Validation Test Report

Simulated evaluation test for the improvement of Alcohol to Acetic acid liver enzyme conversion efficacy with Entropy beverages treatment

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ABSTRACT:

The potential of using Entropy pulsating capacitive field treatment on alcoholic drink to quicken the conversion of alcohol to non-toxic acetic acid for reducing the health risk of the untreated alcoholic drink consumption is demonstrated in the simulated test conducted. In this simulated test, conductivity of the treated alcoholic drink increased more than untreated drink at the end of the reaction, catalyzed by the acetoacetic bacteria from the mother of vinegar. This indicates that acetic acid amount has increased, and GC acetic acid test result further confirmed the nett increase of the acetic acid is 411ppm in untreated whisky and 472ppm in Entropy treated whisky. A 14.8% (61ppm/411ppm x 100%) nett improvement in alcohol to acetic acid conversion rate for the treated whisky over untreated whisky. This is a clear indication that the treated whisky can accelerate the alcohol to acetic acid conversion.

In addition, after addition of hydrogenase enzyme, the untreated whisky has a higher acetaldehyde content, increased from 174 ppm to 185 ppm at end of reaction. For Entropy treated whisky the acetaldehyde content reduced significantly from 174 ppm to 150ppm. The mildly toxic aldehyde reduced at much faster rate in the Entropy treated whisky than the untreated whisky, hence reducing the negative toxic effect of alcohol.

KEY WORDS: Entropy Treatment Process, Conversion of Alcohol to Acetic Acid, Antioxidant Effect, Alcoholic Drink

INTRODUCTION:

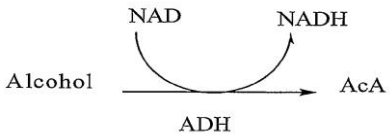
Alcohol Metabolism in Human Body

There are several factors that affect the metabolism of alcohol in human body. Concentration of alcohol, blood flow at site of absorption, irritant properties of alcohol, rate of ingestion all play their own roles in alcohol absorption. In general, less than 10% of alcohol intake is excreted in breath, sweat and urine. More than 90% of them are removed by oxidation. Some of the alcohol ingested orally does not enter the systemic circulation as they are oxidized in the stomach by ADH (alcohol dehydrogenase) isoforms. Lee SL et. al. (2006) revealed that the higher levels of alcohol metabolizing enzymes in the liver compared to the stomach makes the liver the major organ that

metabolizes alcohol. Alcohol is metabolized through a complex catabolic metabolic pathway. Serval enzymes are involved in processing the alcohol (or ethanol) first into acetaldehyde and further into acetic acid. It is finally converted to acetyl-CoA and becomes a substrate for the citric acid (Krebs) cycle, ultimately producing cellular energy, water, and carbon dioxide.
Ethanol has a caloric value of about 7 kcal/g (carbohydrates and protein produce 4 kcal/g and fat produces 9 kcal/g). Unlike carbohydrates (glycogen in liver and muscle) and fat (triglycerides in adipose tissues and liver) which can be stored and utilized in time of need, the alcohol can not be stored in liver. It remains in human body as liquid forms until it is eliminated. There is little hormonal regulation to pace the rate of alcohol elimination. Thus, it is a major burden on the liver to oxidize the alcohol to remove this agent from the body.

The main reaction scheme of alcohol oxidation in the human body is as follows:

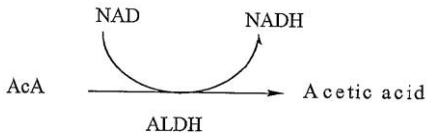
Eriksson and Fukuago (1992) reported that 5% of the ethyl alcohol (ethanol, C_2H_5OH) ingested by a human being is excreted unchanged while the remaining 95% is first degraded to acetaldehyde (CH_3CHO) in the cells of alcohol-metabolizing tissues, mainly in the liver. This reaction 1 as shown below takes place in the cytoplasm of hepatocytes and is catalyzed by the local enzyme alcohol dehydrogenase (ADH). The reaction uses one molecule of the coenzyme nicotinamide-adenine dinucleotide (NAD) per molecule of alcohol.



$$CH_3CH_2OH + NAD^+ \rightarrow CH_3CHO + NADH + H^+$$

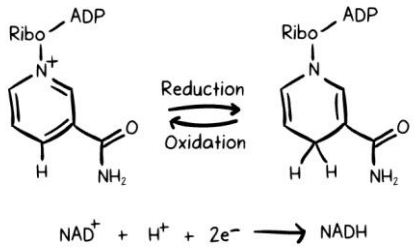
In the reaction, NAD and ADH form an enzyme=coenzyme (ADH-NAD) complex, at the same time NAD being concurrently reduced to NADH. The NADH is then detached and the ADH is ready to repeat the reaction by accepting a new NAD molecule. The cell has a limited capacity to oxidize NADH back to NAD, which determines the maximum velocity of the reaction. A normal liver metabolizes alcohol at the rate of about 8 g/h. This rate is independent of the concentration of alcohol in the blood. There is always an excess of ADH enzyme for this reaction.

