAC) present in *A. aceti* 1023 [E. A. Mullins, J. A. Francois, and T. J. Kappock (2008) *J. Bacteriol.* 190, 4933-40]. Subsequent genome sequencing has indicated that some acetic acid bacteria use the vCAC, some use the normal CAC containing succinyl-CoA synthetase, and some may use either one. This implies some degree of metabolic flexibility in a pathway that is relevant to specific acetic acid resistance phenotypes. We used a combined enzymological and crystallographic approach to arrive at a model that explains how AarC recognizes its substrates. Analysis of *aarC* transcription suggests a combination of inducible and housekeeping expression patterns. In addition, we will

disclose functional assignments for UctB and UctC that expand the scope of likely physiological roles for acyl-CoA: acid interconversions coupled to acetyl-CoA production.

Keywords: acid metabolism, functional annotation, x-ray crystallography.

S3B-O-10
SCREENING OF QUORUM SENSING AND QUORUM QUENCHING ACTIVITIES IN THE GENOME OF A *GLUCONACETOBACTER XYLINUS* STRAIN

M.J. Valera,[1] W. Streit,[2] M.J. Torija,[1] A. Mas,[1] E. Mateo[1*]

[1]Departamento de Bioquímica y Biotecnología. Facultad de Enología. Universitat Rovira i Virgili. C/ Marcel•lí Domingo s/n. E- 43007 Tarragona, Spain; [2]Abteilung für Mikrobiologie und Biotechnologie, Biozentrum Klein Flottbek, Universität Hamburg, Germany.

[*]Corresponding author: estibaliz.mateo@urv.cat Phone: +34 977558688.

Quorum sensing (QS) is a cell density dependent system, which is used to regulate diverse physiological functions, as biofilm formation. N-acylhomoserine lactones (N-AHLs) are autoinducers involved in many QS mechanisms to regulate gene expression in gram-negative bacteria. On the other hand, proteins interfering with these bacterial QS molecules have been found. They can stop the activity of this signalization and cut the communication between cells. It is the Quorum Quenching (QQ) signalization, which has been described, in different microorganisms and their diverse mechanisms involved are being studied.
Some acetic acid bacteria are able to produce cellulose as secondary metabolite and form biofilms. Previously in some species of *Gluconacetobacter* a Quorum Sensing N-AHLs-dependent system (GinI/GinR proteins homologous to LuxI/LuxR described in *Vibrio fischeri*), was reported; however, the opposite process, a Quorum Quenching, has never been studied in this group of bacteria.
The aim of this study was to screen for QS and QQ activities in the genome of one *Gluconacetobacter xylinus* strain, cellulose overproducer and biofilm-former, in order to better understand the mechanisms involved.
A fosmid library of *Ga. xylinus* genome was constructed in *Escherichia coli.* The strain *Agrobacterium tumefaciens* NTL4, carrying a *traI-lacZ* promoter fusion, was used as reporter to make the suitable screening in order to find activities related with the autoinducer AHL. We have obtained 2 clones with putative Quorum Sensing activities and 13 with Quorum Quenching activities. Further studies are underway to characterize the genes involved in production of AHL or anti-AHL molecules.

Keywords: cell-cell communication, homoserine lactone, fosmid library, biofilm.

S3B-P-01
PERFORMING ^{13}C-METABOLIC FLUX ANALYSIS ON ACETIC ACID BACTERIA

S. Ostermann,[1*] W. Wiechert,[1] M. Oldiges[1]

[1]Institute of Bio- and Geosciences IBG1: Biotechnology, Forschungszentrum Jülich, Germany.

[*]Corresponding author: s.ostermann@fz-juelich.de Phone: +49 02461613374.

The acetic acid bacteria have a great industrial production potential, which could be exploited if their biochemical properties are better understood. Their metabolic reactions (flux rates) appear as a result of all cellular control hierarchies and determine the material flows. A reliable and predictive biochemical model would enable directed metabolic engineering to increase efficiency and yield of production processes.
For determining the intracellular metabolic flux rates, the so called Fluxome, a ^{13}C-metabolic flux analysis (^{13}C-MFA) can be performed. A defined isotopically labeled substrate is fed into a bioreactor and all carbon fluxes over the system borders are quantified (i.e. substrate uptake rates and product formation rates). Moreover a characteristic intracellular isotopic labeling pattern in the metabolites emerges and with bioinformatic tools the flux data can be calculated and displayed. Although ^{13}C-MFA was already performed for i.e. *E. coli, S. cerevisiea* or *C. glutamicum* a lot of adaptions to the family of *Acetobacteraceae* have to be performed.
A biochemical network model of the central metabolic pathways (glycolysis, pentose phosphate pathway, anaplerotic reactions, citric cycle) based on the genome sequence of *Gluconobacter oxydans 621H* was created. In this model the faith of every single carbon atom from glucose to intermediate and product is determined. To ensure that all relevant reactions are included the carbon flow of the bioprocess is balanced. Therefore a continuous cultivation mode (chemostat) with ^{13}C-glucose as substrate and sufficient biomass for measurements was established. After 4 residence times the substrate uptake rate (glucose) and product formation rates (CO_2, biomass, gluconate, 2-keto-gluconate, acetate) were measured with a carbon recovery between 90,4 ± 5,76% and 110,4 ± 10,13%. To receive the intracellular labeling information all cellular reactions were stopped instantly by cold methanol quenching and the metabolites were extracted followed by LC-MS/MS analytics for measurement of the ^{13}C labeling distribution. For every sample 48 metabolites with 448 mass traces are considered in total. These raw data were combined with organism specific biochemical assumptions, e.g. fixation of known reaction directionalities or drain of carbon into biomass. Thereof the software 13CFLUX[2] calculates a flux distribution and labeling pattern *in silico*. This pattern is iteratively fitted to the measured labeling pattern to approximate to the *in vivo* metabolic flux distribution.

Keywords: metabolic flux analysis, carbon metabolism, isotopic labeling.

S3B-P-02

OVER-EXPRESSED TRUNCATED C-TERMINAL OF ASPARTYL-TRNA SYNTHETASE FROM *ACETOBACTER PASTEURIANUS* SKU1108 INHIBITS CELL DIVISION OF *ESCHERICHIA COLI* AT HIGH TEMPERATURE

G. Theeragool,[1*] K. Tasanapak,[1] W. Yongmanitchai,[1] K. Matsushita[2]

[1]*Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand;* [2]*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan.*

[*]*Corresponding author: fscignt@ku.ac.th*
Phone: +66 818017710.

Thermotolerant acetic acid bacteria play important role in high temperature vinegar fermentation due to their ability to grow and produce acetic acid at high temperature. In order to clarify the molecular mechanism of thermotolerance, random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was used to investigate the genetic variations between thermotolerant and thermosensitive strains of acetic acid bacteria. Three typical RAPD-PCR amplified DNA fragments of 0.6, 1.0 and 1.3 kb could be clearly observed in 13 different thermotolerant but not in thermosensitive strains. The 1.3 kb DNA fragment carries partial aspS gene encoding truncated 138 amino acid residues of C-terminal aspartyl-tRNA synthetase (AspRS). The entire aspS gene from a thermotolerant acetic acid bacterium, *Acetobacter pasteurianus* SKU1108, has been further cloned and characterized. The cloned aspS gene consists of 1,788 bp coding for 595 amino acid residues. The deduced amino acid sequences showed 99 and 82% identity with the same protein from *A. pasteurianus* NBRC 3283 and *Gluconacetobacter diazotrophicus*, respectively. The strong tetrapeptide Gly-Leu-Asp-Arg motif (motif 3) which acts as ATP binding domain is located at the position 537th -540th amino acid of C-terminal AspRS. Investigation of regulatory region of *aspS* gene by deletion analysis suggested that the tentative promoter is located at around 173 bp upstream from ATG initiation codon. The plasmid pGEM-TAspRS138 or pUCAspRS138 carrying partial *aspS* gene encoding 138 amino acids of truncated C-terminal AspRS (motif 3) caused cell morphological change in *E. coli* from short rod to long filament at 37 and 42°C but not at 30°C. This morphological change was also observed in *E. coli* harboring pGEM-TAspRS316 or pUCAspRS316 which synthesize 316 amino acids of C-terminal AspRS. However, the normal cell shapes were observed in *E. coli* harboring pUCAspRS595 synthesizing 595 amino acids and pGEM-TAspRS138 carrying disrupted *aspS* gene. The results revealed that the over-expressed truncated C-terminal AspRS may cause the titration effect of ATP inside the cell and therefore less amount of ATP is available for cell division. This cell division inhibition effect is more critical at high temperature so more ATP is required for growth at high temperature.

Keywords: aspartyl t-RNA synthetase, aspS gene, truncated C-terminal, ATP-binding, cell division.

SESSION IV. What can we do with them? Biotechnological applications. Bioengineering aspects. New products.

S4-KN

APPLICATIONS, TECHNOLOGIES AND SCALE UP CRITERIA OF FERMENTATION PROCESSES WITH ACETIC ACID BACTERIA

F. Emde[1*]

[1]*Heinrich Frings GmbH & Co. KG, Jonas Cahn Str. 9, 53115 Bonn, Germany.*

[*]*Corresponding author: emde@frings.com*

Acetic Acid Bacteria (AAB) have been used in food manufacturing since ancient times. Until today they are applied in production processes of fermented liquid foods, mainly for vinegar production. Beside these traditional processes in modern food and beverage industry selected strains of AAB became more and more attractive for production purposes or natural processing aids.

In the last century AAB became also of interest for a number of biotechnological production processes, too. Their use in the bio-chemical-synthesis to L-ascorbic acid still has a major economic value. AAB also play an important role in the production of tanning agents like dihydroxyacetone and L-erythrulose in cosmetic industry. Whether the huge potential of AAB to oxidize a broad range of substrates into the corresponding products will find extended use in biotechnological industry will be observed with great interest in the AAB-community.

All processes that will be developed in lab scale must be transferred into a much larger scale to achieve an economically feasible production in a fermentation plant. But how shall the bioreactor and process design look like?

