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4th International Conference on Acetic Acid Bacteria

Vinegar and other products (AAB 2015)

15-19 September 2015
Taiyuan, P. R. China

TABLE OF CONTENTS

ORAL PRESENTATIONS

SECTION III. Process and quality control of vinegars

SECTION IV. Relationship of microbes with vinegars

SECTION V. Equipments and engineering aspects which vinegar companies need

SECTION VI. Others-except vinegar, investigation, technology and product related to acetic acid bacteria

POSTER PRESENTATIONS

4rd International Conference on

Acetic Acid Bacteria

Vinegar and other products (AAB 2015)

15-19 September 2015
Taiyuan, P. R. China

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ORAL PRESENTATIONS

SECTION I. History and culture of vinegars

I-1
HISTORY OF VINEGAR PRODUCTION IN EUROPE AND IN THE WORLD: INDUSTRIAL BIOTECHNOLOGY *AVANT LA LETTER*!

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Vinegar production has a long history worldwide and has evolved over centuries from an art and handicraft into a controlled fermentation process nowadays. Many medieval manuscripts (13th-15th century) with medicinal, alchemy-related or kitchen recipes report on the trade and practice of vinegar production. In Europe, till first half of the 16th century, vinegar was mainly made from wine (as source of ethanol) in the households. Around 1600, poor grape harvests and colder climate conditions led to a switch to brew vinegar rather from mead, apple cider and mainly from beer; also production moved from the households to true vinegar breweries, usually located in the cities. This was the established trade situation till the 19th century, but then it changed drastically, due to scientific and technological developments. The invention of the continuous distillation column and the availability of cheap raw materials (beet sugar, molasses, potatoes, maize, …) for producing distilled alcohol led the yeast, genever and spiritus factories to produce also alcohol vinegar. Famous Dutch (Clusius, Boerhaave), French (Pasteur, Lavoisier) and German (Kutzing, Liebig) scientists resolved in the meantime the secrets of vinegar fermentation. Based on Louis Pasteur's findings since 1858, a broth containing 10-14% alcohol, ammonium salts and autolysed yeast, could be fermented aerobically by acetic acid bacteria into vinegar in high yield. This fermentation alcohol based vinegar process is now worldwide applied. Fruit vinegars dominate in Europe, while cereal vinegars are typical for Asia. Specialty vinegar brands are on the rise again. In addition to Japanese microbiologists (Asai, Yamada, ...), also microbiologists from Belgian universities (Frateur from Louvain; De Ley from Ghent) contributed in the 20th century much to the taxonomy, physiology and genetics of the acetic acid bacteria. Vinegar production is indeed a nice example of industrial biotechnology *avant la lettre*, evolving from small-scale surface culture in barrels (Orleans process) via immobilized cell culture (Boerhaave/Schuzenbach/Frings process) or solid state to currently large-scale (100 m^3) submerged (acetator) fermentation processes. In most cases, a *seed vinegar* (undefined mixed) - starter culture is essential to obtain a desired vinegar brand up till today!

Keywords: Vinegar; production; history.

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I-2
INVESTIGATION AND APPLICATION OF ACETIC ACID BACTERIA IN CHINA - CHINESE CEREAL VINEGAR AS A MAIN EXAMPLE

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Based on the document literature and abstracts collected on The 4th International Conference on Acetic Acid Bacteria (AAB·2015), I prepared this topic - Investigation and Application of Acetic Acid Bacteria (AAB) in China, which includes: Brief introduction of

Chinese cereal vinegar; Research of Chinese cereal vinegar; Other products by AAB in China; Investigation of AAB in China; AAB and their products possessing a bright future in China.

Aspect I. Brief introduction of Chinese cereal vinegars

From the historical literature, we can find that vinegar in China originated more than 3000 years ago. Up to Northern and Southern Dynasties (420-581 AD), the book named *Qi Ming Yao Shu*《齐民要术》 recorded 23 different kinds of methods about how to brew vinegars.

Nowadays, on Chinese markets, there are many kinds of homemade vinegars, most of which are brewed with starchy materials such as sorghum, rice, corn, barley and wheat. This is why Chinese vinegars are normally called as cereal vinegars. Among them, Shanxi mature vinegar, Zhenjiang aromatic vinegar, Sichuan bran vinegar and *Monascus* vinegar, are the main representatives of Chinese vinegars. They are also honored as four traditional China-typical famous vinegars due to their unique flavors, long production histories, massive yields and characteristic fermentation processes.

Traditionally, most Chinese vinegars except *Monascus* vinegar are brewed by solid-state fermentation (SSF), even now, mainly by a mixture of liquid-state or hemi-solid-state fermentation for saccharification and alcohol processes and SSF for acetic acid fermentation.

Aspect II. Research of Chinese cereal vinegars

In this aspect, Chinese scientists have paid more attention on the following areas: Health and main compounds of traditional Chinese cereal vinegars; Process and quality control of vinegars; Microbes related with vinegars; Equipment.

Besides cereal vinegars, there are some other vinegars such as sugarcane vinegar, apple vinegar, orange vinegar, pear vinegar and etc. on Chinese market. Even these vinegars may not be a mainstream of vinegar products in China, this does not hinder Chinese scientists from doing the relative researches.

Aspect III. Other products by AAB in China

As we know, besides being used to produce vinegars and other organic acids, AAB can be applied to produce cellulose and sorbose, fix nitrogen, synthesize surfactants, produce yellow and brown pigments, and eliminate formaldehyde. Chinese scientists have done the related investigation in almost all above-mentioned aspects of AAB. Especially, recently Chinese scientists and companies have successfully developed or modified the technologies to produce nata de coco using cellulose-producing AAB strains; and in the last two decades, a novel type of soft drink-fermented vinegar drink is popular in China. Now on Chinese market, we can see many kinds of vinegar soft drink made by apple, strawberry, pear, pineapple, and etc..

Aspect IV. Investigation of AAB in China

Chinese scientists have been doing the related investigation on AAB, especially from 1970s to now. Chinese scientists have isolated a lot of AAB strains from cereal vinegar paste (*Cupei*, brewing mash of vinegar) and other samples, selected some AAB strains with good characteristics, and applied them into making vinegar and relative products. For example, in 1970s-1980s Chinese scientists isolated *Acetobacter pasteurianus* CICC 20001 (once named *Huniang* 1.01) and CICC 20011 (once named *Acetobacter rancens* As1.41), did a lot of researches on them. Now both of them have been widely used to brew vinegar by solid-state and liquid-state fermentation, and to produce the relative products. Besides *A. pasteurianus* CICC 20001 and CICC 20011, Chinese scientists also isolated and screened many other AAB strains with special properties and utilized them to produce fruit vinegars, nata de coco and vinegar drinks as I mentioned before.

Recently AAB's phylogenetics, ecology, genetics, physiology, biochemistry, molecular biology, genome and so on, have been become a research hotspot in China. Especially Chinese researchers as ones in other countries are paying more and more attention to genomics, transcriptomic, proteomics, metabolomics, and anti-acid mechanism of AAB. For instance, our research group has finished genomic sequencing and comparative genomic analysis of *A. pasteurianus* CICC 20001 and CICC 20011, and now is investigating their transcriptomic and anti-acid mechanism.

Aspect V. AAB and their products possessing a bright future in China

The achievements by Chinese scientists have revealed that AAB and their products possess a bright future in China. The following fields could be of interest for further researches on AAB.

Firstly, although some functional compounds in traditional Chinese cereal vinegars (TCCV) have been found, many other could be further investigated. For instance, besides functional compounds previously reported such as ligustrazine and fumalic acid, our research group found that many other ones might exist in traditional Shanxi mature vinegars.

Secondly, in the area of process and quality control of vinegars, besides TCCV fruit vinegar, sugarcane vinegar, other related products with AAB like nata de coco, are becoming a novel research hotspot in China.

Thirdly, with regard to relationship of microbes with vinegars, AAB biological researches should pay more attention. Besides, molds, yeasts, lactic acid bacteria related with TCCV are worth being investigated, and the effects of microbes from the starters on the vinegar quality should be further studied, too.

Fourthly, about equipment needed by companies, it is worth promoting that a large scale solid-sate tanks are applied for vinegar brewing in order to improve production environment and TCCV quantity and quality.

Finally, with respect to liquid-state fermented cereal vinegars, the following issues may be urgent to further investigate: Increasing their flavor; Developing an effective tail gas recovery technology; Modifying the effective separating and filtrating method.

Keywords: Acetic acid bacteria; Chinese cereal vinegar; research development.

I-3
FACTS AND FICTION, SCIENCE AND TECHNOLOGY: THE BALSAMIC VINEGAR CASE STUDY

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The history of microbial biotransformation is closely associated with vinegar production, which dates back to around 2000 years B.C. However, among fermented foods, vinegar has always been considered a poor commodity because it is not a *food*, lacks significant nutritional value, and it is produced from the transformation of richer and more nutritive fermented foods, such as wine and honey. Vinegar is a flavoring agent, but also a preservative and in some countries it's considered a healthy drink. Vinegar production is regulated by an extensive set of statutes and the definition of vinegar varies from a country to another. FAO/WHO defines vinegar as any liquid, fit for human consumption, produced exclusively from suitable products containing starch and/or sugars by the process of double fermentation, first alcoholic and then acetous. Several botanical species can be used for vinegar production on the condition they satisfy two main basic requirements. Firstly they must be safe for human and animal consumption, and secondly they must provide a direct or indirect source of fermentable sugar. The two fundamental steps in vinegar production are the preparation of the raw materials and the fermentation. The first stage embraces all the necessary operations to ensure the availability of fermentable sugar and protein in solution, including slicing and/or crushing to obtain the fruit juice, enzymatic digestion of starch (for cereals), in some cases cooking and steaming. In general, fruits require less processing than seed crops. Conversely, seed crops are more easily stored and preserved, making their use independent of harvesting time.

In general, vinegars are properly ascribed to the wider category of condiments, and they fall into two different sub categories: with and without Geographical Indication (GI). Under the agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS), GIs are "*indications which identify a good as originating in the territory of a Member, or a region or locality in that territory, where a given quality, reputation or other characteristic of the good is essentially attributable to its geographical origin*". GIs and trademarks are also similar in terms of the rationale behind their legal protection, that is, consumer protection and prevention of unfair competition. Misuse of GIs may be detrimental to the interests of consumers and constitute unfair competition between producers.

Nowadays in the European Community there are four vinegars with the PDO and two with the PGI, the first vinegar with recognized GI status was applied to the two Traditional Balsamic Vinegar of Modena and of Reggio Emilia and it date back to year 2000. The more recent one is the PGI of 镇江香醋 Zhenjiang Xiang Cu, in the year 2012. Third balsamic vinegar had the PGI in 2009 the Aceto Balsamico di Modena (ABM), with the opposition of some state member.

Keywords: Geographical indications; balsamic vinegar; trademarks.

SECTION II.
Health and main compounds of traditional vinegars

II-1
ANALYSIS OF POLYPHENOLS AND FLAVONOIDS, THE KEY INDEX FOR SHANXI EXTRA AGED VINEGAR, AND THEIR CORRELATION WITH ANTIOXIDANT CAPACITY

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Shanxi extra aged vinegar (SEAV), one of the famous Chinese traditional vinegars, is produced by traditional solid-state fermentation technology. It is not only special in taste and flavor but also possesses antioxidant capacity. Compounds of polyphenols and flavonoids that are key indexes for SEAV are considered highly correlated with the antioxidant capacity. However, the work about the variation of the antioxidant capacity and polyphenols and flavonoids content during aging was little captured. For this purpose, the polyphenols and flavonoids of 20 SEAV samples with different aging time, which are from 4 manufacturers, were analyzed. Results showed that the contents of polyphenols and flavonoids increased with the extension of aging time. The content of polyphenols were 1.95±0.55 mg/mL and 4.42±1.44 mg/mL, respectively, for samples aging 2-3 and 8 years. Those were 0.71±0.63 mg/mL and 3.08±1.25 mg/mL, respectively, for flavonoids. The antioxidant capacity of SEAV presented similar trend with that of polyphenols and flavonoids. Assays of ABTS indicated it increased from 4.93±3.12 mmoL/L for aging 2-3 years to 13.23±4.37 mmoL/L for aging 8 years. These values were 4.30±1.78 mmoL/L and 10.95±3.51 mmoL/L, respectively, when analyzed with FRAP assay. The antioxidant capacity is highly correlated with the contents of polyphenols and flavonoids. The correlation coefficients of antioxidant capacity of FRAP with polyphenols and flavonoids were 0.919 and 0.969, respectively. Those were 0.884 and 0.963 when detected with ABTS, while those were 0.107 and 0.395 with DPPH. Furthermore, 8 phenolic and flavonoids compounds, including ferulic acid, gallic acid, catechin, syringic acid, chlorogenic acid, caffeic acid, p-coumaric acid and rutin, were simultaneously detected with the method of HPLC. Concentrations of them, except rutin, increased with the extension of aging time. Thus, aging processes, an important stage for SEAV production, improved the antioxidant capacity. Polyphenols and flavonoids were the important contributors to antioxidant capacity, which accumulated during aging process.

Keywords: Shanxi extra aged vinegar; polyphenols; flavonoids; antioxidant capacity.

II-2
EFFECT OF MATURE PROCESS ON VOLATILE COMPOUNDS OF SHANXI MATURE VINEGAR, A TRADITIONAL CHINESE VINEGAR

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The aroma of vinegar is one of the most important factors that make Shanxi mature vinegar (SMV) as the most famous one in China. Changes in volatile components were investigated during mature process of SMV. Six vinegar samples were produced by Shanxi Mature Vinegar Group co., LTD, and stored for different times. Volatile compounds were analyzed by headspace solid-phase micro-extraction and analyzed by gas chromatography-mass spectrum (GC-MS). After optimizing, the samples were incubated at 45°C for 2 min and extracted with stirring at the same temperature for 5 min prior to injection into a GC-MS. One hundred and thirteen compounds were identified in the samples, mainly including nitrogen-containing, alcohols, acids, esters, aldehydes, ketones, phenols, heterocyclics and others. Mature process of SMV can lead to an increase in the total quantity of volatile components, but long aging time did not work much more efficient, while one year and three year samples performance were better than others. The first factor, which explains 97.8% of total variance, was mainly influenced by 3-hydroxy-2-butanone and ethyl acetate. Multivariate statistical analysis supported the influence of the process on the volatile composition of the final vinegars.

Keywords: Mature; volatile; cereal vinegar; GC-MS.

II-3
DETERMINATION OF RHEOLOGICAL PROPERTIES AND ITS RELATIONSHIP WITH TOTAL AROMA RELEASE IN SHANXI AGED VINEGAR BY RHEOMETER AND SOLID PHASE MICROEXTRACTION COUPLED WITH GC-MS

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Shanxi aged vinegar (SAV) is the most famous traditional vinegar in northern China. The aroma composition of SAV was well determined. However, the aroma perception, which is an important quality factor of SAV, is determined not only by the aroma composition, but also the release of aroma compounds. The aroma release could be influenced by several factors, such as non-volatile compounds and rheological properties of the food matrix. The role of rheological properties of food matrix in aroma release was studied by many authors, while the results were conflicting. Therefore, the aim of our research is to study the relationship between rheological properties and total aroma release in SAV.

The rheological properties of SAV were determined by rheometer, and the total aroma release from a modified SAV system, developed by adding carboxymethylcellulose (CMC), pectin, glucose, fructose, NaCl and tannic acid into SAV. Analyses were conducted by solid phase headspace gas-chromatography mass-spectrum. Nineteen compounds, which accounted for majority aroma profile of SAV, were chosen as target compounds, in order to investigate the change of total aroma release of SAV. The consistency coefficient (K) and flow behavior index (n) of SAV and modified SAV were determined in the rheometer analysis. The K value of SAV ranged from $1.09e^{-5}$ to $3.55e^{-4}$. The n values of SAV were all above 1, indicating SAV exhibited a shear-thickening behavior. The K values of all the modified SAV increased significantly, and the highest K value (0.0428) was acquired in pectin modified SAV. The pectin and CMC modified SAV was turned into shear-thinning behavior ($n<1$). Furthermore, total aroma release in modified SAV was all lower than that of the original SAV, and the lowest total aroma release was acquired in CMC modified SAV. A weak negative correlation was found between K and total aroma release in modified SAV system ($R^2=0.349$), while a weak positive correlation existed between n and total aroma release ($R^2=345$). Moreover, the main aroma compounds of SAV, namely acetic acid and furfural, were more easily influenced by increasing K value.

Our results indicated that rheological properties influence aroma release and the mass transfer mechanism plays the main role in the release of aroma compounds, especially for compounds accounted for the majority of aroma profile. Compared to previous study concerned rheological properties and aroma release, the originality of our study was in the following aspects. In previous studies, the food system investigated was emulsions, or gels system, while aqueous solution system, especially complex aqueous solution system, like vinegar and wine, was rarely studied. Meanwhile, the typical aroma compounds in SAV, such as acetic acid, furfural and pyrazinederivate, were seldom studied in reported studies as well. Additionally, the viscosity of SAV could vary in a large range, due to different raw material or ageing time.

Keywords: Shanxi aged vinegar; total aroma release; rheological properties; consistency index; SPME.

SECTION III.
Process and quality control of vinegars

III-1
MIXED CULTURE OF *SACCHAROMYCES CEREVISIAE* AND *ACETOBACTER PASTEURIANUS* FOR ACETIC ACID PRODUCTION

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Mixed culture of *Saccharomyces cerevisiae* and *Acetobacter pasteurianus* was carried out for high yield of acetic acid. Acetic acid production process was divided into three stages. The first stage was the growth of *S. cerevisiae* and ethanol production, fermentation temperature and aeration rate were controlled at 32°C and 0.2 vvm, respectively. The second stage was the co-culture of *S. cerevisiae* and *A. pasteurianus*, fermentation temperature and aeration rate were maintained at 34°C and 0.4 vvm, respectively. The third stage was the growth of *A. pasteurianus* and production of acetic acid, fermentation temperature and aeration rate were controlled at 32°C and 0.2 vvm, respectively. Inoculation volume of *A. pasteurianus* and *S. cerevisiae* was 16% and 0.06%, respectively. The average acetic acid yield was 52.51 g/L under these optimum conditions. To enhance acetic acid production, a glucose feeding strategy was subsequently employed. When the initial concentration of glucose was 90 g/L and final glucose concentration was increased to 210 g/L by feeding twice, acetic acid yield reached 66.0 g/L.

Keywords: Acetic acid; Acetobacter; yeast; mixed culture; orthogonal experimental design; fed-batch culture.

III-2
PRODUCTION AND CHARACTERISTICS OF HIGH QUALITY VINEGAR FROM SUGARCANE JUICE

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A high quality sugarcane original vinegar drink was produced from fresh sugarcane juice using the wine yeast and LB acetate bacteria by submerged alcoholic fermentation followed by acetic fermentation at room temperature. The quality parameters of vinegar were investigated during the process of submerged fermentation. It was observed that the alcoholic fermentation period of 9 to 20 days and an acetic fermentation period of 15 to 21 days each in succession, produced the sugarcane quality vinegar with 3.04% (w/v) total acid and 4% (v/v) alcohol. Besides the vinegar consisted of many saccharides (fructose, glucose, sucrose) and organic acids (oxalic acid, tartaric acid, citric acid, acetic acid). The prominent ingredient of acetic acid ranged from 8.16 to 13.65 mg/g. Vinegar produced by this process yielded a yellow-brown color with full wine aromas and cane flavor, mild and mellow, low-alcohol and strong odor of vinegar. The present study provides a new approach to process sugarcane byproducts, which contributes to the value-added production and processing of sugarcane.

Keywords: Sugarcane juice; submerged fermentation; original vinegar; yeast.

III-3
ACETIC ACID FERMENTATION AT HIGH TEMPERATURE BY A THERMOTOLERANT *ACETOBACTER PASTEURIANUS* T24 FROM SELF-ADAPTION EXPERIMENT

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The aim of the present work was to improve the thermotolerance of acetic acid bacteria. In this study, a thermotolerant *Acetobacter pasteurianus* T24 was obtained through adaptive experiments. The strain T24 exhibited better growth at 40°C in the solid-state medium. The strain T24 exhibited rapid growth and the wild strain HN101 exhibited a longer lag phase at 40°C in acetic acid fermentation than strain T24. Under the condition of low ethanol concentration, the highest acetic acid concentration produced by T24 at 40°C increased by 18.05% over the wild stain HN101. These results indicated that the adapted strain had acquired thermotolerance over the course of adaptation. Thus, this strain was used for acetic acid fermentation at high temperature. This work reveals the potential value for improvement in industrial vinegar production.

Keywords: Acetobacter pasteurianus; thermotolerance; acetic acid fermentation.

SECTION IV.
Relationship of microbes with vinegars

IV-1
THE OCCURRENCE OF ACETIC ACID BACTERIA: UBIQUITOUS OR NICHE-SPECIFIC?

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Acetic acid bacteria (AAB) are Gram-negative, aerobic bacteria that belong to the *Acetobacteraceae*. They are well-adapted to carbohydrate/ethanol-rich commodities and naturally occur in habitats associated with plants, flowers, and fruits as well as in insect intestines. Currently, AAB are mainly associated with the biotechnological process of vinegar and cellulose production. Although their occurrence in natural fermentation ecosystems has been substantiated, their distribution and role remains rather unclear. Members of the *Acetobacteraceae* have been reported to contribute to a range of spontaneous food and beverage fermentations, such as the cocoa and coffee bean fermentation processes, the manufacturing of acidic beers, and the production of several other slightly acidic beverages (*e.g.*, kombucha, milk kefir, and water kefir). Also, AAB have been reported as spoiling bacteria in wine, cider, and beer fermentations.
In general, AAB are fastidious and obligate aerobic microorganisms. However, they can remain in a viable but non-culturable state when oxygen levels are low, explaining their survival in and isolation from an increasing number of food fermentation ecosystems. Similarly, they are regarded cumbersome to cultivate and/or isolate in the laboratory using conventional microbiological media. Currently, with the implementation of culture-independent methods as well as the introduction of high-throughput sequencing technologies and metagenomics, the abundance and functionalities of AAB in food commodities are being unravelled. Despite their sporadic isolation or incidence in food fermentation ecosystems, the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter* constitute the most frequently encountered taxa. The complete genome sequencing of strains of representative AAB species has elucidated the gene repertoires related to interesting metabolic features, facilitating the understanding of the key role AAB play in natural fermentation ecosystems.