For a wide field of different fermentation processes we have developed a "scale up toolbox" based on expert knowledge, analytical, experimental and computerized methods and a "decision tree". The input parameters include kinetic data, rheological data, biosystem data and data influencing the risk of contamination. In kinetic analysis the parameters of specific rates, yield coefficients, inhibition and limitation boundaries, heat dissipation, consumption rates, K_M-values and the formation of by-products will be investigated. A number of biosystem data like viscosity, shear sensitivity, ability to form biofilms or polymers, foam formation and mixing time requirement are also to be taken into account in the "decision tree" for process and bioreactor design. Additionally characteristics like pH and temperature range, osmotic pressure, cytotoxicity of the media components and mutation rate of the active biomass are indicating under which conditions the process must be operated in regard to sterility.

It is unusual that most of the relevant information is available from general lab trials to get sufficient data to follow the "decision tree" to the output with scaling, design and bioprocess engineering data. Therefore specifically elaborated experimental and bioprocess engineering tools

are used additionally. One example is the installation of inhomogeneous mixing zones in mini plant bioreactors. Experiments with non ideal mixed vessels are helpful to anticipate the performance of large scale systems. Tools for final design like CFD, cell models or dimension analysis based on the determined critical mixing parameters and the necessary mass transfer coefficient k_La are used subsequently. Other methods will be presented to decide about the process design and the use of batch, fed-batch and repeated-(fed)-batch processes depending on the above criteria.

Keywords: acetic acid bacteria, technology, industrial processes, scale up.

S4-O-01
4-KETO-D-ALDOPENTOSES AND 4-PENTULOSONATES, NEW PRODUCTS WITH ACETIC ACID BACTERIA

O. Adachi,[1*] R.A. Hours,[2] E. Shinagawa,[3] Y. Akakabe,[1] T. Yakushi,[1] K. Matsushita[1]

[1]Dept. Biol. Chem., Fac. Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan; [2]CINDEFI (CONICET, UNLP). School of Science, La Plata National University, Argentina; [3]Dept. Chem. Biol. Eng., Ube Natl. Coll. Technol., Ube 755-8555, Japan.

[*]Corresponding author: osao@yamaguchi-u.ac.jp Phone: +81 839335857.

4-Keto-D-aldopentoses and 4-pentulosonates (4-keto-D-pentonates) have been identified for the first time. These are entirely new sugars and sugar acids involving a ketone group at the C4 position in their molecules, while ketopentoses so far known are limited to those having a ketone group at the C2 position, such as D-, L-ribulose and D, L-xylulose. A novel strain, *Gluconacetobacter liquefaciens* RCTMR 10, was isolated from a water-kefir, traditional fermented healthy drink in Argentina. The strain rapidly converted 2,5-diketo-D-gluconate (25DKA) to a new compound with high yield, which was identified to be 4-keto-D-arabonate (4KAB). It appeared that decarboxylation and dehydrogenation reactions took place at a time in the reaction. Finally, 4-keto-D-arabinose (4KAR) was confirmed as the direct precursor of 4KAB and that two novel membrane-bound enzymes, 25DKA decarboxylase and 4-keto-D-aldopentose 1-dehydrogenase, were involved in the reaction. Alternatively, D-arabonate was oxidized to 4KAB with another membrane-bound enzyme, D-pentonate 4-dehydrogenase.
Extensive studies have revealed that such a novel sugars and sugar acids can be produced ubiquitously by oxidative fermentation with most strains of *Gluconobacter* and *Gluconacetobacter*. 4KAR and 4KAB were produced from D-glucose via 25DKA and also from D-arabinose. Likewise, D-ribose was oxidized to 4-keto-D-ribose and it was oxidized further to 4-keto-D-ribonate. In D-aldopentose oxidation, D-aldopentose 4-dehydrogenase has been confirmed in the membrane fraction of acetic acid bacteria. In this study, four different novel compounds and four different new membrane-bound enzymes have been identified. Among the membrane-bound enzymes characterized, D-aldopentose 4-dehydrogenase has been confirmed as a new quinoprotein, PQQ-dependent enzyme.

Keywords: D-aldopentose 4-dehydrogenase, 4-keto-D-aldopentose 1-dehydrogenase, 2,5-diketo-D-gluconate decarboxylase, 4-keto-D-aldopentose, 4-keto-D-pentonate.

S4-O-02
COMPARISON OF NOVEL GH 68 LEVANSUCRASES OF LEVAN- OVERPRODUCING *GLUCONOBACTER* SPECIES

F. Jakob,[1] D. Meißner,[1] R.F. Vogel[1*]

[1]Lehrstuhl für Mikrobiologie Technische Universität München, Emil-Ramann-Str. 4, D-85354 Freising, Germany.

[*]Corresponding author: rudi.vogel@wzw.tum.de Phone: +49 8161715169.

Strains of different *Gluconobacter* species are capable of incompletely oxidizing a wide range of carbohydrates, alcohols and sugars, whose end products can be used for various (food) biotechnological applications. We recently demonstrated that also levans isolated from exopolysaccharide (EPS)-overproducing *Gluconobacter* species are promising functional compounds for food applications, e.g. to improve volume, texture and shelf-life of breads. Fructan production strongly depends on the corresponding fructosyltransferases (Ftfs), which catalyze the formation of these polymers from sucrose. Therefore, we characterized novel, highly active Ftfs from three EPS-overproducing food-grade strains, i.e. *Gluconobacter sp.* TMW 2.767 and *Gluconobacter sp.* TMW 2.1191 isolated from water kefir, and *Gluconobacter cerinus* DSM 9533T isolated from cherries.
Several PCR techniques including degenerate gradient temperature PCR, modified and standard inverse PCR, modified site-finding PCR and modified single primer PCR, were used to finally detect complete open reading frames coding for Ftfs. The prospective *ftf*-gene sequences were heterologously expressed in *Escherichia coli* Top 10 cells, which were cultivated on solid media supplemented with sucrose (substrate) and arabinose (inductor of gene expression). *E. coli* transformants harbouring one of the three different ftf-genes produced polysaccharides from sucrose in contrast to the *E. coli* wildtype. Each of the heterologously expressed proteins encoded a levansucrase, catalyzing the formation of β-(2→6)-linked fructose polymers, which corresponded to our previous analyses of the chemical nature of the isolated polymers formed by the *Gluconobacter* strains. This suggests that the expressed genes are responsible for the production of these fructans. Structurally, these enzymes belong to the glycoside hydrolase 68 family (GH 68), sharing the typical modular topology of gram-negative levansucrases (no predictable signal peptides, conserved motifs involved in fructan polymerization). Based on similarity calculations, levansucrases of *G. sp.* TMW 2.767 and *G. cerinus* DSM 9533T (91% positives in 445 amino acids) as well as levansucrases of *G. sp.* TMW 2.1191 and *G. oxydans* 621

H (94% positives in 436 amino acids) can phylogenetically be grouped in different sub-clusters. In conclusion, we could identify novel levansucrases, which can be used for *ex situ* (enzymatic catalyses) or *in situ* (fermentation) production of functional fructan polymers by *Gluconobacter* strains for food and other applications.

Keywords: Gluconobacter, functional levans, PCR, levansucrases, GH 68.

S4-O-03
GLUCONIC ACID PRODUCTION IN STRAWBERRY BY ACETIC ACID BACTERIA

F. Sainz,[1] E. Mateo,[1] A. Mas,[1] M.J. Torija[1*]

[1]Departamento de Bioquímica y Biotecnología. Facultad de Enología. Universitat Rovira i Virgili. C/ Marcel•lí Domingo s/n. E- 43007 Tarragona, Spain.

[*]Corresponding author: mjesus.torija@urv.cat Phone: +34 977558442.

Acetic acid bacteria (AAB) are microorganisms naturally present on grapes and therefore, in wine. Even though studies on AAB have focused on ethanol to acetic acid transformation in wine and vinegar, AAB have other physiological characteristics that make them biotechnologically important. For instance, they can perform incomplete oxidation of sugars, alcohols and acids accumulating the corresponding products in the medium. One product that can be obtained by the incomplete oxidation of glucose is gluconic acid. This acid can destabilize wines during aging and storage, increasing the risk of final spoilage. However, in other beverages the presence of gluconic acid enhances natural sweetness and acts as a buffer for other acids.

Gluconic acid production can be carried out by some AAB species belonging to *Acetobacter* and *Gluconobacter* genera, more specifically by the latter genus since glucose is its preferred carbon source. The aim of this study was to carry out the gluconic acid production from glucose in strawberry pulp concentrate without fructose consumption to preserve the natural sweetness of the fruit. For this reason, different strains of *Gluconobacter* genus, as well as the strain SS8 of *Acetobacter malorum*, the unique AAB strain isolated from strawberry pulp, were tested in order to choose the best for gluconic acid production. Furthermore, in order to find out the best conditions, two different pHs were tested: 3.3, the natural pH of strawberry pulp concentrate and 4.8, the optimal pH for gluconic acid according to the literature.

The results showed that all the strains were capable of producing gluconic acid but some of them also consumed the fructose. Furthermore, at the strawberry pH, although not being the optimal pH, strains produced an important amount of gluconic acid.

Keywords: Gluconobacter, Acetobacter malorum, glucose, fructose.

S4-O-04
THE RESPONSE OF *ACETOBACTER SENEGALENSIS* TO STRESSORS: A STUDY TOWARDS IMPROVEMENT OF VINEGAR STARTER PRODUCTION

R. Shafiei,[1,2*] P. Thonart[1]

[1]Wallonia Center of Industrial Biology (CWBI), University of Liége, Liége, Belgium; [2]Department of biology, Faculty of science, University of Isfahan, Isfahan, Iran.

[*]Corresponding author: r.shafiei@student.ulg.ac.be Phone: +32 43663994.

Acetic acid bacteria encounter various harsh conditions during acetic acid fermentation. Ethanol as the main substrate and acetic acid as the major product at low pH can influence deeply on the cellular functions of acetic acid bacteria. In previous studies in CWBI, *Acetobacter senegalensis* was used for production of dried vinegar starters; however the impact of stressors (ethanol and acetic acid) on *A. senegalensis* remained unclear. In this study, different techniques such as flow cytometry, culturability on solid medium and 2-DiGE were used comparatively to investigate the effect of carbon sources of inoculum media on the tolerance of *A. senegalensis* to stressors.

Analysis of respiration system by flow cytometric methods showed that the presence of 2% (v/v) acetic acid in inoculum medium, in one hand, causes 80% of cells to continue to do respiration after a sudden exposure to 1-3% (v/v) acetic acid in stress media while 89.7% of cells grown in glucose appeared as dead cells after an abrupt exposure to 3%(v/v) of acetic acid. On the other hand, 59.2% and 49.33% of cells grown in the presence of 2% (v/v) of acetic acid could maintain their entire membrane integrity after exposure to 1% and 3% (v/v) of acetic acid, respectively.

Inoculum medium contained 5% (v/v) of ethanol as a carbon source enabled about 90% of cells to keep their growing capacities after a sudden exposure to 3% acetic acid. In contrast, just 40% of cells grown in glucose as a carbon source maintained their culturability on solid medium after exposure to 1% acetic acid. A similar profile of culturability was observed for the cells grown in 5% (v/v) ethanol or 2% (v/v) of acetic acid.

A proteomic approach (2-DiGE) was used to analyze proteins expressed in the presence of different carbon sources. Differentially expressed proteins were mainly associated with energy metabolism, carbohydrate metabolisms, folding, sorting and degradation processes. The relative abundance of proteins was extensively different for cell grown in glucose compared with protein contents of cells grown in ethanol or acetic acid.

In conclusion, production of a cost effective vinegar starter needs a qualified biomass which tolerates ethanol and acetic acid. Tolerance of *A. senegalensis* to acetic acid depends to a great extent on the composition of the medium which cells grow in. In spite of low adaption to acetic acid for cell grown in glucose, using ethanol or acetic acid in inoculum media renders a physiological state in *A. senegelensis* which enables it to cope with

higher concentration of acetic acid readily, this biomass has a potential to be used as a starter.

Keywords: starter, 2-DiGE, respiration, membrane integrity, viability, Acetobacter senegalensis.

S4-O-05
CHEESE WHEY RECYCLING IN DAIRY FOOD CHAIN: EFFECTS OF VINEGAR FROM WHEY IN DAIRY COW NUTRITION

C. Belli,[1] E. Salimei,[2] G. Alfano,[1] G. Lustrato,[1] C. Maglieri,[2] F. Fantuz,[3] L. Grazia,[4] G. Ranalli[1*]

[1]DiSTAT, University of Molise, 86090 Pesche (IS) Italy; [2]DiSTAAM, University of Molise, 86100 Campobasso, Italy; [3]Dip. Scienze Ambientali, University of Camerino, 62024 Matelica, Italy; [4]DiPROVAL, University of Bologna, 40127 Bologna, Italy.

[*]Corresponding author: ranalli@unimol.it
Phone: +39 874404155.

Selected yeast (*Kluyveromyces marxianus* Y102 strain) and an acetic acid bacterium (*Acetobacter aceti*, DSM G3508 strain) were used as inocula respectively in cheese whey for alcoholic and acetic fermentations. The experimental tests were carried out at both laboratory and pilot plant (20 l and 2,000 l) levels. The data from the trials (working period 28 days) show increased ethanol production, increased acetic acid yield (whey vinegar = wheynegar), and greater fermentation stability with biomass recycling (18.6 g/l). Batch and feed-batch fermentation tests resulted in increased and standardized alcoholic fermentation, and allowed acetic acid recovery (average lactose consumption 56%, ethanol 6.7 g/l per day and acetic acid production 4.35 g/l per day). The effects of wheynegar administration were then investigated on milk yield and composition, nutritional status of dairy cows and physical characteristics of total mixed ration (TMR). Twenty Holstein cows were divided into two groups; group C, receiving the traditional TMR, and group W, receiving the TMR plus 10 l wheynegar. The dietary treatment, lasted 35 days, did not affect milk yield and composition except for the urea content, significantly lowered in group W. The selection of coarse (<19 mm), medium (8-19 mm) and fine (< 8 mm) dietary particles was not influenced by the wheynegar administration however a tendential lower selection against coarse particles was noted in W group. The results clearly highlight that microbial biotechnologies may significantly contribute to both the reduction of the polluting load of whey and the development of a stable nutrient recycling system within the dairy food chain.

Keywords: whey, microbial fermentation, wheynegar, dairy cow nutrition.

S4-O-06
INFLUENCE OF DIFFERENT HIGH-MOLECULAR WEIGHT LEVANS PRODUCED BY SELECTED ACETIC ACID BACTERIA ON THE VOLUME AND TEXTURE OF BREADS

F. Jakob,[1] H. Rübsam,[2] T. Becker,[2] A. Pfaff,[3] R. Novoa-Carballal,[3] A.H.E. Müller,[3] R.F. Vogel[1*]

[1]Lehrstuhl für Mikrobiologie Technische Universität München, Emil-Ramann-Str. 4, D-85354 Freising, Germany; [2]Technische Universität München, Lehrstuhl für Brauereitechnologie, Germany; [3]Universität Bayreuth, Lehrstuhl für Makromolekulare Chemie II, Germany.

[*]Corresponding author: rudi.vogel@wzw.tum.de
Phone: + 49 8161715169.

Acetic acid bacteria (AAB) are applied in several food fermentations like vinegar or kombucha mainly for their ability to synthesize flavouring acids. Some AAB strains are also known to produce exopolysaccharides (EPS) like gluconacetan or levan. Hydrocolloids such as polysaccharides can be used to improve the textural properties of bakery products acting as thickening and gelling agents. However, the potential of positive functional effects of novel EPS types produced by AAB strains for such applications has not been explored yet.
Therefore, we screened 21 AAB strains for their ability to produce hompolysaccharides from sucrose. *Gluconobacter sp.* TMW 2.767, *Gluconobacter cerinus* DSM 9533T, *Neoasaia chiangmaiensis* NBRC 101099 and *Kozakia baliensis* DSM 14400 synthesized high EPS yields ranging from 6-12 g/l in liquid gluconate media supplemented with 8 % (w/v) sucrose. The isolated, lyophilized EPS of each strain were characterized as β-(2→6)-linked fructofuranosyl polymers (levans) using HPLC and NMR (^{1}H, ^{13}C, HMQC, COSY) analysis and subsequently used as baking ingredients for wheat breads. The addition of each tested fructan in the lowest tested dosage (1% w/w flour) caused an increased volume and a softer crumb of fresh wheat breads. Furthermore, a retarded staling of the EPS breads was observed during 1-week storage. Since these positive functional effects were comparatively higher upon addition of EPS from *N. chiangmaiensis* and *K. baliensis*, we furthermore investigated the isolated polymer fractions of each strain in terms of their molecular weight distribution using asymmetric field flow fractionation (AF4). Whereas the isolated levan polymers of *G. frateurii* and *G. cerinus* had an average molecular mass (M_w) in the range of 10^7 Dalton (Da), fructans from *N. chiangmaiensis* and *K. baliensis* mainly consisted of fractions with a M_w of 10^8 and 10^9 Da, respectively. Conformation plots of all investigated levans revealed highly similar, continuously increasing slopes indicating highly similar distributions of branching. Combined with data of NMR analyses (no detectable signals indicating branching) we suggest that these fructans are linear. Consequently, linear levan fractions synthesized by the selected AAB strains caused increased volume, softer crumb and antistaling of wheat breads more effectively with increasing molecular weight. In conclusion,

we could demonstrate for the first time a novel application and structure/function relation of AAB EPSs in baking.

Keywords: acetic acid bacteria, levans, functional effects, breads.

S4-O-07
BREWAGE OF NOVEL MULTIFUNCTION CHINESE QUINCE VINEGAR

B. Gao,[1] L.P. He[2*]

[1]School of Biological and Pharmaceutical Engineering, Wuhan Polytechnic University, No.1 West Zhonghuan Road, Changqing Garden, Wuhan 430023, Hubei Province, P.R. China; [2]School of Life Sciences of Guizhou University, Huaxi, Guiyang 550025, Guizhou Province, P.R. China.

[*]Corresponding author: sh409gb@163.com

The demand for multifunctional vinegar is increasing nowadays. Based on the demand, in this study, a kind of novel multifunction vinegar was prepared rich in -aminobutyric acid and amino acids using Chinese quince and germinated rice as main raw materials through a series of processes of liquefaction, saccharification, alcohol fermentation and acetic acid fermentation. Pre-treatment of Chinese quince was conducted by pectinase and cellulose under the condition of time 8 h and temperature 55 °C. The liquefaction process of germinated rice was catalyzed by amylase at pH 7.5 and 92 °C for 30 min, followed by the saccharification 8h at pH 5.5 and 55 °C by addition of glucoamylase and pre-treatment of Chinese quince. Subsequently, alcohol fermentation was conducted by addition of active dry yeast at pH 4.5 to 5.5 and 38 °C for 60 h. Then, acetic acid fermentation was carried out by cycle liquid-solid process using its solution as reflux for 96 h. Thus, novel multifunction Chinese quince vinegar was obtained with the multifunctional ingredients of 3.7% acetic acid, 7 mg/100 mL γ-aminobutyric acid and 340 mg/100 mL amino acids through the series of processes above.

Keywords: vinegar, Chinese quince, germinated rice, -aminobutyric acid, cycle liquid-solid fermentation.

S4-P-01
BALSAMIC HONEY VINEGAR ELABORATION USING OAK, CHESTNUT, MULBERRY, CHERRY AND ASH CHIPS

I. Anton,[1] W. Tesfaye,[1*] A. Morata,[1] F. Palomero,[1] S. Benito,[1] J.A. Suárez-Lepe[1]

[1]Dept. Tecnología de Alimentos, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, Ciudad Universitaria S/N, 28040 Madrid, Spain.

[*]Corresponding author: wendu.tesfaye@upm.es
Phone: +34 913363987.