Keywords: Acetic acid bacteria; Acetobacteraceae; cocoa; coffee; acidic beers; (meta)genomics.

IV-2
IDENTIFICATION OF ACETIC ACID BACTERIA THROUGH MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

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The present classification of acetic acid bacteria (AAB) is the result of the continuous application of new and improved identification techniques. Traditionally, AAB identifications have been performed by physiological and chemo-taxonomical characterization. These time-consuming and often unreliable methods have been either complemented gradually or have been replaced completely by molecular identification methods, such as PCR of repetitive DNA elements in genomic DNA [*e.g.*, $(GTG)_5$-PCR fingerprinting], amplified fragment length polymorphism DNA fingerprinting, and sequencing of 16S-23S internal transcribed spacer regions or housekeeping genes. These molecular methods resulted in great improvement of AAB classification and correct identifications. Yet, all these methods have drawbacks too, particularly concerning the speed and cost to construct and keep up-to-date identification databases as well as to identify new isolates. Therefore, a more rapid, cost-effective, and accurate method for AAB identification can be offered by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS mass spectra based on whole bacterial cells or whole bacterial cell extracts typically contain peaks of ribosomal or nucleic acid-binding proteins that can be used for identification.
Few biodiversity studies have applied MALDI-TOF MS without the initial construction of a reference database. Yet, MALDI-TOF MS is also suitable as a dereplication tool to investigate isolates from complex communities and recognize those representing the same species. This application coupled with subsequent identification of representative strains through comparative sequence analysis of 16S rRNA or housekeeping genes allows the build-up of niche- or product-specific databases for their use in biodiversity studies. After obtaining a reliable MALI-TOF MS database using optimal universal sample preparation protocols, the method could be used as a fast, thanks to its high-throughput capacity, and reliable dereplication and identification tool for AAB. Both applications will be illustrated with examples of numerous AAB isolates from cocoa bean and lambic beer fermentation processes.

Keywords: Acetic acid bacteria; bacterial identification; bacterial dereplication; MALDI-TOF MS; cocoa; lambic beer.

IV-3
STUDY ON MICROORGANISMS INVOLVED IN SHANXI AGED VINEGAR FERMENTATION

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Shanxi aged vinegar is one of the most famous traditional Chinese vinegar, which is fermented with traditional solid-state fermentation technique and microbial population enriched from the raw material and producing environment. The products have the characteristics such as high content of amino acid, rich in organic acid composition, good taste and flavor. All of these features are not only related to the raw material and fermenting technique, but also connected with the microorganisms involved in the fermentation. However, the background information about the fermenting microorganisms is quite limited till now. Studies on microorganisms involved in Shanxi aged vinegar fermentation and the microbial population variation during the whole fermenting stages are helpful for starter selection, regulation process parameter; control the hazards of the harmful microbe. Here, the investigations on microorganisms involved in Shanxi aged vinegar fermentation based on the molecular marker and molecular ecology techniques were introduced.

Keywords: *Shanxi aged vinegar; fermentation; microorganisms.*

IV-4
BATCH-TO-BATCH UNIFORMITY OF BACTERIAL AND FUNGAL COMMUNITY SUCCESSION IN THE ACETIC ACID FERMENTATION OF ZHENJIANG AROMATIC VINEGAR

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Solid-state fermentation of traditional Chinese vinegar is a mixed-culture refreshment process that proceeds for many centuries without spoilage. Here, we applied metagenomic approach to investigate bacterial and fungal community succession in three batches of acetic acid fermentation (AAF) for Zhenjiang aromatic vinegar. First, Temporal patterns of bacterial and fungal succession in the *Pei* (solid-state vinegar culture) showed no significant difference ($P>0.05$) among three batches of AAF. The average number of bacterial community operational taxonomic units (OTUs) decreased dramatically from 119±11 on day 1 to 48±16 on day 3, and then maintained in the range of 61±9 from day 5 to the end of fermentation. *Lactobacillus* and *Acetobacter* were dominated in bacterial community, while *Aspergillus* and *Alternaria* were dominated in fungal community during the fermentation process. The relative abundance dynamics of *Lactobacillus* and *Acetobacter* showed high correlation (coefficient as 0.90 and 0.98 respectively) among different batches. Second, within a batch of fermentation process, the patterns of bacterial diversity between the starter (took from the last batch of vinegar culture on day 7) and the *Pei* on day 7 were highly similar (90%), which significantly contributed to uniformity of microbial succession. These findings validate the batch-to-batch uniformity of bacterial and fungal community succession, which accounts for the quality of Zhenjiang aromatic vinegar. Based on our understanding, this is the first study helps to explain the rationality of age-old artistry from a scientific perspective.

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Keywords: Batch uniformity; bacterial community; fungal community; solid-state fermentation; Zhenjiang aromatic vinegar.

IV-5
SCREENING AND IDENTIFICATION OF ANTAGONISTIC BACTERIA FROM SHANXI MATURE VINEGAR AND ANALYSIS OF ANTIBACTERIAL SUBSTANCES

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Shanxi mature vinegar has been using solid fermentation by local microorganisms. During long period producing process, it has its unique microbial community in the fermentation material and environment which guaranteed the distinctive flavor, quality and many other functions. It is important to research the function of the different microorganisms which participated the vinegar producing. The preceding research showed that the acetic acid of the Shanxi mature vinegar has the disinfect function.
By using plate isolation and cylinder-plate method, one

strain has been screened. The sample was isolated from the Cupei (vinegar mash) of Shanxi mature vinegar. The phenotype, bio-chemical tests and 16S rDNA sequence analysis revealed that the strain was *Bacillus subtilis*, and named *B. subtilis* HJD.A32. By excluding of organic acids, hydrogen peroxide and testing of proteinase digestion, the property of the substance produced by the strain was determined, which including H_2O_2, protein or peptides, which molecular weight were around 1KDa, 3-10KDa and greater than 10KDa separately.
The property of the antibacterial substance produced by the strain has the strongest ability of inhibiting Gram-positive bacteria, like *B. coagulans, Staphyloccocus aureus,* and Gram-negative bacteria, like *Escherichia coli* and *Salmonella* sp.
The titers to the indicator bacteria *B. subtilis* SH108 was 256AU/mL. The inhibitory activity has decreased only 8% after 100°C for 20 min.
Therefore, the sterilization of the Shanxi mature vinegar, in addition to acetate from acetic acid bacteria, also comes from bacteriocins of protein or peptide produced by *Bacillus subtilis* in the vinegar mash.

Keywords: Shanxi mature vinegar; Cupei (vinegar mash); Bacillus subtilis; bacteriocin; antimicrobial spectrum.

IV-6
USE OF GENETIC TOOLS FOR THE PHYSIOLOGICAL CHARACTERIZATION, ISOLATION AND APPLICATION OF MEMBRANE-BOUND DEHYDROGENASES FROM ACETIC ACID BACTERIA

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Acetic acid bacteria such as *Gluconobacter oxydans* are used in various biotechnological processes due to their ability to efficiently carry out regio- and stereo-selective oxidations of carbohydrates, alcohols, aldehydes, carbohydrates, and derivative compounds by their membrane-bound dehydrogenases.
In recent years, we have developed and improved genetic tools for acetic acid bacteria, including conjugative gene transfer and selection/counter selection strategies, which now allow highly efficient genetic modification such as the markerless chromosomal insertion, deletion or exchange of genes in acetic acid bacteria. These tools are used to construct *G. oxydans* strains with targeted deletions in one or several membrane-bound dehydrogenases, introduction of homologous or heterologous genes for membrane-bound dehydrogenases, including NADH-dehydrogenase. In this way, by studying the effects of the genomic alterations on the physiology and whole-cell biocatalytic function of cells of acetic acid bacteria, the substrate specificities, physiological roles and application potential of selected membrane-bound dehydrogenases can be studied in depth. Also, these genetic tools and strains constructed with their aid are useful for the isolation and *in vivo* enzymatic characterization of new membrane-bound dehydrogenases from the natural biodiversity of cultured as well as uncultured acetic acid bacteria.

Keywords: Gluconobacter oxydans; genetic tools; membrane-bound; dehydrogenases; physiological characterization.

IV-7
SPECIFIC FEATURES CONFERRING HIGH ACID RESISTANCE TO *KOMAGATAEIBACTER* SPP.

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Acetic acid bacteria (AAB) are used in many biotechnological applications, such as the production of bacterial cellulose and important industrial chemicals like 2-keto-L-gluconic acid, vitamin C, L-sorbose or D-sorbitol. The performance of AAB to transform alcohol into acetic acid with high efficiency at low pH differentiates them from other microorganisms. Due to this characteristic, specialized strains are also involved in the food and beverages industry, such as chocolate, vinegar and kombucha.
Several species of the genus *Komagataeibacter,* which are implicated in the industrial production of vinegar, resist acetic acid concentrations of up to 20%, while a concentration as low as 0.5% is bacteriostatic for most microorganisms. However, mechanisms that confer resistance or adaptation to acetic acid are still poorly understood, the loss of such resistance when the cells are grown in non-stringent conditions indicates that the molecular mechanisms involved are both inducible and transient. During evolution, the genome of these microorganisms has been subjected to mutations and rearrangements making them able to acquire their intrinsic resistance to these extreme environmental conditions: high acetic acid and alcohol concentrations and low pH. This intrinsic tolerance to these parameters is usually a result of the regulation of the outer membrane modification influencing phospholipids, fatty acid and polysaccharides composition. The acquired tolerance through metabolic adaptation must be the result of activation and deactivation of the expression of specific genes, and may involve gene transfer of mobile elements (plasmids/transposons). To investigate the genetic basis that confers resistance to acetic acid to AAB, we chose to compare the genome of a set of reference strains and bacterial isolates obtained in our laboratory. Using Illumina and Pacific Bioscience (Pac-Bio) sequencing technologies, the genomes of vinegar producers strains *Komagataeibacter europaeus* 5P3 (LMG 26311), *Komagataeibacter oboediens* 174Bp2 (LMG 26312) as well as the reference strain *Komagataeibacter europaeus* LMG 18494 and LMG

18890 were analyzed. In addition, draft genomes of the *Acetobacter pasteurianus* 3P3 (LMG 25310) and *A. pasteurianus* LMG 1262^T were also obtained for comparative purposes.
The *Komagataeibacter* genome carried around 900 additional genes as compared to that of the species *A. pasteurianus*. The Core genome of *K. europaeus* 5P3 contained 1751 genes, 355 of which were not present in *A. pasteurianus*, including several ABC transporters. Interestingly, *Komagataeibacter* lacked amino acid and O-antigen exporter systems that were found in *A. pasteurianus*. The DNA/RNA methylation analysis of *K. europaeus* 5P3 strains revealed several DNA specific methyltransferases with known and new target sequences. Together these results provide new insights into the molecular basis of AAB resistance to high concentrations of acetic acid.

Keywords: Acetic acid bacteria; Komagataeibacter; acetic acid resistance; core genome; methylation.

IV-8
ZINC AND COPPER INDUCE OVEREXPRESSION OF BACTERIAL CELLULOSE SYNTHASE OPERON IN *KOMAGATAEIBACTER EUROPAEUS* 5P3

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Bacterial cellulose (BC) provides to bacteria advantageous conditions for growing under unfavourable environments. The *bcs* operon contains four genes involved in the bacterial cellulose synthesis. The *bcsA* gene is responsible for the UPD-glucose synthesis. The *bcsB* gene is the regulatory sub-unit, while *bcsC* gene encodes the outer membrane protein implicated in the BC secretion and *bcsD* gene is involved in the crystallization of BC.
Some Acetic acid bacteria (AAB) are the most efficient cellulose producers among prokaryotes. In liquid environments, AAB synthetize BC as a strategy to remain on the surface and reach aerobic conditions. In solid media, bacterial cellulose provides moisture and surface adherence. BC also confers protection against UV radiation and predators. Nevertheless, the role of BC synthesis during bacteria exposition to metals has never been assessed. Several species of the genus *Komagataeibacter europaeus* are high acetic acid resistant and powerful cellulose producers. The aim of this research is to assess the role of BC synthesis in *K. europaeus* exposed to trace metals. First, *K. europaeus* 5P3 was exposed to several concentrations of Zn^{2+} or Cu^{2+}. Then, the gene expression of the *bcs* operon was assessed during the metal exposure. In addition to bacterial cellulose synthesis, the expression of the *czcCBA* operon, which codes for the metal efflux system CzcCBA, was also evaluated. Preliminary analysis by qRT-PCR demonstrated overexpression of the genes *bcsA*, *bcsB*, *bcsC* and *bcsD* when *K. europaeus* 5P3 was exposed to 0.5 mM of Zn^{2+} or 1 mM Cu^{2+}. Furthermore, bacteria exposed to the 0.5 mM Zn^{2+} also shown overexpression of genes *czcC*, *czcB* and *czcA*, which are involved in zinc resistance.
Because of the low-cost and the renewable resource property, BC has been reported as a new metal adsorbent material for industrial purposes. High cellulose production and metal tolerance suggest that the strain *K. europaeus* 5P3 could be a powerful cellulose producer for the biotechnological industry. The outcomes of this research could help to understand the resistance mechanisms of AAB against unfavourable environments.

Keywords: Acetic acid bacteria; bacterial cellulose; metal resistance.

IV-9
HIGH STRENGTH ACETIC ACID PRODUCTION IN *ACETOBACTER PASTEURIANUS* BY TRANSCRIPTIONAL ANALYSIS AND ENHANCEMENT OF ALCOHOL RESPIRATORY CHAIN RUN

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Nowadays, achieving high strength vinegar fermentation is still important to vinegar producers. The molecular mechanism of acetic acid production and resistance is hotspotting this area. We disclose and improved by transcriptional analysis and enhancing alcohol respiratory chain (ARC) run of *Acetobacter pasteurianus*.
A better strain *Acetobacter pasteurianus* FS2-4 which is improved from HS-1 was got through a combination of modified UV irradiation and nitrous acid mutation after acetic acid shock. The transcript profile about acetic acid production and resistance of *Acetobacter pasterurinaus* is examined by qRT-PCR. Twenty genes related to tricarboxylic acid (TCA) cycle, acetic acid production and resistance system are chosen. Based on above analysis, the ARC was chosen to be enhanced to improve the production.
The maximum acetic acid yield of *Acetobacter pasteurianus* FS2-4 increased 26.6% and the initial acidity tolerance increased from 20 g/L to 50 g/L. The transcriptional result shows that acetic acid production and resistance system is up regulated while TCA cycle is down regulated. Among acid resistance systems, it shows kdpA, clpB, ompA are enhanced and fapA,B, frbC, purE are down regulated while aatA, Gro EL, Gro ES aren't changed. Except suc B, all other gene in TCA cycle are down regulated. We also found acidification rate showed strong positive correlation with ARC activity. So, activity of ARC enzymes, ubiquinone biosynthesis and oxygen availability were taken into account to improve ARC run. Results disclosed that acidification could not be fully inspired until the above three aspects being balanced properly. Therefore, to achieve high strength fermentation, some precursors of alcohol respiration associated factors were added along

with an appropriate aeration rate for repeated batches. Obviously, average acidification rate was enhanced to 2.21±0.02 g/L/h with the optimized method, while the original rate was only 1.78±0.02 g/L/h.

Keywords: Acetobacter pasteurianus; alcohol respiratory chain; *transcriptional analysis; acetification rate.*

IV-10
LEVANSUCRASE DIVERSITY AMONG DIFFERENT GENERA OF ACETIC ACID BACTERIA

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Acetic acid bacteria (AAB) produce extracellular polysaccharides such as cellulose, acetan or levan. Levans (*β*-2,6-linked *β*-(D)-fructose-polymers) are built up by secreted levansucrases (LS) and exhibit great potential as prebiotics, hydrocolloids or for the development of biodegradable materials.

Nevertheless, little is actually known about the diversity of LS among different genera of AAB, while current knowledge about AAB LS is actually restricted to the N_2-fixing AAB species *Gluconacetobacter (Ga.) diazotrophicus*. The aim of our study was, therefore, to investigate the diversity of LS among AAB on genetic level for identification of further promising levan producing strains and LS.

In this way, we at first identified LS gene sequences in 3 known levan overproducing *Gluconobacter* strains as well as in *N. chiangmaiensis* NBRC 101099 and *K. baliensis* DSM 14400 *via* different degenerate and inverse PCR methods. Based on these identified nucleotide sequences and some available LS nucleotide sequences obtained from recently sequenced AAB genomes, two PCR primer (screening) sets were developed, which targeted to the *Gluconobacter* (*cluster 1: C1*) like LS type and the *Ga. diazotrophicus* (*cluster 2: C2*) like LS type, respectively. In this way, a total of 18 new (partial) LS gene sequences (14 x C1; 4 x C2) were identified during this work.

Taken together, based on available nucleotide sequences, C1-LS are actually found in different strains of the genera *Gluconobacter* (19), *Kozakia* (2), *Komagataeibacter* (1) and *Acetobacter* (1), whereas C2-LS are present in different strains of the genera *Gluconacetobacter* (2), *Asaia (A.)* (2), *Neoasaia* (1) and *Kozakia* (1). Noticeably, all so far genetically investigated *Gluconobacter* strains harbor C1-LS genes, which confirms our previous findings about levan production among AAB and implies the adaptation of these strains to sugar/sucrose-rich environments. On the contrary, C2-LS are found in a few AAB strains, some of which are able to fix N_2 (*Ga. diazotrophicus*, *Ga. azotocaptans*, *A. species* SF2.1) and whose LS share structural similarity to those of N_2-fixing *Burkholderia* strains.

In conclusion, we (i) provide a general view on LS diversity among physiologically/metabolically different AAB, (ii) have identified AAB as the most potent and versatile levan producers among bacteria and (iii) have developed primer sets, which can be used to identify and to phylogenetically differentiate LS producers among AAB.

Keywords: Acetic acid bacteria; levan; levansucrase; diversity.

IV-11
DETERMINATION OF DEHYDROGENASE ACTIVITY IN *GLUCONOBACTER* AND *ACETOBACTER* STRAINS

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The acetic acid bacteria (AAB) are known for the ability to perform quick and incomplete oxidations of a high number of alcohols and carbohydrates, resulting in the accumulation of organic acids as final products. Sugar and sugar alcohols are the substrates preferred by *Gluconobacter* species, while *Acetobacter* species show higher preference for ethanol. Examples of this oxidation activity are the production of acetic acid from ethanol or D-gluconic acid and derivatives from glucose. Two different groups of enzymes, which differ in the localization and the substrate specificity, are responsible to carry out D-glucose oxidation. The main pathway is the one linked to the bacterial membrane, where D-glucose is directly oxidized to D-gluconic acid by membrane-bound pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (mGDH). This acid could be further oxidized to 2-keto-D-gluconate (2-KGA) and 2,5-diketo-D-gluconate by a flavin-dependent gluconate-2-dehydrogenase (GA2DH) and 2-keto-D-gluconate dehydrogenase (2KGDH), respectively. Additionally, D-gluconate could also be oxidized to 5-ketogluconic acid (5-KGA) by a membrane-bound PQQ-dependent glycerol dehydrogenase which could be called as gluconate-5-dehydrogenase (GA5DH) by focusing on gluconate metabolism.

The aim of this work was to determine the enzymatic activity of these four membrane- bound enzymes in 3 AAB strains isolated from strawberry and grape, compared with each type strains. Thus six strains of AAB (2 strains of *Gluconobacter oxydans,* 2 of *Gluconobacter japonicus* and 2 of *Acetobacter malorum*) were grown in synthetic medium with 30 g/L of D-glucose and 40 g/L of fructose at different growth phases (24, 48 and 96 h). In general, strains of *Gluconobacter* genus presented higher activity in these enzymes than the *A. malorum* strains. In *Gluconobacter* strains, the activity of mGDH and GA2DH was extremely high and reasonable, respectively, while in *A. malorum* strains, reasonable mGDH activity and low but significant GA2DH activity were detected. Additionally, *G. oxydans* strains were the only ones that showed a reasonable GA5DH activity while *G. japonicus* and *A. malorum* pre-

sented a low and very low enzyme activity. Finally, none of the strains showed 2KGDH activity.

Keywords: D-glucose; D-gluconic; 2-ketogluconic acid; 5-ketogluconic acid.

IV-12
ACETIC ACID BACTERIA OF UNIMORE MICROBIAL CULTURE COLLECTION: FROM STRAINS PRESERVATION TO SELECTED STARTER CULTURES DISTRIBUTION

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Increased knowledge on physiological, biochemical and genetic properties of acetic acid bacteria (AAB) is driving advancements in biotechnology and innovative fermentation processes. AAB scalable fermentations require robust strains able to maintain their functional traits over processes and to dominate unsterile environments.
Accordingly, microbial collections of well-characterized AAB strains are fundamental sources to select cultures with desired properties for biotechnological applications. The University of Modena and Reggio Emilia Microbial Culture Collection (UMCC), specialized in the selection of functional microorganisms for both academic and industrial purposes, holds several AAB strains collected from must, wine, vinegars and *kombucha tea*. Selective strain isolation, molecular typing, polyphasic identification, and technological selection are the core of UMCC activity finalized to build up appropriate AAB starters for different industrial needs.
Shift from preservation status to active AAB cultures is optimized to maintain high cell viability and to reduce genetic drift or strain instability. Technological screening of strains, design and development of selected starters at pilot scale, in static and submerged conditions, are performed to provide industrial cultures for vinegar production. Moreover, consumer demand for high added-value products, including fermented and low sour beverages indicates potential applications for novel and functional starter cultures.
UMCC strain information is managed by the BioloMICS NET software (BioAware/ http://biolomics.umcc.unimore.it), which acts as a comprehensive platform able to combine phenotypic and molecular traits with industrial strain performance.

Keywords: Culture collection; acetic acid bacteria; starter culture; vinegar production.