Vinegar may be defined as a condiment made from different sugary and starchy raw materials by alcoholic and subsequent acetic fermentation. Vinegar production ranges from traditional methods employing wood casks and surface culture to submerged fermentation in acetators. Actually the Honey market in Spain and Europe is loss making because of the cheapest imported honeys from the third countries. One of the main reasons for making honey vinegar is to provide an outlet for honey which would otherwise not profitable to the bee keepers. In many industries the margin of profit is realized by transforming such a material into sub products in this case to vinegar.
The balsamic honey vinegar samples are withdrawn periodically after submitting the cooked mead to alcoholic and acetic fermentation in an oak laboratory scale barrel. A blend of oak, chestnut, mulberry, cherry and ash chips filled in a plastic strainer, and was immersed periodically during the surface culture acetification process.
The evolutions of volatile compounds are monitored during 124 days. The analysis of variance pointed to a clear relationship ($p < 0.05$) between type of vinegar and volatile compounds content. During the acetification process methanol, diacetyl, acetoin, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-3, butanediol, hexanol and 2-Phenylethyl acetate increased their concentration. Meanwhile, ethanol, 1-propanolethyl acetate, isobutanol, ethyl lactate and isoamyl acetate decreased their concentrations. The level of furfural, furfuryl alcohol, 5-hydroximethyl furfural are higher in the Balsamic honey vinegar compared to vinegars elaborated without cooking the mead as it was expected. Indeed, whiskylactone, syringaldehyde and vainillin increase their concentration during aging either with chips or in barrel. The sensory analysis data revealed that the overall aroma and flavour intensity of this balsamic honey vinegar is higher than the rest of honey vinegars without the chips or those with only oak chips.

Keywords: balsamic honey vinegar, surface culture acetification, volatile compounds, sensory analysis.

S4-P-02
COINOCULATION OF ACETIC ACID BACTERIA IN MICROACETIFICATIONS OF STRAWBERRY WINE

D. García,[1] E. Mateo,[1] A. Mas,[1] M.J. Torija[1*]

[1]Departamento de Bioquímica y Biotecnología. Facultad de Enología. Universitat Rovira i Virgili. C/ Marcel•lí Domingo s/n. E- 43007 Tarragona, Spain.

[*]Corresponding author: mjesus.torija@urv.cat
Phone: +34 977558442.

Traditional vinegar is a process usually initiated by a vinegar mother, an undefined starter culture obtained from previous vinegar. But, this practice does not ensure a total control of the acetification or the quality of the product. For this reason, some studies have been carried out to test the possibility of using pure strains of acetic acid bacteria (AAB) as starters for the production of vinegar by traditional methods. These few studies have demonstrated the difficulty for a unique AAB strain to start and finish the process.

In previous studies, only one strain (SS8) of *Acetobacter malorum* was isolated during strawberry spontaneous acetifications and the use of this strain as pure starter showed a lack of imposition at the end of the process. Furthermore, a succession of species or genera has been reported in vinegar production, suggesting that a mixed inoculum of *Acetobacter* and *Gluconacetobacter* species could be used as a good starter culture.
Therefore, the aim of this study was to analyse the effect of coinoculation of two strains of different AAB species to produce strawberry vinegar. Different mixed cultures were tested by using the strain SS8 of *A. malorum* and another strain from *Gluconacetobacter europaeus*. These processes were compared with acetifications carried out with these strains inoculated separately. Microacetifications were performed in 250 mL-erlenmeyers with 100 mL of strawberry wine (6.5 % (v/v) Ethanol) and were considered finished when the acidity achieved 5.5-6% (w/v).
Microacetifications with mixed cultures finished in 12 days while, none of the microacetifications inoculated with only one strain achieved the expected acidity. Furthermore, important differences in the acetification behavior were observed depending on the strain tested.
Although more assays should be done, AAB coinoculation seems to be a good solution to improve the production of vinegars by traditional methods.

Keywords: mixed culture, Acetobacter malorum, Gluconacetobacter europaeus, surface acetification.

S4-P-03
DEPENDENCE OF VIABLE CELL CONCENTRATION ON OPERATIONAL VARIABLES DURING AN ACETIFICATION PROCESS IN A FED-BATCH PILOT FERMENTER

I.M. Santos-Dueñas,[1*] J.E. Jiménez-Hornero,[2]
A.M. Cañete-Rodríguez,[1] J.C. Mauricio,[3]
T. García-Martínez,[3] S. Baena-Ruano,[1] I. García-García[1]

[1]Department of Chemical Engineering, Marie Curie Building, University of Córdoba. Spain; [2]Department of Computing and Numerical Analysis, Leonardo da Vinci Building, University of Córdoba, Spain; [3]Department of Microbiology, Severo Ochoa Building, University of Córdoba, Spain.

*[*]Corresponding author: ines.santos@uco.es
Phone: +34 957218586.*

The operational variables in a fed-batch acetification fermenter can affect significantly the mean reaction rate as well as the final productivity. As it is known, different values in such variables can affect the mean values for ethanol and acetic acid concentrations. Considering the bacterial dependence on these compounds, it would be interesting to know how the bacterial cell concentration changes throughout the cycles. The comparison of this information with the bio-reaction rates could throw light on the complex influence of ethanol and acetic acid on both cell concentration and their activity.
In this work, in the scope of a study aimed to maximize the productivity of the acetic acid wine fermentation in a fed-batch pilot fermenter, the evolution of viable and total cell concentration have been found. The influence of three operational variables has been studied: ethanol concentration at unloading (E), unloaded volume (V) and loading flow rate (R). The variables ranged between 0.5-3.5% v/v, 25-75% of total volume and 0.01-0.06 mL·min^{-1} for E, V and R respectively.
The results show that mean values for bacterial cell concentrations in the cycles are not depending on the studied operational variables, nevertheless, instantaneous cell concentration profiles throughout the cycles could be quite dependent on the working conditions, particularly when different values for V are used. So, when V has a low value, a flat profile is obtained; on the other side, the unloading of 75 % of total volume results in an important variation on the instantaneous cell concentration throughout the cycle. Also, it is interesting to find that neither E nor R have significant influence on the instantaneous cell concentration profiles.
On the other hand, taking into account that the highest and the lowest bio-reaction rates are obtained when a low value of V is used, as well as high and low values of E respectively, it is clear that cells activity is quite dependent on the mean ethanol and acetic acid concentrations during the cycles.
Finally, the variation on the cells activity does not mean a similar change in cell viability, in fact, the percentage of cell viability has been found constant (roughly 92% of total cell concentration) and independent of the operating conditions.

Keywords: wine vinegar, acetic acid bacteria, viability, submerged culture, operational variables.

S4-P-04
EFFECT OF YEAST AND ACETIC ACID BACTERIA INOCULATION IN STRAWBERRY VINEGAR PRODUCTION BY TRADITIONAL METHODS

C. Hidalgo,[1] M.J. Torija,[1*] A. Mas,[1] E. Mateo[1]

[1]Departamento de Bioquímica y Biotecnología. Facultad de Enología. Universitat Rovira i Virgili. C/ Marcel•lí Domingo s/n. E- 43007 Tarragona, Spain.

*[*]Corresponding author: mjesus.torija@urv.cat
Phone: +34 977558688.*

Strawberry (*Fragaria ananasa*) vinegar production is a good alternative to take advantage of this seasonal fruit with important health benefits. The aim of this study was to analyze the effect of the inoculation on the production of strawberry vinegar using pure cultures of yeast and acetic acid bacteria (AAB) strains in order to improve the vinegar elaboration process. These strains were previously isolated from strawberry. Fruit vinegar was produced by a two-step bioprocess: alcoholic fermentation and acetification.
The alcoholic fermentations were inoculated with the strain RP1 of *Saccharomyces cerevisiae* in 100 L stainless vats. Sugars (120 g/L) from strawberry pulp concentrate yielded about 6.5% (v/v) of ethanol in 3 days. The inoculated strain took over the fermentation, being the only strain recovered and identified by molecular methods.

On the other hand, the acetification was conducted by traditional method in 60 L cherry barrels designed to increase the air-contact surface. A pure "vinegar mother" was produced using the AAB strain, SS8 of *Acetobacter malorum*. This vinegar mother was used to carry out the acetification process, which was performed in different steps, increasing the volume by strawberry wine addition when the titratable acidity reached 3% (w/v), till a final volume of 45 L. The final yield of acetic acid was 6.3% (w/v) after 44 days. The inoculated AAB strain was molecularly identified at the beginning of the process; however, two different genotypes (Ap1 and Ap2) of *Acetobacter pasteurianus* appeared in the middle of the process and one of them (Ap2) took over the acetification.
Results showed that the use of selected microbiota from strawberry allowed a successful process. Despite the AAB inoculation was not as expected, it allowed to shorten the length of the process.

Keywords: wine, Saccharomyces cerevisiae, Acetobacter malorum, starter cultures.

S4-P-05
KINETIC MODELLING OF ACETIC FERMENTATION OF ONION JUICE BY GENETIC ALGORITHMS

J.M. González-Sáiz,[1] C. Pizarro,[1] I. Esteban-Díez,[1] N. Pérez del Notario,[1] A. Sáenz-Mateo[1]

[1]*Department of Chemistry. University of La Rioja, C/Madre de Dios 51, 26006 Logroño (La Rioja), Spain.*

[*]*Corresponding author: josemaria.gonzalez@unirioja.es Phone: +34 941299634.*

Wastes and by-products of the onion-processing industry pose an increasing disposal and environmental problem and represent a loss of valuable sources of nutrients. Recycling, reprocessing and alternative utilization of onion processing residues offer potential of converting the waste materials into useful products of added value. The overall purpose of the study was to examine the feasibility of onion waste as a support-substrate for the profitable production of vinegar.
The basic tool to simulate the evolution of a bioprocess is the kinetic model. The model must be simple and have a high predictive ability to give results capable of explaining the real behaviour. The difficulties in the kinetic modelling of biological processes are mainly related to the description of the bacterial growth. In this work, a genetic algorithm is designed to obtain a set of kinetic parameters for the specific growth rate that enables the model to explain the industrial fermentation. Only acetic concentration data from pilot fermentator are required. The codification of the different kinetic parameters that form a chromosome in a binary one, the response chosen and the out-of-range parameters generated during the execution of the GA are critical points in the algorithm. A four-composed desirability function was developed as the evaluation function to minimized.

Keywords: acetic fermentation, onion juice, kinetic model, genetic algorithm.

S4-P-06
OXIDATION OF SUGARS AND POLYALCOHOLS BY ACETIC ACID BACTERIA DURING SURFACE CULTURE FERMENTATIONS

P. Giudici,[1*] M. Gullo,[1] L. De Vero[1]

[1]*Department of Agricultural and Food Sciences (DipSAA), University of Modena and Reggio Emilia, via Amendola 2 (Padiglione Besta), 42122 Reggio Emilia, Italy.*

[*]*Corresponding author: paolo.giudici@unimore.it Phone: +39 0522522034.*

Acetic Acid Bacteria (AAB) are well known for their ability to oxidize alcohols, aldehydes, sugars, polyalcohols and others molecules with ketonic or aldehydic functional groups. Species of *Acetobacter* and *Gluconobacter* lack a functional Embden-Meyerhof-Parnas pathway and are unable to metabolize hexose sugars by this route. Hexose and pentose sugars are oxidatively metabolized by the hexose monophosphate pathway to acetic and lactic acids. In some cases, hexose sugars may be directly oxidized to gluconate and ketogluconates without further catabolism, leading to an accumulation of these end products in the culture medium. Furthermore, *Gluconobacter oxydans* has been used to oxidize various sugars and sugar alcohols to substances of industrial significance, such as sorbose, gluconic acid and ketogluconic acids. Dihydroxyacetone, 2,3-butanediol, and acetoin are also significant products of carbohydrate metabolism evolved by AAB.
Our trials of surface static fermentation was carried out on complex media such as base wine (BW) for Traditional Balsamic Vinegar, where sugars and alcohols are present in different amount. In this conditions AAB sequentially oxidized the several carbon sources, first ethanol, then glucose and glycerol. In particular the oxidation of glucose to gluconate occurred when the ethanol was exhausted, then high amount of gluconate was accumulated in the medium. BW had an initial composition of around 7% (v/v) of ethanol and 25% of sugars with glucose and fructose in the ratio 1/1. At the end of the fermentation the glucose/fructose ratio was in favour of fructose, titratable acidity was very high due to the gluconate formed, while volatile acidity decreased. In summary, when surface fermentation is extended afterward the ethanol run out, other substrates are oxidized and give origin to a vinegar with a completely different composition. The extension of the oxidative step is a tool for increasing vinegar's sweetness and acidity without the acetic acid sensorial pungency.

Keywords: gluconic acid, sugar oxidation, surface static acetification.

S4-P-07
POLYNOMIAL MODELLING FOR THE OPTIMIZATION OF ACETIC ACID WINE FERMENTATION IN A FED-BATCH PILOT FERMENTER

I.M. Santos-Dueñas,[1*] J.E. Jiménez-Hornero,[2] A.M. Cañete-Rodríguez,[1] S. Baena-Ruano,[1] D. Cantero-Moreno,[3] I. García-García[1]

[1]Department of Chemical Engineering, Marie Curie Building, University of Córdoba, Spain; [2]Department of Computing and Numerical Analysis, Leonardo da Vinci Building, University of Córdoba, Spain; [3]Department of Chemical Engineering. University of Cadiz, Spain.

[*]Corresponding author: ines.santos@uco.es
Phone: +34 957218586.

The operational complexity in biological industries demands for models which facilitate the optimization and control of their processes. Throughout the years many models, particularly deterministic semi-structured ones, have been proposed to describe experimental evidence on the influence of diverse variables of the process. However such a modelling approach makes difficult to succeed because of the complex interrelations between the different variables as well as the arisen mathematical problems. Indeed, this type of biotechnological models are difficult to solve because of the high-order non-linear multi-parametric equations systems as well as the variation in experimental data, both hinder the estimation of the system parameters. When this model-based approach is followed, one of the first problems to solve is then ascertain whether the parameters concerned can be unambiguously determined; this is a very complex analysis known as study of "identifiability" that rarely gives a positive answer for biological models. In this way, no meaningful physical conclusions can be drawn from the parameters – main advantage for these models.
For these reasons, different alternatives are considered in order to modelling the process at the same time that the minimization of the costly experimental work is looked for. Among these methods, stands out the response surface methodology by quadratic models. This alternative has been successfully used for the optimization of fermentation processes.
In this work, a 8 L Frings pilot fermenter working in fed-batch mode has been used for studying the influence of the main operational variables: ethanol concentration at unloading time (E), ranging between 0.5-3.5% v/v, unloaded volume (V), ranging between 25-75% of total volume and loading flow rate (R), ranging between 0.01-0.06 $mL \cdot min^{-1}$. As objective functions, mean acetification rate (A) and productivity (P) have been evaluated. After using a second order polynomial and a Doehlert design for three variables, next conclusions can be drawn: both acetification rate and productivity are mainly depending on E and V, their maximum values are reached for low values of V and high ones for E.

Keywords: wine vinegar, acetification.

S4-P-08
PRELIMINARY EVALUATION OF HONEY VINEGAR PRODUCTION IN A SUBMERGED CULTURE FED-BATCH PILOT FERMENTER

A.M. Cañete-Rodríguez,[1*] I.M. Santos-Dueñas,[1] J. Peinado-Peinado,[2] J.M. Flores-Serrano,[3] F. Padilla-Álvarez,[3] I. García-García[1]

[1]Department of Chemical Engineering, Marie Curie Building, University of Córdoba, Spain; [2]Department of Biochemistry, Severo Ochoa Building, University of Córdoba, Spain; [3]Department of Zoology, Charles Darwin Building, University of Córdoba, Spain.

[*]Corresponding author: b42carom@uco.es
Phone: +34 957218586.

The honey is an important product for the European Union (EU) from both the consumption and production points of view. Approximately, EU consumes 22% of the world's annual production and its own production accounts for 13% of the global one, but in any case, only produces around half the honey it consumes. Inside EU the leading markets are: Germany, Spain, UK, France, Italy and The Netherlands, but the main producer is Spain. Among the world's leading producers, Spain is in the thirteenth place. Though with large fluctuations, Spanish honey production has increased in roughly a 30% since 1990, amounting to 32 thousand tonnes in 2009 (FAOSTAT). Then, the previous figures, beside the fact that honey is a natural product which complies with many food trends such as health, taste and convenience, make honey and its by-products an important issue for this country.
Though an estimated 85% of all honey is for direct consumption, other uses for the rest, such as raw material for mead and subsequent biotransformation, could be profitable alternatives for using the honey which does not comply with the legislative requirements for a direct use. For instant, honey containing a Hydroxymethylfurfural (HMF) concentration higher than 40 mg/kg cannot be marketed for direct consumption, only industrial use is allowed. At this point, a study on different options to appreciate as much as possible this "residual" honey seems to be quite interesting. Honey vinegar might stand out among the possible alternatives. This condiment could have a high added value beside many of the desired features found in the original honey. In this work the behaviour of previously produced mead [9.4±0.2%(v/v) ethanol concentration] has been studied as a raw medium for vinegar elaboration; a fed-batch process in submerged culture similar to that used in other industrial types of vinegars has been followed: 31°C, aeration rate of 7.5 $L \cdot L^{-1} \cdot h^{-1}$, unloading 50% of total volume at the end of each cycle when ethanol concentration reached a value of 1%(v/v) and a loading stage to the final working volume (8 L) without exceeding a preset ethanol concentration [4% (v/v)] at any time. The low nitrogen content in honey entails the addition of some nitrogen sources previously the alcoholic fermentation stage, apart from that the mead is easily produced. The acetification results show that an stable process can be obtained with an acetification rate of 0.15±0.01%(v/v)$\cdot h^{-1}$ and a productivity of 10.3±0.2 g acetic acid$\cdot h^{-1}$, further optimization could improve these figures.

Keywords: honey, mead, acetification, honey vinegar.

S4-P-09

PRODUCTION OF STRAWBERRY VINEGAR AS A BASE PRODUCT IN THE DEVELOPMENT OF NEW HEALTHY BEVERAGES

A.M. Cañete-Rodríguez,[1] I.M. Santos-Dueñas,[1] J.L. Bonilla-Venceslada,[1] J.E. Jiménez-Hornero,[2] I. García-García[1*]

[1]Department of Chemical Engineering, Marie Curie Building, University of Córdoba, Spain; [2]Department of Computing and Numerical Analysis, Leonardo da Vinci Building, University of Córdoba, Spain.

[*]Corresponding author: isidoro.garcia@uco.es Phone: +34 957218589.

Modern societies are not only concerned with the production of high quality traditional foods but with the development of new healthy products. On the other hand, many developed countries have important levels of different foods productions as well as very restrictive regulations for obtaining prime commercial quality. Then, important amounts of many products can be qualified as lower quality category, increasing in this way, the risk of becoming wastes, an example is strawberry. Spain is the third main strawberry producer in the world after United States and Turkey (FAOSTAT, 2009); an estimation of 20% of the whole production can be lost unless used as raw material for other uses despite of being a percentage of healthy and nutritionally valuable fruit. The development of strawberry based new fermented beverages is an interesting alternative for using these leftovers with benefits from the commercial and nutritional points of view. The oxidation capacity of acetic acid bacteria on different type of substrates make them key microorganisms for taking advantage of this surplus fruit. In this work, some basic aspects regarding the production in submerged culture of strawberry vinegar are considered; this vinegar would be mixed with other strawberry fermented products for obtaining innovative beverages.

The main aim is evaluate, at bench scale, the possibilities of developing the two staged bio-transformation process: alcoholic fermentation followed of acetification. Different concentrated strawberry pulps with total sugars concentrations ranging between 30 - 100 gL^{-1} are used. Preliminary studies show that a concentrated pulp with a sugar concentration around 55 g L^{-1} may yields a final acetic acid concentration (25 g L^{-1}) suitable for the subsequent mixtures to prepare; additionally, its viscosity and emulsifying features can be properly dealt with inside the bioreactor. The alcoholic fermentation by *Saccharomyces cerevisiae* yields 25 g of ethanol L^{-1} approximately in less than 24 hours. Once finished the alcoholic fermentation, the conversion of ethanol into acetic acid was carried out in the same bioreactor; an inoculum from a Frings acetator producing alcohol vinegar supplied a fully active mixture of acetic acid bacteria for the process. Though a lag phase is observed, this procedure guarantees that in less than 70 hours all the ethanol is eliminated. In collaboration with other research groups, the behaviour of inoculating specific acetic acid bacteria strains is being studied.

Keywords: strawberry, vinegar, submerged culture, new beverages.

S4-P-10

SMALL SCALE CULTIVATION OF ACETIC ACID BACTERIA FOR VINEGAR PRODUCTION

T. Schlepütz,[1*] J. Büchs[1]

[1]Aachener Verfahrenstechnik - Biochemical Engineering, RWTH Aachen University, Germany.

[*]Corresponding author: tino.schlepuetz@avt.rwth-aachen.de Phone: +49 02418027179.