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SECTION V.
Equipments and engineering aspects which vinegar companies need

V-1
SOLID STATE FERMENTATION PROCESS ENHANCED BY VIBRO-FLUIDIZATION

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Solid state fermentation (SSF), with the advantages of energy and water saving, abundant metabolites and unique flavor of the product, it is widely used in China liquor brewing, vinegar and soy sauce production. However, there are two basic strengthening ways for traditional SSF: static or artificial turnover, wherein heat and mass transfer in fermentation process are both difficult. Besides, mechanical stirring as the current mainstream approach is not suitable for solid phase system, resulting in long fermentation period and high labor intensity in traditional vinegar production. In view of this, this work proposed the novel vibro-fluidization manner to strengthen the substrate mixing and heat and mass transfer in SSF process of vinegar production. By periodically dislocation or tilting movement of the tray, the stress state of substrate was switched between active stress and passive stress, so as to continuously break its force balance and promote the substrate scattered and mixed. Mechanical vibro-fluidization manner, not only improves the mixing uniformity of substrate in SSF process, but also reduces the shear damage to microbial cells. It provides an effective way for large-scale industrial production of vinegar, soy sauce and liquor under SSF conditions.

Keywords: Vinegar production; solid-state fermentation; vibro-fluidization; heat and mass transfer.

V-2
STUDY ON-SELF-INDUCING REACTOR AND ITS APPLICATION IN VINEGAR AND YEAST PRODUCTION

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Currently the most important international acetic fermentation equipment is self-inducing reactor. It has many advantages, such as uniform bubble distribution, short production cycle, high utilization of raw materials, high conversion rate, small area of production plant, and you can easily do large scale in production and automation management, with cost saving. As the similar reasons of acetic acid fermentation, self-inducing reactor is also widely used in yeast production. In this system the impeller rotates at a sufficiently high speed, the gas in the headspace starts to be sucked and distributed into the liquid through the orifices on the blades. The gas is dispersed into small bubbles (diameter of about 200μm), forming a huge gas-liquid mass transfer interface, while the gas-liquid mixture flow through the jet way on a stator to the fermentation broth, achieving a perfect oxygen transfer rate (OTR up to 3.5 - 7.0kg.m-3.hr-1).

A method has been established for the scale-up of self-inducing reactor by combining semi-theoretical model and CFD simulation, wherein the semi-theoretical model is derived from energy conservation, and parameters in the model were corrected by the experimental data. The CFD model was conducted coupled with gas-inducing principle that can reliable predict the gas induction rate. The first step of scale-up process is to calculate the impeller characteristic parameters according to the semi-theoretical formula, then results are analysed by CFD simulation. To verify the reliability of the method, experiments were carried out in 50L, 300L and 2300L reactors. The average deviation of the gas induction rate was between predicted values and experimental ones were less than 15%.

A 3D transient CFD model was also established to apply in yeast fermentation process in a gas-inducing reactor, in which biochemical reaction, hydrodynamics and mass transfer are coupled together. The particle image velocimetry (PIV) and yeast fermentation experiments have been used to validate the CFD model, a good agreement has been achieved. Currently the CFD model can be used to predict the transient distribution of liquid velocities, air volume fraction and component concentration of cell, residual sugar and dissolved oxygen within the logarithmic growth period.

Since 2004, our research team has scaled up self-inducing reactor from 10L to 20L, 50L, 100L, 200L, 300L, 2300L, $5M^3$, $8M^3$, $10M^3$, $15M^3$, $20M^3$, $30M^3$, $50M^3$, $70M^3$, $100M^3$, $150M^3$, $180M^3$, $200M^3$. Our self-inducing reactors have been used in more than 60 vinegar factories and more than 10 yeast factories.

Keywords: Self-inducing reactor; scale-up; yeast; vinegar.

V-3
OVERVIEW OF ENGINEERING TASKS AND TYPICAL EQUIPMENT DESIGN FOR INDUSTRIAL VINEGAR PLANTS

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Vinegar fermentation plants can be found in a huge variety regarding production sizes and concepts, technological standards, raw materials used and integration into the industrial value chain. While highest quality vinegars are pro-

duced and matured in plants with surface or solid-state fermentation systems most of the worldwide consumed vinegar is produced in plants with submerged fermentation.
The main sections and units of such a vinegar plant are:
- Raw material storage and handling section
- Raw material processing
- Nutrient preparation
- Process water purification
- Fermentation section
- Exhaust gas treatment
- Unfiltered vinegar discharge and maturation/storage tanks
- Filtration unit or precipitation tanks
- Filtered vinegar tanks
- Maturation/storage and blending tank section

In regard to engineering and design aspects most of the sections are not critical and system design can be carried out according to general liquid (and solid) processing units and utility supply. However, more attention must be paid on the critical fermentation and filtration parts of the plants.
This overview should highlight the most important and critical aspects in these unit operations. In particular the influences on yields, productivity, acidity, heat removal, process stability and quality relevant extracts and volatiles depending on fermenter design and process operation will be presented. Scientific and practical fermenter design examples from the last decades to state of the art systems will be shown. On the basis of the fundamental calculations for mixing, oxygen transfer, microbial kinetics and for the liquid/gas-mass-balance in different fermenter types their general differences and influences on production performance and product quality will be discussed.

Keywords: Submerged fermentation; productivity; yield, oxygen transfer; mass balance.

V-4
A GLIMPSE INTO THE FUTURE OF VINEGAR PRODUCTION TECHNOLOGY AND THE DEVELOPMENT OF A VIRTUAL BREWING ASSISTANT

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In recent years a growing demand for vinegar with concentrations above 18% has led to new developments in the vinegar industry especially with regards to an optimized and integrated measurement and control system.
High concentrations of alcohol and acid inhibit bacteria growth. Therefore it is absolutely necessary to create the optimal and especially stable environment for the bacteria enabling them to withstand ever growing acidity concentrations.
Cetotec has pushed the boundaries in high-strength vinegar production and is currently achieving concentrations >22% in large-scale industrial vinegar plants.

The development of a new to the world innovation called *ACETOLINE* offers the opportunity of an integrated process control enabling the on-line and simultaneous measurement of ethanol and acetic acid.
For the first time in vinegar production technology, acidity and ethanol can be measured automatically on-line during fermentation without any manual interference.
The new system operates on near infra-red (NIR) technology and spectroscopic analysis.
Almost every substance has a spectral *fingerprint*, which is generated by the adsorption of the penetrating light, which can be used to quantify the concentration in a mixture of substances.
The on-line system ACETOLINE can be directly attached to the fermenter and integrated into the Cetotec fed-batch process control system CETO-Pro. This software has been optimized for high strength vinegar production enabling fully automatic process control including the exact achievement of a *target concentration* with the help of the ACETOLINE.
Frequent laboratory measurements for process monitoring become obsolete as the system takes care of this task, also time-consuming titration in the laboratory for determining acidity or distillation for determining alcohol are not necessary anymore. Automatic control including remote control and monitoring of the entire plant from afar are already feasible today, these technological advances combined with on-going integration will keep on changing the face of vinegar production technology and will lead to immense optimizations with regards to day-to-day operations in vinegar plants across the world.

Keywords: Acetoline; on-line measurement of ethanol and acetic acid; integrated process control; near-infrared technology; high-strength vinegar production.

SECTION VI.
Others-except vinegar, investigation, technology and product related to acetic acid bacteria

VI-1
HOW TO MAKE *GLUCONOBACTER OXYDANS* FIT FOR THE FUTURE

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G. oxydans is specialized in the oxidation of monosaccharides, while growth with disaccharides as sole carbon source is either very low (*e.g.*, sucrose) or impossible (*e.g.*, lactose) because disaccharide hydrolyzing enzymes are missing. In addition, growth on polysaccharides (*e.g.*, xylan, cellulose) is not possible because extracellular hydrolases are not encoded in the genome of *Gluconobacter* strains. Due to the great biotechnological and industrial usage of *G. oxydans* and the fact that microbial hydrolysis of renewable raw materials as cheap and prevalent substrates for the production of high value chemicals is becoming increasingly important, an extension of the substrate spectra of this organism for polysaccharides would be highly desirable. The first step to design a *G. oxydans* strain that can hydrolyze disaccharides was described previously.[1] The corresponding mutant was able to cleave trehalose by heterologous expression and production of a trehalase in the periplasm. The resulting glucose molecules were used as substrate. In further studies, we took advantage of the same system for the heterologous expression, production and translocation of the *Bacillus subtilis* endoxylanase XynA in *G. oxydans*. To overcome the outer membrane as a barrier between active XynA in the periplasm and its substrate xylan, a leaky outer membrane strain of *G. oxydans* was generated that released more than 70% of active XynA into the medium. We are now at the stage of generating *G. oxydans* strains that should be able to secrete different polysaccharide-hydrolyzing exoenzymes into the supernatant of the culture medium to extend the substrate spectrum for the utilization of polysaccharides as carbon and energy source and for the production of value added compounds such as sugar acids and keto-sugars. *G. oxydans* has the ability to grow in high osmotic sugar solutions. However, it is still unknown how the organism adapts to highly osmotic sugar rich environments. Therefore, the mechanisms of osmoprotection in *G. oxydans* were investigated. We found an accumulation of mannitol as a potent osmoprotectant inside the stressed.[2] This intracellular mannitol accumulation correlated with increased extracellular osmolarities of the medium. Furthermore, mannitol alleviated the osmotic stress of sucrose on cellular growth. Moreover, the positive effect of exogenous mannitol on the rate of glucose consumption and gluconate formation was monitored. These results may be helpful to optimize the processes of industrial product formation in high concentrated sugar solutions.

Keywords: Substrate spectrum; exoenzymes; renewable raw materials; osmoprotection.

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VI-2
GLUCONIC ACID BIOPRODUCTION BY ACETIC ACID BACTERIA, A DIFFICULT EQUILIBRIUM TO REACH

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Fermentation continues being more efficient than chemical procedures for obtaining gluconic acid. Though fungi are widely used for this purpose, the risks of producing mycotoxyins may not recommend its use for food-related products. Then, some acetic acid bacteria (AAB) including *Gluconobacter oxydans* are becoming increasingly important in this respect. As it is well known, one of the most salient features of *Gluconobacter* spp. is their overflow metabolism[1] by which a substantial fraction of glucose is rapidly oxidized to gluconic acid and scarcely assimilated by these microbes as a result. The bacteria can also use glucose via the pentose phosphate pathway their efficiency, however, is strongly dependent on pH and on the glucose concentration of the medium. Apparently, the pentose phosphate pathway is conderably inhibited at pH≤3.5 and glucose concentrations over 15 mM.[2]

On the other side, gluconic acid is known to be further oxidized to keto-D-gluconic acids.[3] Without pH control, pH rapidly may drops to values lower than 3 and under these conditions glucose is converted virtually exclusively to gluconic acid; nevertheless, at pH 5-6, the process leads preferentially to keto-acids. At the same time, a low initial concentration of glucose in the medium also appears to favour the formation of keto-acids. Additionally, the presence of $CaCO_3$ in the medium also seems to favour the formation of keto-acids.[3]

Finally, the concentration of dissolved oxygen also affects the process;[4] apparently an oxygen concentration of at least 30% of the saturation level is required to maximize the formation of 2,5-diketogluconic acid.

Therefore, selectively converting glucose into gluconic acid and preserving the latter it is rather a complex process and its outcome depends to a greater or lesser extent on the particular microorganism, cultivation medium and fermentation conditions used.