Fermentations for the production of vinegar by acetic acid bacteria are typically initiated with inoculation vinegar - undefined residues of former fermentations. These bacteria are obligatory aerobic and have to survive at high concentrations of ethanol and acetic acid in the vinegar production. Already short interruptions of the oxygen supply lead to severe harm of the bacteria in submerged fermentation. Hence, typical inoculation cascades are not possible with these bacteria, so that vinegar is industrially produced in repeated batch processes. Furthermore, isolation and preservation of acetic acid bacteria from vinegar is only possible with much effort in diluted media. Accordingly, studies on the production of vinegar are generally done in fully instrumented lab scale bioreactors. This hampers efficiency and throughput of investigations on that process.

In this contribution we present the scale down of the fermentation for spirit vinegar production into milli- and microliter scale shaken cultures. Realizing a high degree of parallelization, less workload and high throughput are achieved. Typical inoculation procedures of shake flask cultures by pipetting result in lack of reproducibility of the bacterial activities. This was demonstrated by respiration measurements in a RAMOS® device. Good reproducibility of respiration curves was achieved with an aerated transport of the inoculum by a bubble column during the inoculation of the shaken cultures.

Vinegar fermentation was examined in microtiter plates. The extraordinary low aeration rates of conventional bioreactors for vinegar production were mimicked by a customized lid. The design of that lid was guided by a diffusion model. The fermentations were then monitored using microtiter plates with optodes for dissolved oxygen tension and the BioLector® device.

Finally, a method for the repeated batch operation in special shake flasks of the COSBIOS® device was developed. These flasks are characterized by a precise outlet at the reactor wall so that in shaken operation culture broth can leave the reactor if fresh medium is supplied. A flushing method was applied which matched the draining and filling phase of repeated batch fermentations in conventional bioreactors. With the help of semiconductor ethanol sensors in the head space of the COSBIOS® reactors to monitor the fermentation and to initiate the medium exchange parallel repeated batch fermentation for the production of vinegar could be established.

Keywords: small scale cultivation, microtiter plate, shake flask, vinegar, parallelization, repeated batch.

S4-P-11
STUDY OF THE VOLATILE AND POLYPHENOLIC CONTENT OF A SHERRY WINE VINEGAR ENRICHED WITH DIETARY FIBRE

A. Marrufo,[1] E. Durán,[1] M.J. Cejudo,[1] R. Castro,[1*] R. Natera,[1] C.G. Barroso[1]

[1]*Analytical Chemistry Department, Faculty of Sciences-CAIV. University of Cádiz. Agrifood Campus of International Excellence, Spain.*

[*]*Corresponding author: remedios.castro@uca.es Phone: +34 956016456.*

Nowadays, the beverage market is turning towards diversification of traditional beverage categories, looking for different and improved sensory properties in relation to the conventional products. Moreover, the development of new products with functional properties is becoming an incentive to raise consumers' consciousness about their consumption. In this context, several experiences of enrichment of a Sherry wine vinegar with dietary fibre from orange and lemon have been carried out. Different doses of lemon and orange fibre have been studied (0-10 g/L). Two storage conditions were studied: room temperature (25°C) and 4°C. Polyphenols were analysed by UPLC-DAD whereas volatile compounds were determined by SBSE-GC-MS. Both methodologies had been previously optimizated and validated. Enriched vinegars exhibited higher polyphenolic contents. Naringin, narirutin and neohesperidin were only found in the enriched vinegars. For volatile compounds, some new compounds were also found in the enriched samples, standing out valencene and limonene for vinegars enriched with orange and lemon fibres, respectively. Both facts influenced positively on the final quality of new products.

Keywords: sherry vinegar, dietary fibre, polyphenols, volatile compounds, new products.

S4-P-12
SUBMERGED CULTURES OF *GLUCONOBACTER GENUS* IN STRAWBERRY PULP FOR MAKING A GLUCONIC ACID BASED PRODUCT. AN INGREDIENT FOR NEW HEALTHY BEVERAGES

A.M. Cañete-Rodríguez,[1] I.M. Santos-Dueñas,[1] J.C. Mauricio,[2] C. Martínez-Pedrajas,[1] I. García-García[1*]

[1]*Department of Chemical Engineering, Marie Curie Building, University of Córdoba, Spain;* [2]*Department of Microbiology, Severo Ochoa Building, University of Córdoba, Spain.*

[*]*Corresponding author: isidoro.garcia@uco.es Phone: +34 957218589.*

In the scope of the development of new healthy foods at the same time that a proper use of the overproduction of some fruits is made, in this work, a study about the production of gluconic acid from concentrated strawberry pulp in submerged culture is carried out.

The resulting product would be used as a base product for mixing with other fermented products into new potentially healthy beverages. In other communication (*Production of strawberry vinegar as a base product*...) authors refer to the production of strawberry vinegar with the same aim. The whole project is the resulting effort with two additional complementary research groups (Microbiology: Group Oenological Biotechnology, University Rovira i Virgili - URV; and Chemical and Sensory Analysis: Group AGR-167, University of Sevilla; both in Spain). The project looks for a new beverage with all the original health properties from the strawberry and a complex equilibrium between acetic and gluconic acids as well as a natural sweetness from residual fructose during the gluconic acid fermentation. Then, the biotransformation by a microorganism that uses mainly glucose and no fructose is a key issue.

In this work, the behaviour in a 5 L fermenter (Sartorious) of two *Gluconobacter japonicus* strains (F9 and E1) selected by the URV Group, is studied. Taking into account the final intended use as well as that the strawberry pulp was pasteurised during the production process, no sterilization operation was carried out; then, contamination by other microorganisms, particularly yeast, must be checked. An adequate pulp concentrate must be used in order to avoid emulsion problems if no pectolytic enzyme preparation is added. So, the proper strawberry pulp has a total sugars content of 55 g L^{-1} approximately. Previous results show that both strains produce a gluconic acid concentration of roughly 20 g L^{-1} in 26 hours. During this period also fructose consumption is observed but this is lower for the strain E1, additionally, no yeast growth was observed until the glucose and most of the fructose are exhausted. For the strain F9, final ethanol concentrations around 1% (v/v) were founds, this suggest some yeast activity which is confirmed by the increasing in yeast cell concentration.

The obtained results for strain E1 suggest that this microorganism might be able to use only glucose. The effect of several operational variables must be studied in order to find the optima conditions for a selective use of glucose.

Keywords: strawberry, gluconic acid, submerged culture, new beverages.

S4-P-13
USING A SERIES OF TWO FED-BATCH PILOT FERMENTERS FOR OPTIMIZING THE WINE VINEGAR PRODUCTION PROCESS

C. Álvarez-Cáliz,[1*] I.M. Santos-Dueñas,[1] A.M. Cañete-Rodríguez,[1] M.C. Millán-Perez,[2] J.L. Bonilla-Venceslada,[1] I. García-García[1]

[1]*Department of Chemical Engineering, Marie Curie Building, University of Córdoba, Spain;* [2]*Department of Microbiology, Severo Ochoa Building, University of Córdoba, Spain.*

[*]*Corresponding author: q42alcac@uco.es Phone: +34 957218586.*

In the scope of the optimization of the wine vinegar pro-

duction, the authors have carried out a vast experimental work previously to several modelling approaches aimed to maximise acetification rate as well as productivity.
If just one fermenter is used, simulations by different models coincide in the impossibility of getting high rates and productivities values when a maximum use of substrate is also looked for. Since, for many industries, the proper use of the substrate as well as obtaining high productivities are issues of great importance from an economical point of view, it would be desirable to find an operational mode able to achieve both objectives simultaneously. Among the possible alternatives, stands out the use of a series of two fermenters by which the second one could ensures the ethanol exhaustion without hindering the possibility of reaching a global high productivity. Indeed, meanwhile the operational variables in the first fermenter would offer a proper environment for bacteria to work in optimal conditions, the unloaded medium - containing an important ethanol concentration, will be depleted in the second one.
The independent operational variables to be considered in the first fermenter are: maximum ethanol concentration to be reached at any time (Ec1), ethanol concentration at unloading time (Ed1), unloaded volume (Vd1) and working temperature (T1). On the other side, for the second fermenter the variables are: maximum ethanol concentration to be reached at any time (Ec2) and temperature (T2).
The number of operational variables involved in each fermenter as well as the relationship among some of them because of the physical connection between both bioreactors, entail a complex modelling approach for allowing the optima conditions to be found. In this case, a response surface methodology by quadratic models and a fractional Doehlert design for six variables have been used. Setting the ranges of variation for Ec1, Ed1, Vd1 and Ec2 involve a previous complex analysis the result of which is: Ec1 [6-4%(v/v)], Ed1 [2-4%(v/v)], Vd1 [4.25-5.75 L] and Ec2 [2.5-4.5%(v/v)]. Additionally, T1 and T2 ranged between 28-32°C.
The model predictions suggest that maximum productivity is obtained when low and high temperatures are set in the first and second fermenter respectively; additionally, low values of Vd1 and high ones for Ed1, Ec1 and Ec2 must simultaneously be used.

Keywords: wine vinegar, acetification.

SESSION V. How do we know what they are up to? Measurement techniques. Biosensors. Quality control. Other aspects.

S5-KN
CHEMICAL CHARACTERIZATION AND HEALTH CLAIMS AND AAB FERMENTED PRODUCTS

M.C. García-Parrilla,[1*] A.B. Cerezo,[1] W.M. Tesfaye,[2] A.M. Troncoso-González[1]

[1]Nutrition and Food Science. Faculty of Pharmacy, University de Sevilla, Spain; [2]Food Technology, Politechnical University of Madrid, Spain.

[*]Corresponding author: mcparrilla@us.es
Phone: +34 954556760.

The diversity of fermented products is increasing to satisfy the requirements in the market. The innovations focus on the development of vinegars with high sensorial quality. Additionally to those vinegars protected by a Denomination of Origin, the challenge is to offer original vinegars elaborated from uncommon substrates as honey, strawberries or other fruits.
Chemical characterization is a powerful tool to establish quality control. It permits to discriminate vinegars for authentication purposes, useful for the Denominations of Origin. The elaboration processes (fermentation or aging) play a crucial role on chemical composition. New strategies of production must consider these aspects to ensure high quality products. More recently, health claims of food or food ingredients is an outstanding topic in the EU. Therefore, the assessment of bioactive compounds is a new feature required.
This communication presents more relevant results of the research group in the authentication of vinegars differentiating products by the substrate, elaboration method involved or aging. Special attention was devoted to Sherry vinegars.
Additionally more recent work aims to study innovative vinegars elaborated in acacia, oak, cherry or chstnut woods thanks to the Winegar European Project.
Polyphenolic compounds are a useful tool to characterize vinegars. Criteria to discriminate these products according to their production area, fermentation process or aging period were achieved.
The identification of polyphenolic compounds with antioxidant properties as (+)-taxifolin or dihydrorobinetin is also presented.

Keywords: vinegar, polyphenol, aging, wood, authentication.

S5-O-01
USE OF DENATURING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR RAPID DETECTION AND IDENTIFICATION OF ACETIC ACID BACTERIA

N. Sagarzazu,[1*] M. Martinez,[1] R. Virto,[1] C. Gonzalez[1]

[1]CNTA (National Centre for Technology and Food Safety), Spain.

[*]Corresponding author: nsagarzazu@cnta.es
Phone: +34 948670159.

Modern vinegar production is carried out using a standard and automated process technology. However, the microbiology of vinegar fermentation is not adequately developed with regard to a thorough knowledge of the taxonomic-systematic position of the organisms involved, their response to ecological factors, their physiology and genetics. The lack of defined pure cultures is due to problems in strain isolation, cultivation and preservation of the vinegar bacteria. Numerous strategies have been used for microbial community analysis, including traditional culture methods, microscopic techniques and nucleic-acid based methods. The identification methods based on analysis of the phenotypic characteristics of the acetic acid bacteria, are not only inaccurate, but also very time-consuming. Therefore, the application of molecular methods, based on the identification/characterization of specific DNA segments, could be a proper solution for the quick and accurate identification of these microorganisms. Denaturing high-performance liquid chromatography (DHPLC) is a new and promising approach for bacterial community analysis, monitoring and identification. This technique has primarily used to detect DNA sequence variations such as insertions, deletions and SNPs in various applications such as clinical diagnostics, gene cloning and microbial investigations. The major advantages of the DHPLC method for community analysis compared with other molecular-based techniques include the use of automated instrumentation, high sensitivity and less laborious and time-consuming operations. DHPLC is used in this study to discriminate genetic differences in the 16S rRNA and Adh (Alcohol dehydrogenase) genes among bacterial species based on its efficiency and sensitivity to enable the detection and discrimination of different genetic sequences. To optimize DHPLC protocols for the analysis of 16S rRNA gene fragments amplified we analyzed DNA isolated from 10 different reference strains representing main bacterial groups of interest (pathogens and spoilage microorganisms, lactic and acetic acid bacteria). Variations of technical DHPLC parameters (acetonitrile gradient, column temperature and flow rate) resulted in a protocol that allowed effective and reproducible separation. For the discrimitation of different strains of *Acetobacter malorum* (DSM 14337 as reference strain and two wild strains) the Adh gene were used.

Keywords: denaturing high performance liquid chromatography (DHPLC), acetic acid bacteria identification, vinegar fermentation.

S5-O-02
SENSORY ANALYSIS OF SHERRY VINEGARS ENRICHED WITH DIETARY FIBRE

A. Marrufo,[1] M.C. Rodríguez,[1] E. Durán,[1*]
M.J. Cejudo,[1] R. Castro,[1] R. Natera,[1] C.G. Barroso[1]

[1]Analytical Chemistry Department, Faculty of Sciences-CAIV. University of Cádiz. Agrifood Campus of International Excellence, Spain.

[*]Corresponding author: enrique.duranguerrero@uca.es
Phone: +34 956016456.

Nowadays, oenological industry is turning to the development of new products with healthy benefits due to the increasing consumers' demand. In addition, these new products should present positive organoleptic properties in order to assure the acceptance from consumers. Therefore sensory analysis of new products is an essential step prior to their possible commercialisation.
In this research, a new type of Sherry vinegar enriched with different dietary fibres (orange and lemon) has been developed and sensory analysed. Different amounts of fibre (0-10 g/L) and storage temperatures (25°C and 4°C) have been studied, and an overall olfactory and gustatory analysis of the vinegars enriched with fibre has been carried out. Moreover, the effect of storage temperature, type of added fibre and amount of fibre on sensory characteristics of the developed vinegars has been studied.
The addition of fibre to the vinegars increased both olfactory and gustatory impressions, so enriched vinegars presented better sensory characteristics.
No preference between both types of fibres was observed. Neither storage temperature was a significant parameter on sensory preference, so the cheapest storage should be preferred, i.e. storage at room temperature. In relation to amount of fibre, 0.5 g/L for lemon and 5 g/L for orange fibre were the optimal values taking into account olfactory and gustatory impression.

Keywords: sherry vinegar, dietary fibre, sensory analysis, new products.

S5-O-03
CHANGES IN AMINO ACIDS CONTENT DURING THE PRODUCTION OF STRAWBERRY AND PERSIMMON VINEGARS

R.M. Callejón,[1] C. Ubeda,[1*] C. Hidalgo,[2] E. Mateo,[2]
A.M. Troncoso-González,[1] M.L. Morales[1]

[1]Área de Nutrición y Bromatología. Facultad de Farmacia. Universidad de Sevilla. C/ P. García González nº2, E- 41012 Sevilla, Spain; [2]Departamento de Bioquímica y Biotecnología. Facultad de Enología. Universitat Rovira i Virgili. C/ Marcel•lí Domingo s/n. E- 43007 Tarragona, Spain.

[*]Corresponding author: c_ubeda@us.es
Phone: +34 954556761.

The evolution of the amino acids and ammonium contents during the production of strawberry and persimmon vinegars was investigated. Different conditions were tested at each stage along the production of these vinegars. A total of 22 amino acids and ammonium were determined by high-performance liquid chromatography (HPLC), employing 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) as precolumn derivatization reagent.
The major amino acids found in strawberry puree were

asparagine, glutamine and proline, while in persimmon were -aminobutyric acid, glutamine, threonine and tyrosine. Although some amino acids increased during the acetifications, many of them were consumed by acetic bacteria. Some differences between inoculated and spontaneous acetifications were observed respect to the most consumed amino acids, which were, in most cases, the most abundant in wine substrate.
GABA and Pro were the major amino acids in final persimmon vinegars. However, there were differences regarding the major ones in the strawberry vinegars. Hence, GABA and Pro were the most abundant compounds in 2008 strawberry vinegars obtained from inoculated wines, Cys in those from spontaneous wines, and Arg in strawberry vinegars from 2009 and 2010 harvests.
The Principal Component Analysis reveals that samples of the same harvest can be separated in substrates, wines and final vinegars by using amino acid compounds as variables. In addition, this statistic analysis allowed us to separate the final vinegars according to the type of fruit, year of harvest and fresh fruit or commercial puree.

Keywords: amino acid, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, strawberry vinegar, persimmon vinegar, acetification.

S5-P-01
CHARACTERIZATION OF COMMERCIAL VINEGAR OF MODENA BY MULTIVARIATE ANALYSIS

M. Labbé,[1] P. López-Colom,[1] F. López[1*]

[1]Departament d'Enginyeria Química, Facultat d'Enologia, Universitat Rovira I Virgili, Av. Països Catalans 26, 43007 Tarragona, Spain.

[*]Corresponding author: francisco.lopez@urv.cat
Phone: +34 977558503.

In recent years consumption of Italian vinegar of Modena has increased in Spain. Under the generic name of balsamic, this type of vinegar represents 7.7% of the volume of all sales and 37.7% of the value. In the Spanish market there are two kinds of Modena vinegar: bottled in origin and bottled in Spain.
This study analyzes the differences between various commercial vinegars of Modena with respect to their sugar, alcohol and organic acid composition. To do so, statistical analysis is applied using the technique of principal components and discriminant analysis.
Forty-five samples of industrial balsamic vinegars of Modena have been used. They were purchased in local markets in Catalonia (Spain). The compounds determined by HPLC were: glucose, fructose, sucrose, glycerol, ethanol, methanol, acetic acid, citric acid, tartaric acid, malic acid, succinic acid and lactic acid. The statistical package SPSS (version 14.0) was used to carry out principal components analysis (PCA) and discriminant analysis.
On the basis of the concentrations of some organic acids-tartaric, citric, malic, lactic and succinic-Modena vinegars can be identified as belonging to a group from a particular producer.
Principal component analysis and discriminant analysis make it possible to distinguish two types of vinegars: one group that presents a lower concentration of citric acid and tartaric acid and a higher concentration of succinic acid, and another group that has a higher concentration of citric acid and tartaric acid and a lower concentration of succinic acid.

Keywords: Modena vinegar, organic acid composition, multivariate analysis.

S5-P-02
DETERMINATION OF BIOGENIC AMINES IN VINEGARS USING SOLID-PHASE EXTRACTION AND 6-AMINOQUINOLYL-N-HYDROXYSUCCINIMIDYL CARBAMATE AS DERIVATIZING AGENT

J.L. Ordóñez,[1*] R.M. Callejón,[1] M.L. Morales,[1] M.C. García-Parrilla[1]

[1]Área de Nutrición y Bromatología. Facultad de Farmacia. Universidad de Sevilla. C/ P. García González nº2, 41012 Sevilla, Spain.

[*]Corresponding author: josorddia@alum.us.es
Phone: +34 954556761.

Biogenic amines ingested in high amounts can have toxicological effects and can cause problems in consumers. These have been reported in a wide range of food products including wines, but the determination of biogenic amines has not been deeply studied in vinegars. In this study, main amines were determined by high-performance liquid chromatography (HPLC) after derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) in different types of vinegars: balsamic, apple, and red, white, and Sherry wine vinegars. A solid-phase extraction (SPE) with mixed-mode resins method was used before analysis. The total content of biogenic amines in vinegars ranged from 0.023 mg/L to 1.445 mg/L, being lower than those found in wines. Although Cadaverine was present in a higher number of samples, Putrescine followed by Histamine were the amines that showed highest concentrations, reaching values up to 0.525 and 0.309 mg/L, respectively. However, methylamine and phenylethylamine were not determined in any vinegar. Balsamic and "Pedro Ximenez" Sherry vinegars reached the highest amounts of biogenic amines. On the other hand, apple, white and Sherry wine vinegars had the lowest concentrations. Principal component analysis using the biogenic amines as variables, allowed to separate the different kind of vinegars, excepting red vinegars.