Keywords: Gluconic acid; acetic acid bacteria; gluconobacter oxydans; keto-D-gluconates.

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VI-3
THE FORMATION PROCESS OF SPHERE-LIKE BACTERIAL CELLULOSE PRODUCED IN AGITATED CULTURE AND ITS CHARACTERIZATION

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Sphere-like bacterial cellulose (BC) was a fascinating and renewable natural biopolymer with many unique properties and could be used in some area such as heavy metal ions absorption, bioseparation, immobilized reaction, cell suspension culture and an adsorbent for sewage treatment. The purpose of this study was to elucidate the formation process of sphere-like bacterial cellulose in agitated culture by *Komagataeibacter nataicola* Y19 and the characterization of BC particles obtained during the fermentation. The results showed the morphology changed from filament BC on the 1st day to final solid sphere-like BC on the 7th day, and accordingly the size of inside pore decreased gradually, degree of crystallinity increased gradually from 80.44% to 88.23%, I_α decreased gradually from 76.63% to 70.84% and I_β increased gradually, the thermal stability increased and the maximum decomposition temperature (T_{max}) increased from 316.92°C to 339.08°C. The research could contribute to elucidate the formation mechanism of solid spheres-like BC.

Keywords: Sphere-like bacterial cellulose; formation process; agitated culture; Komagataeibacter nataicola; characterization.

VI-4
CHARACTERIZATION OF A NOVEL HETEROPOLYSACCHARIDE PRODUCED BY *KOZAKIA BALIENSIS*

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*Kozakia baliens*is is a relatively new member of the family *Acetobacteraceae*. It produces levan from sucrose, a fructan-type exopolysaccharide (EPS) that is widely used in food applications. Throughout cultivation of two *K. baliensis* strains (DSM 14400, NBRC 16680) on sucrose-deficient media, we found that both strains still produce high amounts of mucous, water-soluble substances from mannitol and glycerol as (main) carbon sources. This indicates that they additionally produce new classes of so far not characterized EPSs.
The aim of our study was to elucidate the nature of these new classes of polysaccharides via comparative genomics. Specific EPS-clusters should be identified, to make a prediction of the EPS-structure, to finally simplify the chemical analysis of the formed EPSs.
To examine the unique structure of the EPS, the presumed sugar composition of the EPS was determined upon acid hydrolysis via HPLC and followed by whole genome sequencing of the two selected *Kozakia* strains. Circularized genomes could be established for each strain and with the information of the investigated sugar composition of the EPS, EPS forming clusters were identified. In both strains complete ORFs for *levansucrase*-genes could be detected. Surprisingly, we were able to identify in both *K. baliensis* strains a *gum*-like cluster, similar to the *gum*-cluster of the commercial xanthan producer *Xanthomonas campestris*. In agreement with these genomic results, both *Kozakia* strains produced a heteropolysaccharide (HePS) composed of glucose, galactose and mannose in a molar ratio of 6:2:1 suggesting that they have *xanthan-like* structures and properties. We found eight *gum*-like genes, gum *B*, *C*, *D*, *E* , *H*, *J*, *K* and *M*, that are supposed to be involved in the addition of the sugar monomers and export of the final polymer. With regards to the sugar composition of the investigated HePS and correlation of the associated cluster with a similar cluster of *Gluconacetobacter diazotrophicus*, we assigned *in silico* specific enzyme functions. By this stepwise suggestion of the enzyme functions, a future structural analysis is simplified, because sugar linkages could be predicted. In comparison to the *gum*-cluster of *X. campestris* the genes *gum F* and *G* are not present in both *Kozakia* genomes, which indicates the absence of acetyl-residues in the produced HePS.
In conclusion, we were able to identify a new HePS produced from *K. baliensis*, which shows highly viscosifying properties and could be used in food applications or as a new biotechnologically relevant polysaccharide. By whole genome analysis of these strains we were able to identify all the important genes and clusters, which are possibly involved in the biosynthesis and regulation of

the investigated HePS. Our data can therefore in general be used for the knowledge-based optimization of EPS production by selected AAB.

Keywords: Acetic acid bacteria; heteropolysaccharide; EPS-cluster; genomics.

VI-5
ENGINEERING *GLUCONOBACTER OXYDANS* FOR VALUE-ADDED PRODUCT PRODUCTION AND SIMPLIFIED PRODUCT RECOVERY

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Acetic acid bacteria are incomplete oxidation specialists able to oxidize various sugars, sugar acids, polyols, and alcohols with regio- and stereo-specificity under normal growth conditions. The natural ability of acetic acid bacteria to produce chiral molecules is of great advantage and is used for the combined biotechnological-chemical synthesis of sugar derivatives that would otherwise require complex and expensive chemistry (*e.g.*, vitamin C, miglitol). The genome sequences of many species within this group are known. However, few methods are available to metabolically engineer industrial strains for improved or novel product production. To this end, a surface display system was designed to anchor enzymes to the outer membrane of acetic acid bacteria. Surface display provides greater enzyme stability, greater access to substrates by avoiding membrane barriers, often increases catalytic rates, and allows simple product recovery. Furthermore, acetic acid bacteria contain many periplasmically-oriented membrane-bound oxidoreductases that can produce a large number of value-added chiral products in nearly quantitative yields. Addition of foreign oxidoreductases and isomerases/epimerases are ideal candidates to complement the natural metabolism of acetic acid bacteria, and are expected to increase the production capacity of industrial synthons and precursors. Consequently, a library of surface display anchor proteins was examined for their ability to deliver proteins to the outer membrane of the model acetic acid bacterium *Gluconobacter oxydans*. These expression vectors were based on the broad-host-range plasmid pBBR1MCS-2, and contained different anchor proteins that were translationally fused to the reporter protein alkaline phosphatase from *Escherichia coli*. To date, best reporter activities were observed for the OprF188 surface display anchor, representing a truncated version of the major nonspecific porin of *Pseudomonas aeruginosa*. This truncated protein is therefore a promising anchoring motif to display various enzymes at the cell surface of acetic acid bacteria. This display system will be used to exploit and expand the natural ability of acetic acid bacteria to produce value-added compounds by whole-cell biocatalysis, for example by *Izumoring* rare sugars.

Keywords: Surface display; value-added production; metabolic engineering; Izumoring.

POSTER PRESENTATIONS

P-1
ANALYSES OF SHANXI AGED VINEGAR CONSTITUENTS AND THEIR EFFECTS ON ANTI-CANCER ACTIVITIES

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Shanxi aged vinegar (SAV) is the most famous vinegar product among the four well known vinegars in China. SAV is produced by spontaneous solid-state fermentation using sorghum, bran, millet chaff and *Daqu* (starter culture). The main constituents of the raw material of SAV and the constituents of SAV in fermentation and aging were analyzed in the study. Seven different polar extracts (V1-V7) were isolated from the ethyl acetate extract of SAV by preparative chromatograph. The extracts inhibition effect on the growth of cancer cells (A549, Hep-G2, MDA-MDB-231, Hela and HCT116) and normal cell (HUVEC) were studied by S16 Cell Analyzer (ACEA Biosciences Inc, USA) which is based on a latest Real Time Cellular Analysis (RTCA) technique. The results revealed that the saccharides of SAV were mainly from sorghum (36.1%), *Daqu* (29.4%) and bran (23.7%); the proteins were mainly from *Daqu* (40.6%) and bran (38.0%); while the polyphenols and flavonoids were mainly from sorghum (65.4% and 96.4%). The organic acids of SAV mostly consist of acetic acid, lactic acid, citric acid, succinic acid, tartaric acid and malic acid. About 58 kinds of volatile flavor compounds were identified from the end product of SAV which are largely affected by the process of fumigation. The contents of most constituents of SAV were increased with the aging time. The maximum contents of these constituents were: total acid (9.92 g/100 mL), reducing sugar (7.86 g/100 mL), total amino (223.77 mmol/L), polyphenols (790.27 mg/100 mL) and flavonoids (106.21 mg/100 mL). The results of RTCA show that extract V2 can significantly inhibit the growth of tested cells. The inhibition ratios of 100 μg/mL V2 on the cells were: A549 (61.5%), Hep-G2 (49.1%), MDA-MDB-231 (44.4%), HUVEC (33.5%), Hela (11.5%) and HCT116 (no inhibition effect). And the IC50 of V2 on A549 was 18.3 μg/mL (R^2=0.9888) far below the normal cell [HUVEC, 281 μg/mL (R^2=0.9820)]. This study suggests that SAV is rich in various organic acid, amino acids, flavor and phenolic compounds. The extract V2 can greatly inhibit the growth of A549 *in vitro*.

Keywords: Shanxi aged vinegar; anticancer activity; real time cellular analysis.

P-2
ANALYSIS ON THE CHARACTERISTIC COMPOSITION OF SHANXI AGED VINEGAR

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Shanxi vinegar, the first of four famous vinegar brands, enjoys a good reputation at the every corner of China. As the pillar industry, it is extremely important to the development of the economic transformation of Shanxi Province. At present time, it has been bedevilled with many problems such as the shortage of production monitoring, the instability of product quality and the lack of related regulatory controls and effective technical means. Meanwhile, a string of fraud in recent years, seriously affected the reputation of Shanxi vinegar.

This paper has an attempt to solve these problems by exploring a new vinegar quality evaluation method. It employs the modern testing instruments and technology to make a comprehensive analysis of different vinegar varieties and then finds out the characteristic components by using the grey system theory.

The study, on the one hand, provides data support for improving the quality and stability of Shanxi vinegar products and lays a solid foundation for its further healthy and sustainable progress. On the other hand, it has far-reaching theoretical impact on the revision and perfection of Shanxi vinegar manufacturing standards.

Keywords: Shanxi aged vinegar; characteristic composition; analysis.

P-3
STUDY ON IMPROVING IDENTIFICATION OF VINEGAR BY ELECTRONIC NOSE

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Two different brands of Shanxi vinegar are chose as samples. The patterns have been built for the determination of electronic nose data range. Sample handling and sensors optimization by orthogonal experiment method improved the recognition rate (from 81.8% up to 94.6%) and misjudgment rate was significantly reduced (from 18.2% down to 5.4%) in EUCLID, COR- RELATION, MAHALANOBIS, DFA.

Keywords: Vinegar; electronic nose; identification.

P-4
RESEARCH OF DIFFERENT STERILIZED METHODS SOLVING AGED VINEGAR GAS PRODUCTION

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The purpose of this work is to solve the problem of aged vinegar gas was production and bottles explosion. The vinegar of gas production subjected to sterilization (80°C for 20 min, 100°C for 20 min) and autoclaving (121°C for 20 min). Then we used Durham's fermentation tube to detect the gas production of the processed vinegar. Results showed that the treatment group and contrast group of vinegar neither had gas production after culturing 3 days at room temperature. Then the vinegar samples were cultured at 30°C constant temperature for 3 days, producing gas phenomenon still had not been found. It proved that using Durham's fermentation tube couldn't detect the gas production of the sample vinegar and processed vinegar.

Keywords: Aged vinegar; gas production; sterilized methods.

P-5
SPROUTED RICE IN THE HUSK AS A SUBSTRATE OF YONGCHUN *MONASCUS* VINEGAR

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Yongchun *Monascus* vinegar (YMV), fermented by black skin red koji, is known as one of the most famous vinegar in China. In this study, we investigated on the effects of sprouted rice in the husk as a substrate on fermentation process and filtration of YMV. After 9 days of alcoholic fermentation, there was a considerable decrease on amylase activity from 3463.6 U/L to 417.5 U/L, whereas the alcohol content reached 5.41%. The broth pH decreased from 5.81 to 3.41 while the acidity increased from 0.13% to 1.3%. During the acetic acid fermentation, there was no significant change in pH, nevertheless the acidity increased from 1.3% to 5.21%. The filtering layer formed by chaff from rice in the husk can play a positive role in improving filtration efficiency.

Keywords: Yongchun Monascus vinegar; filtration; sprouted rice in the husk.

P-6
SCREENING OF *ACETOBACTER PASTEURIANUS* MUTANT WITH ETHANOL TOLERANCE BY A NEW ATMOSPHERIC AND ROOM TEMPERATURE PLASMA

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A novel atmospheric and room temperature plasma (ARTP) which used helium as the working gas was employed to generate mutants of *Acetobacter pasteurianus* with improved ethanol tolerance. The best strain U1-1 was selected after mutagenesis. U1-1 could grow in liquid medium with 11% ethanol. The production of acetic acid reached 32.83±0.75 g/L, 385.7% higher than that of the parent strain, meanwhile, U1-1 has stable production. Moreover, the cell membrane permeability were measured by PI assay and the results showed that the cell membrane permeability of starting strain (AP-1.01) is significantly higher than U1-1. The better ethanol tolerance of strain U1-1 was maybe due to the decreased membrane permeability.

Keywords: Acetobacter pasteurianus; *ARTP*; *ethanol tolerance*; *mutant.*

P-7
COMPARATIVE ANALYSIS FOR OPERONS BASED ON *ACETOBACTER PASTEURIANUS* GENOME

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Operon is the basic transcriptional and functional units in prokaryotes. *Acetobacter pasteurianus* (*Ap*) strains are used for vinegar fermentations worldwide. They possess strong abilities to tolerate and produce acetic acid. Thermo-tolerance are even gifted to some of these strains Nowadays, *Ap* strains have been widely studied from multiple aspects, but analysis for operons covering their genomes are still absent.

In this study, operon data of 9 *Ap* strains whose complete genome have been published, that is, 7 IFO 3283 substrains (represented by IFO 3283-01), IFO 3283-01-42C, a mutated IFO 3283-01 strain with thermo-tolerance acquired and 386B, a strain isolated from Ghana with inherent thermo-tolerance, were retrieved from DOOR 2.0 database. Then, a comparative analysis was conducted to detect the differences of operon structure among these strains. Operon structures of 7 IFO 3283 substrains were found almost the same, thus IFO 3283-01, the strain with more detailed genome annotations, was chosen to represent all the IFO 3283 substrains to accomplish the study. The results showed that IFO 3283-01 and 386B have their own unique operons respectively, and operon structures of IFO 3283-01-42C are the same as those of IFO 3283-01 except the absent of 16 operons, which are located in the 92-kb-deleted segment reported by the previous study conducted by Azuma *et al*. (2009). It seems that the absent of 16 operons leads to the acquisition of thermo-tolerance of IFO 3283-01-42C, however, 11 of the 16 operons were found exist in the genome of 386B, the inherent thermo-tolerant strain, indicating the complexity in physiological activities responsible for thermo-tolerance. IFO 3283-01-42C was found lose its fermentation abilities at higher temperature as reported by Matsutani *et al*. Thus the 11 operons that are absent only in IFO 3283-01-42C were believed to be responsible for the fermentation abilities, especially at higher temperature. Besides, the 5 operons almost consist of transposase which only exist in IFO 3283-01 were believed to make this strain more thermo-sensitive than the two thermo-tolerant strains (although whether transposase can be the member of an operon is unknown). Meanwhile, another 11 operons carrying out multiple functions seem to be interrupted by transposase in IFO 3283-01, while their complete structures were seen in the genome of 386B. At last, some operons (5 in each genome), of which the functions were unknown, were found be shuffled between IFO 3283-01 and 386B.

Keywords: Operon; Acetobacter pasteurianus; genome.

P-8
GLOBAL INSIGHTS ON ACETIC ACID RESISTANCE MECHANISMS AND GENETIC STABILITY OF *ACETOBACTER PASTEURIANUS* STRAINS BY COMPARATIVE GENOMICS

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Acetobacter pasteurianus (*Ap*) CICC 20001 and CGMCC 1.41 are two acetic acid bacteria strains with strong abilities in producing and tolerating high concentrations of acetic acid, which have been widely used to brew vinegar in China. To globally understand the fermentation characteristics, acid-tolerant mechanisms and genetic stabilities of these two *Ap* strains, their genomes were sequenced. The genome comparisons with other 9 sequenced *Ap* species reveal that chromosomes of *Ap* CICC 20001 and CGMCC 1.41 are evolutionarily conserved, while their plasmids are individual. The acid-tolerant metabolic pathway analysis indicates that some amino acid metabolisms, such as degradations of threonine, glycine and ornithine, and mechanisms of acetic acid tolerance, such as ADH, citrate synthase and aconitate hydratase, collaboratively contribute to acetic acid resistance in *Ap*. The balance of instability factors, such as transposase, mobile element and long tandem repeats, and stability factors, such as the restriction-modification system and the CRISPER-Cas system, in genomes of these two *Ap* strains provides the basis of their genetic stabilities, in accordance with their industrial performance. These observations provide important insights into acid resistant mechanism and genetic stability of *Ap* and lay a foundation for future genetic manipulation and engineering of these strains.

Keywords: Comparative genomics; Acetobacter pasteurianus; acetic acid resistance; genetic stability.

P-9
OPTIMIZATION OF TWO-DIMENSIONAL ELECTROPHORESIS PROCESS FOR PROTEOMIC ANALYSIS OF *ACETOBACTER PASTEURIANUS* INDUSTRIAL PELLETS

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Acetobacter pasteurianus is a predominant industrial bacterium in the rice vinegar production. To investigate the molecular mechanism of acetic acid tolerance at proteome level, a set of two-dimensional gel electrophoresis (2-DE) operation for profiling proteome of *A. pasteurianus* samples from high acidity manufacture was optimized. Considering that the acidic and complicated characteristic of industrial pellets (pH 3.2), a combination of sonication and TCA/acetone protein extraction methods showed a good performance on releasing proteins and trashing non-protein fraction interferences. In the first dimension isoelectric focusing electrophoresis process, an extended desalting time would be necessary to these industrial samples. Dissolution buffer affected greatly the proteins isolation efficiency inside the gel. Plus the optimizations of loading quantity (120 μg/strip), IPG strips (pH 4-7) and isoelectric focusing set-points (80,000 Vh), a protocol of 2-DE for *A. pasteurianus* industrial pellets was concluded. Appling for the pellets

from a high acidity vinegar fermentation (>9%, w/v), more than 1000 protein spots can be obtained, and the obtained differential spots were suitable for the requirement of protein identification by Mass Spectrum.

Keywords: Acetobacter pasteurianus; proteomics; two-dimensional gel electrophoresis; protein extract.

P-10
SEPARATION AND CULTURE OF SUPERIOR ACETIC ACID BACTERIA FROM HAINING YUFENG TRADITIONAL ROSE VINEGAR

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As_4, Af_6, Ad_2 acetic acid bacteria strains with high acid producing efficiency were isolated from vinegar mash of Yufeng traditional rose rice vinegar produced in Haining, Zhejiang province. They were identified as *Acetobacter* (*A.*) accordingly to their morphological characteristics, culturing characteristics and physiological and biochemical characteristics. As_4 was characterized as *A. pasteurianus,* Ad_2 belonged to *A. aceti,* Af_6 was identified as *A. rancens. A. aceti* Ad_2 had the highest acid production and could reach 1.02 g/mol within 32 h. In the future, this strain should be mutagenized in practice to improve its acid-producing rate.

Keywords: Rose vinegar; A. pasteurianus; A. rancens; A. aceti.

P-11
ISOLATION AND IDENTIFICATION OF GAS-PRODUCING MICROORGANISM IN AGED VINEGAR

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This work is aimed to study microorganisms which cause Shanxi Aged Vinegar gas production, drawing and other phenomena. First we used blood agar media to isolate and purify microbes from aged vinegar, finally found a suspected strain.

Keywords: Vinegar; gas-producing microorganisms; isolate.

P-12
ISOLATION AND PRELIMINARY IDENTIFICATION OF *BACILLUS* IN DAQU OF SHANXI MATURE VINEGAR

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In this study, Daqu, a kind of starter for Shanxi mature vinegar fermentation, was investigated. *Bacillus* (*B.*) in Daqu was isolated by spread plate method, and identified by general microbe testing method. A total of 26 strains were isolated. According to morphological observation and physiological characteristics, the main *Bacillus* can be preliminarily determined in Daqu including *B. amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. circulans, B. firmus*. This study revealed the diversity of *Bacillus* in Daqu, laid the foundation for the physiological and biological characteristics research of strains in fermentation.

Keywords: Shanxi mature vinegar; Daqu; Bacillus; identification.

P-13
COMPARATIVE ANALYSIS OF CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS (CRISPR) LOCI IN THE ACETIC ACID BACTERIA

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The clustered regularly interspaced short palindromic repeats (CRISPR) is a widespread adaptive immunity system that exists in most Archaea and many bacteria against foreign DNA such as phages, viruses and plasmids. In general, The CRISPR system consists of direct repeat, leader sequence, spacer sequence and CRISPR-associated sequence. Acetic acid bacteria (AAB) are gram-negative strictly aerobic and belong to the family of the *Acetobacteraceae* of the α-proteobacteria. They could be isolated from variety nature fields such as fruits, flowers, and fermented foods. AAB play an important role in industrial fermentation of vinegar and bioelectrochemistry. To investigate the polymorphism and evolution pattern of CRISPR loci in AAB, we selected 48 species from three main genera *Acetobacter*, *Gluconacetobacte*r, and *Gluconobacter* with the whole genome sequences available from the NCBI database. The bioinformatics analysis of leader sequence, direct sequence, spacer sequence were performed. The results showed that the CRISPR system existed in 32 species of 48 strains tested. Most of the CRISPR-Cas system in AAB belonged to type I CRISPR-Cas system (containing *Cas 3* gene and *Cas 7* gene), subtype E and C.

Besides, type II CRISPR-Cas system which contain *Cas 9* gene was also found in the genus *Acetobacter* and *Gluconacetobacter*. The repeat sequences of some CRISPR were highly conserved among species from different genera, and the leader sequence of some CRISPR possessed conservative motif. Furthermore, the evolutionary analysis of Cas 1 and DR demonstrated that they evolved independently, and were not suitable for classification of species. Interestingly, the conservation of *cas1* genes were associated with that of repeat sequences among different strains, suggesting they were subjected to similar functional constraints. Specially, the number of spacer was positively correlated with the number of prophage and IS, implying that the acetic acid bacteria may be continually invaded by new foreign DNA. The comparative analysis of CRISR loci will add the basal insights into the molecular mechanism of acetic acid tolerance and genome stability in AAB.