Keywords: HPLC, AQC, solid-phase extraction, biogenic amines, vinegar.

S5-P-03
EFFECTS OF SACCHARIFICATION METHODS ON THE ORGANOLEPTIC PROPERTIES OF RICE VINEGAR

A. Mori,[1*] S. Shiratori,[2] N. Nakamura[3]

[1]Mori Akihiko Consulting Engineers Office, Japan; [2]Niigata University, Japan; [3]Kewpie Jyozo Co.Ltd, Japan.

*Corresponding author: stephano.mori@nifty.ne.jp
Phone: +81 426767020.

Vinegar is one of oldest brewed seasoning from racial favorite drinks. Rice vinegar is made from Japanese sake in Japan and its saccharification is used koji mold or enzyme (amylase). We wished to find out difference of taste among the effects of the saccharification methods,.by chemical analyses and organoleptic tests.

Rice vinegar used in these tests were acetified with Acetobacter aceti IFO 3283 strain. Analytical methods used were chemical analyses [total acid, nonvolatile acid, ethyl alcohol, direct producing sugar, soluble solid, protein, folmol nitrogen, total nitrogen, pH, optical density, common salt, total phosphorus], organic acid (by HPLC) flavoring substance (by GC)], and organoleptic tests namely pungent, thickness (strong or weak), refreshing, sweetness, sourness, synthetic favorite.

Multiple regression equation was gained. Level of significance was less than 1%, R^2 was 93%, analysis of variance was less than 1%. Results of factor analysis among difference of saccharifying material gave little effects. As the results, difference of sacchrification method gave significant effects.

Keywords: Acetobacter aceti, organoleptic test, rice vinegar, saccharificaion.

S5-P-04
EVALUATION OF BIOGENIC AMINES IN PRODUCTS OBTAINED BY ALCOHOLIC AND GLUCONIC FERMENTATION OF STRAWBERRIES

J.L. Ordóñez,[1*] M.A. Álvarez-Fernández,[1]
R.M. Callejón,[1] M.L. Morales,[1] M.C. García-Parrilla[1]

[1]Área de Nutrición y Bromatología. Facultad de Farmacia. Universidad de Sevilla. C/ P. García González nº2, 41012 Sevilla, Spain.

*Corresponding author: josorddia@alum.us.es
Phone: +34 954556761.

Biogenic amines were determined in different alcoholic and gluconic fermentations of strawberries purees by high-performance liquid chromatography (HPLC) using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) as derivatization agent. A solid-phase extraction (SPE) with mixed-mode resins method was used before analysis. This resin is composed of reverse-phase (C18) and ion exchange sorbents (sulfonic groups). The method was successfully validated obtaining adequate values of selectivity, response linearity, precision, accuracy, and low detection and quantification limits. The total content of biogenic amines in samples ranged from 0.115 mg/L to 0.237 mg/L, being lower than those found in others fermentation products. Most of biogenic amines such as histamine, agmatine, methylamine, tyramine, phenylethylamine and spermidine were not detected in any alcoholic and gluconic products. Only spermine was present in all samples reaching the highest concentrations. Some differences were observed in the gluconic products. Hence cadaverine was only present in those samples fermented by acetobacter strains.

Keywords: HPLC, AQC, solid-phase extraction, biogenic amines, strawberry.

S5-P-05
STUDY OF THE VOLATILE PROFILE OF HIGH QUALITY VINEGARS BY STIR BAR SORPTIVE EXTRACTION

A. Marrufo,[1] M.J. Cejudo,[1] E. Durán,[1] R. Castro,[1]
R. Natera,[1*] F. Chinnici,[2] C.G. Barroso[1]

[1]Analytical Chemistry Department, Faculty of Sciences-CAIV. University of Cádiz. Agrifood Campus of International Excellence, Post Office Box 40, Pol. Río San Pedro, Puerto Real 11510, Cádiz, Spain; [2]Food Science Department, University of Bologna, Viale Fanin 40, Bologna 40127, Italy.

*Corresponding author: ramon.natera@uca.es
Phone: +34 686121125.

A stir bar sorptive extraction gas chromatography-mass spectrometry (SBSE-GC-MS) method has been used for the characterization of the volatile composition of 26 high quality vinegars of three different protected geographical indications (traditional balsamic vinegar of Modena, balsamic vinegar of Modena, and Sherry vinegar).

In this research work, a total of 113 individual volatile compounds have been identified in different vinegars by SBSE-GC-MS. Some of the studied compounds have been previously identified in wine but 39 tentatively identified volatile compounds have not been previously reported in vinegars from this origin.

From the results obtained, it can be seen that the numerous volatile compounds identified in vinegar samples have allowed an optimal differentiation of the vinegars studied on the basis of their raw material, aging time and aging type. So, a close relationship can be established between these last ones and the volatile content of vinegar, which undergoes significant changes during the aging in wood. The analytical parameters selected are suitable descriptors to differentiate vinegar samples according to the raw material employed and the aging in wood. Furanes and terpenes together with acids and alkanes were the most discriminant variables for raw material and aging time, respectively, whereas for aging type, these ones were acetates and esters.

Keywords: traditional balsamic vinegar of Modena, balsamic vinegar of Modena, sherry vinegar, volatile compounds, SBSE.

S5-P-06
VINEGAR CLASSIFICATION BASED ON UV-VIS AND NIR DATA FUSION AND VARIABLE SELECTION

J.M. González-Sáiz,[1*] I. Esteban-Díez,[1]
A. Sáenz-Mateo,[1] C. Pizarro[1]

[1]*Department of Chemistry, University of La Rioja, c/Madre de Dios 51, 26006 Logroño, La Rioja, Spain.*

[*]*Corresponding author: josemaria.gonzalez@unirioja.es Phone: +34 941299634.*

Quality control and food authentication are tasks of great importance to increase consumer awareness of food safety issues, to protect genuine food processors and to fight frauds and unfair competition. In particular, considering the great diversity of vinegars available nowadays on the market and the differences in prices depending on the declared quality of the products, fast and reliable methods of quality assurance are needed. In this context, the use of spectroscopic fingerprints has gained attention in last years as an effective approach for authentication purposes. However, the discrimination of different types of vinegar is still a complex problem and data fusion from different spectroscopic techniques could help to improve classification performance.

The aim of this study was to develop a class model to discriminate between vinegar samples (n=31) belonging to 4 categories (cider, Sherry, red wine and white wine vinegar), using chemometrics to combine the information from multivariate and unspecific data obtained by UV-Visible and NIR spectroscopy.

The wavelength ranges 400-700 nm, and 1100-1880 and 2080-2300 nm were used as the working regions for UV-Visible and NIR spectra, respectively. Several pre-processing methods were applied to data to try to minimise physical effects on spectra. The first derivatives of the UV-Vis spectra and the SNV NIR spectra were selected for use in the subsequent analyses.

As a first step, the SIMCA method was applied to develop a model for each data set separately, using 10 cross-validation groups for internal prediction, 2 PCs for the inner space of each class and separate category centring as scaling option (full spectrum approaches). Next, the two data sets were joined (low level data fusion approach) and SELECT, as feature selection technique, was applied to extract a subset of discriminant variables. To ensure a suitable ratio of samples to variables and to obtain a reliable model, only 5 variables were retained. Finally, SIMCA was performed using the 5 selected and autoscaled variables. The results obtained by data fusion were compared with those obtained using the two data sets separately. The adoption of a data fusion approach together with the application of a variable selection method provided improved results, particularly in terms of prediction ability and model sensitivity.

Data set	Variables	% Class.	% Pred. (10 CV)	% Sensitivity	% Specificity
SNV NIR	502	100	80.7	67.7	96.8
1st Der. UV-Vis	301	100	93.6	61.3	100
Fused dataset	5	100	100	93.6	100

Keywords: chemometrics, data fusion, UV-Visible, NIR, vinegar classification.

Closing Session

ADAPTIVE EVOLUTION AND ADAPTIVE BREEDING OF ACETIC ACID BACTERIA, AND ITS FUTURE PROSPECT FOR AAB RESEARCH AND DEVELOPMENT

K. Matsushita[1*]

[1]*Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan.*

[*]*Corresponding author: kazunobu@yamaguchi-u.ac.jp Phone: +81 839335858.*

Acetic acid bacteria (AAB) inhabit sugar-rich niche, especially fruits and flowers, and thus to have an ability to utilize sugars or sugar alcohols for their energy sources. The strategy of sugar utilization is rather unique compared with others; they use such the sugars or sugar alcohols mainly by "oxidative fermentation", which is carried out by the respiratory chain comprising (primary) quinoprotein dehydrogenases and (terminal) ubiquinol oxidases, both located on the cytoplasmic membrane. The unique respiratory chain seems to be acquired by adaptive evolution of AAB to such the sugar-rich niche by interacting with other microbes living at the same habitat. Genetic instability of AAB, which is an ability well known for a long time, and recently has been confirmed by the genome study of *Acetobacter pasteurianus*, may contribute the prompt adaption of AAB.

Since the fermentation with AAB is usually done below or around 30°C, the optimum growth temperature, a large cooling expense is required in the industrial fermentation processes, especially nowadays by global climate warming. Thus, the development of thermotolerant AAB is expected to release us from such a strict temperature control, and thus to enable us to perform stable fermentation without serious care. We have isolated many thermotolerant AAB adapted to tropical habitat from nature, and further aquired thermo-adapted AAB strains *in vitro* by repeated cultivations at the upper limited growth temperature under acetic acid fermentation condition. These thermotolerant or thermo-adapted strains were shown to be advantageous for high temperature fermentation compared with mesophilic or non-adapted strains. By comparative genome analysis between each counterparts, several important genes have emerged to be potentially involved in such a high-temperature growth or fermentation.

In this conference, I would like to summarize a notion how oxidative fermentation is evolved in AAB and also an achivement how AAB is adapted to high-temperature fermentation. Furthermore, I will present what kinds of subjects remain to be clarified for understanding AAB physiology and how the adaptability of AAB and comparative genomics are useful for AAB research and development.

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Keywords: adaptive evolution, adaptive breeding, thermotolerance, comparative genomics, Acetobacter pasteurianus.

Index of Authors