Keywords: Acetic acid bacteria; CRISPR; repeat; Cas.

P-14
ISOLATION, SCREENING AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM DAQU OF SHANXI AGED VINEGAR

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Lactic acid bacteria (LAB) is a collectively called which could produce lactic acid using fermentable sugars. Lactic acid is an important kind of non-volatile acid in vinegar, it can give vinegar a gentle acidity, a better taste. In this research, the relationship between dynamic changes of LAB and the content of non-volatile acid during the production of Shanxi aged vinegar were firstly studied. Sample grains in different periods of Shanxi aged vinegar fermentation were collected. The count of LAB was obtained by means of anaerobic MRS plate culture and content of non-volatile acid was measured through a single-boiling distillation apparatus. The study found that in fermented grains, the content of non-volatile acid changed with the number of LAB.
In this study through traditional culture and purification methods, single LAB colonies were isolated and purified from Daqu. Strains were preliminarily identified by physiological and biochemical tests. After that, superiorlactic acid bacteria were selected by alcohol tolerance and acid resistance experiments. The results were as followings, among 21 strains of LAB, 7 strains of LAB were selected, numbering L7, L8, L9, L10, L12, L14, L18, which could produce more acid and live in higher alcohol and acidity environment. After culturing 18 h in MRS liquid medium at their optimum growth temperature, 35°C, their acid production were 0.598 g/100 mL, 0.346 g/100 mL, 0.490 g/100 mL, 0.482 g/100 mL, 0.436 g/100 mL, 0.517 g/100 mL, 1.310 g/100 mL. The seven strains of LAB all could live and metabolize normally in medium with an alcohol concentration of 12% (V/), and pH 3.0.

Keywords: Shanxi aged vinegar; Daqu; lactic acid bacteria; screening.

P-15
STRAWBERRY PURÉE: SELECTIVE BIO-TRANSFORMATION OF ITS GLUCOSE CONTENT INTO GLUCONIC ACID. INFLUENCE OF THE INITIAL CONCENTRATION OF SUBSTRATE ON THE STABILITY OF THE PRODUCT

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Developed countries use to produce food surpluses that in some cases are exposed to a high risk of being turned into wastes and frequently are not made the most of. Fruit surpluses in general and those from strawberry in particular could be an example of what is happening in this regard. An important fraction of strawberry production cannot be sold as whole fruit and results in considerable economic losses in producing regions; for instance, in Spain, strawberry surpluses account for about 20% of the whole national production and are largely transformed into strawberry purées. The composition of these purées makes of them excellent cultivation media for many bioconversions aimed to the development of either new and/or higher added value products.[1] An interesting option is the production of a gluconic acid ferment, by acetic acid bacteria (AAB), as a base component for new beverages. Nevertheless the complex metabolism of AAB[2,3] makes gluconic acid stability quite dependent on several variables among which the initial glucose concentration is specially important.
Working with a culture of *Gluconobacter japonicus* CECT 8843 at freely evolving pH, a selective biotransformation of glucose into gluconic acid retaining the fructose is found. The gluconic acid formed in strawberry purée containing no added sugars started to disappear after glucose depletion, but the acid remained stable if sugar-enriched purée was used. Additionally, preserving the sensory properties of the raw material requires using pasteurized rather that sterilized strawberry purée so that the AAB strain must be able to prevail over other, unwanted, microorganisms inevitably present in industrial pasteurized strawberry purée, then the inoculum preparation is also a key issue.

Keywords: Strawberry purée; acetic acid bacteria; gluconobacter oxydans CECT 8843; gluconic acid; fructose.

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P-16
KOMAGATAEIBACTER HAINANENSIS SP. NOV., A NOVEL CELLULOSE-PRODUCING STRAIN ISOLATED FROM COCONUT MILK

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The phylogenetic position of strain WE7, a cellulose-producing acetic acid bacterium isolated from naturally fermented coconut milk, was investigated. The strain WE7 was allocated to a novel species in the genus *Komagataeibacter* or *Gluconacetobacter* by analysis using nearly complete 16S rRNA gene sequences, as well as concatenated partial sequences of the housekeeping genes *dna*K, *gro*EL and *rpo*B. The strain WE7 could not form γ-pyrone compounds and a water-soluble brown pigment, and showed closer relationship with *Komagataeibacter* species than *Gluconacetobacter* species in a 16S rRNA gene sequence phylogenetic tree, and therefore it could be differentiated from its closely related *Gluconacetobacter* genus. The strain WE7 could also be differentiated from its closely related *Komagataeibacter* species and *Gluconacetobacter entanii*, by the ability to grow on the only carbon source D-mannitol, D-gluconic acid sodium salt and glycerol respectively, the ability to form acetic acid from D-fructose, sucrose, D-mannitol, D-galactose and ethanol, and the ability to grow without acetic acid. Moreover, the major fatty acid of strain WE7 was $C_{18:1}$(52.28%). The DNA G + C content of strain WE7 was 63.2 mol%. The name *Komagataeibacter hainanensis* sp. nov. WE7 was proposed.

Keywords: Acetic acid bacteria; Komagataeibacter hainanensis sp. *nov.; multilocus sequence analysis; 16S rRNA gene sequence; polyphasic classification; coconut milk.*

P-17
ACETATE NOT HYDRION CAN PROMOTE ACETIC ACID OF ACETIC ACID BACTERIA DURING LIQUID-STATE FERMENTATION

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In this study we found that *Acetobacter* spp. grew slowly during liquid-state fermentation, which biomass (wet weight) at the end of fermentation only reached about 0.4%, but the acetic acid production rate could reach 15.21 gram of acetic acid per a gram of wet biomass. In the research, we also found that the production rate of acetic acid of acetic acid bacteria could be obviously accelerated by adding 1%~2% acetic acid to the medium at the beginning of fermentation. Besides acetic acid, propanoic acid and acetate also showed the abilities to accelerate acetic acid production, but the inorganic acid such as hydrochloric acid did not do. These results imply that the promotive role of initial acid on acetic acid produced by acetic acid bacteria might come from the acetate, not from the hydrion [H^+].

Keywords: Acetic acid bacteria; acetate; vinegar; liquid-state fermentation.

P-18
DEVELOPMENT OF TARTARY BUCKWHEAT VINEGAR WITH HIGH GABA AND D-CI

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The previous researches have shown that functional component contents in buckwheat grain can be significantly improved during germination. In this study, in order to develop a buckwheat vinegar with high contents of functional components such as GABA and D-CI, we used the germinated tartary buckwheat grain as raw material, and combined uncooked materials fermentation process to brewed the buckwheat vinegar following the traditional process of Shanxi mature vinegar. The results showed that the buckwheat vinegar developed in this study possessed pure hamony color, mellow taste, with 234 mg/100 mL GABA, and 75.05 mg/100 mL D-CI, which were 3 and 10 times higher than ones in the vinegar products on the market, respectively. So the physiological functions of these products were enhanced remarkably such as antioxidant, soften blood vessels, lowering blood sugar, blood lipid.

Keywords: Vinegar; tartary buckwheat; germination; GABA; D-CI.

P-19
PRODUCING MATURE VINEGAR WITH WHOLE INTELLIGENT EQUIPMENT

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Traditional ways of producing mature vinegar cannot meet the growing demand of quantity and quality therefore industrialized fermentation is the inevitable trend of vinegar industry development. However, there are still some issues that current industrialized ways cannot solve, such as loss of acetic acid, choking environment and expensive labour cost. In the past 8 years, Shanxi Liangfen Vinegar Co., Ltd has developed a whole intelligent producing line. With the technology of Complex Probiotics and targeted control, Shanxi Liangfen Vinegar Co., Ltd can provide personalized vinegar for different consumers, increasing the influence of Chinese traditional grain brewing technique.

Development of Complex Probiotics: microbial species and quantities is the foundation for industrialized solid-state fermentation. Accordingly, the company has created a samples database with more than 60 microbial species, which can be classified into six function groups: alcohol-production, acetic acid-production, aroma-production, organic acid-production, amino acid-production and others. Through analysis of bacterial community structure and variation by using DNA fingerprinting patterns, the company can provide safety, delicious and personalized vinegar for different consumers.

Technology of targeted control on solid-state fermentation: after 8 years continuous improvement, we finally realized the goal to produce vinegar under a standardization, intelligent, modularization solid-state fermentation line. This achievement will make us one of the technological leaders within vinegar industry, as well as will allow as to initiate a new sealed solid-state fermentation pattern. We believe the new fermentation pattern will allow to produce different vinegar products more easily, to build different factories more quickly, to save energy and assure higher quality.

Keywords: Industrialized fermentation; personalized vinegar; Shanxi Liangfen.

P-20
RESEARCH ON FERMENTATION WITH ONE LIQUID-PHASE AND DOUBLE SOLID-PHASES

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Shanxi aged vinegar is one of Chinese four famous vinegar, it has a long history, known as "the best in all the land of vinegar". Its main brewing process includes "cooking, fermentation, smoking, pouring and aging".

The traditional alcohol fermentation of Shanxi aged vinegar is liquid-state fermentation. The process of Fermentation with One Liquid-Phase and Double Solid-Phases includes two phases, one liquid and one solid.

The process is an innovation based on the traditional vinegar technology, namely increasing a solid alcohol fermentation phase before the traditional acetic acid fermentation phase.

This technology can reduce the alcohol concentration before the acetic acid fermentation phase with the synergy of *Daqu* and bran koji. Finally it reduces the traditional fermentation time by half, improves the production rate, the amino acid and nitrogen content and the flavor substances of the final product.

Keywords: Vinegar; aged vinegar; liquid-stae fermentation; solid-state fermentation.

P-21
CELLULOSE PRODUCING ACETIC ACID BACTERIA: SCREENING TOWARDS APPLICATIONS

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Bacterial cellulose is an insoluble, extracellular polysaccharide that is produced by different bacteria species. It is an attractive biomaterial having potential use in many fields as natural polymer or in composite materials due to its highly pure form, ultrafine reticulated structure and high tensile strength.

Among cellulose producing acetic acid bacteria (AAB), *Komagataeibacter xylinus* is a representative species of cellulose synthesis. In this study a pool of 50 strains were screened for their ability to produce cellulose. These strains were isolated from Kombucha tea, a slightly acid and sparkling beverage obtained by the fermentation of black tea through a symbiotic culture of yeasts and AAB. An exopolysaccharidic network floating at the surface of the liquid sustains microbial growth. In this study Kombucha tea was chosen as a selective source of cellulose producing AAB. First a molecular typing by (GTG) 5 fingerprinting was conducted and then strains were identified by a polyphasic approach.

Candidate strains were pre-selected on the basis of qualitative traits such as rapid growth on basic culture media containing glucose, ability to produce cellulose as a layer or deposit on the surface of tubes.

Quantitative assays were conducted by microscale tests to select the best candidate for optimised cellulose production in different culture conditions.

Results suggest that selective strain isolation was an appropriate strategy to recover high cellulose producer strains. Selected strains showed variability in cellulose yield in static and modified culture conditions. The pool

of strains collected and typed are a microbial resource to study mechanisms of cellulose synthesis, to develop tailored cellulose processes and further optimization aimed to increase cellulose yield.

Keywords: Biomaterial; cellulose; Komagataeibacter xylinus.

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Index of Authors