The acetaldehyde molecules converted from the alcohol will then move into cytoplasmic organelles known as mitochondria where they are oxidized to acetic acid (CH_3COOH) in the reaction 2 as shown below. This acetaldehyde oxidation reaction is catalyzed by the enzyme aldehyde dehydrogenase (ALDH).



$$CH_3CHO + NAD^+ + H_2O \rightarrow CH_3COOH + NADH + H^+$$

In this reaction, one molecule of the coenzyme NAD is reduced to NADH. Both the latter and the NADH previously accumulated in the cytoplasm are re-oxidized to NAD in the mitochondrial respiratory chain at the maximum capacity of this system. The capacity of the mitochondrial respiratory chain depends on the overall level of the body metabolism rate.



The metabolically harmless acetic acid that derived from the above-mentioned alcohol oxidization reaction will be further oxidized to carbon dioxide and water mainly in extrahepatic tissues. When the capacity of cells to oxidize NADH back to NAD is exceeded during alcohol degradation according to Reaction 1 and 2, cells will accumulate an excess of NADH compared with NAD. This change in the cellular oxidation -reduction equilibrium, which always takes place in connection with alcohol metabolism, it causes inhabitation of NAD-mediated enzyme reaction in a typical normal metabolism of hepatocyte. This will impact the citric acid cycle when there is a positive NADH/NAD ration. This is believed to be the most important reason for the development of alcohol-induced fatty liver.

In a normal liver, 99% of the alcohol brought by blood circulation is metabolized to acetic acid. The remaining 1% is released as acetaldehyde into the circulation. The capacity of the alcohol-metabolizing tissues is not sufficient to oxidize all the acetaldehyde formed in reaction 1 to acetic acid by reaction 2. This is supported by Eriksson and Fukunaga (1992) work which reported that the venous blood flowing out of the liver during alcohol metabolism carried a 15 μM of acetaldehyde.

High consumption of the alcohol will develop the hangover symptoms and major part of this symptom is due to the toxic effects of acetaldehyde. Other unpleasant symptoms associated with alcohol drinking that last for several hours include headache, flushed skin, dyspnea, and nausea. These are due to the acetaldehyde accumulation in the body. Biochemical and medical research also suggests that acetaldehyde plays a major role in the development of alcohol dependence. This is based on the finding that acetaldehyde can induce changes in the structures of cerebral neurotransmitters. It can inhibit enzymes involved in protein synthesis and alter the immunological properties of tissues. They are believed to play a more significant role than alcohol in the aetiology of many alcohol-related diseases, such as brain damage, hepatic cirrhosis, and compulsive drinking. In view of this, some acetaldehyde binding compounds have developed to reduce the amount of acetaldehyde released into the systemic circulation and lessen the consequences of such release. However, Tabakoff et al. (1989) had reported oral administration of such binding agent like methionine has yield 20% reduction in blood acetaldehyde concentrations. It also cautions that this binding complex may late detach, and they do not affect the rate of alcohol metabolism nor the NADH/NAD ratio.

From the viewpoint of lowering the health risk of alcohol drinking, the desirable alcohol oxidization process shall be the conversion from alcohol straight to acetic acid avoiding stopping at the intermediate conversion to mildly toxic Acetaldehyde stage or accumulation of acetaldehyde. This incomplete enzyme conversion product can cause headache and hangover. In serious cases, it may lead to liver damages including alcohol fatty liver disease and cirrhosis. Any ways of boosting liver enzyme metabolism and allowing better conversion of alcohol to non-toxic acetic acid is worth exploring for a healthier way of alcohol drinking.

Simulated Test Using AAB as Liver Enzyme in Alcohol to Acetic Acid Conversion Process

A practical simulation test for the evaluation of the liver enzyme conversion rate for alcohol to acetic acid is to use the acetoacetic bacteria which contains the same enzyme as in the liver. Both the liver cell and acetoacetic bacteria are single cell in nature and produces the same alcohol enzymes. As such, simulated test employing the addition of known amount of acetoacetic bacteria to the untreated and treated alcohol drink is an appropriate lab based simulation.

Acetic acid bacteria (AAB) first described as "Vinegar bacteria" by Louis Pasteur over 150 year ago, are important and diverse group of bacteria involved in the production of acetic acid (ethanoic acid) in the making of vinegar. AAB are characterized by their ability to perform the oxidative fermentation process from which they are gleaning metabolic energy (Taban & Saichana, 2017). This process is carried out by membrane dehydrogenases. AAB are gram-negative or gram-variable, obligate aerobes, and are classified in the family *Acetobacteraceae*. Malimas, et al. (2017) describes that AAB are non-spore forming, ellipsoidal to rod-shaped cells that can occur singly or in short chains. They have a strong ability to produce acetic acid and show high resistance to high ethanol and acetic acid levels. The ethanol oxidation performed by them is catalyzed by two membrane-bound enzymes located on the outer surface of the cytoplasmic membrane, Ethanol is first oxidized to acetaldehyde by a PQQ-dependent alcohol dehydrogenase (ADH) and acetaldehyde is further oxidized to acetic acid by aldehyde dehydrogenase (ALDH). ADH is stable over a broad pH range of 2.3 to 8.0 and retained more than 90% activity when incubated on ice for 30 min. ALDH is also stable at acidic pH (optimum pH 4 to 5) and more heat stable than ADH (retaining more than 50% activity after 30 min at 60 ^{o}C as reported by Kanchanarach et al (2010)). Thus, it is ideal to use AAB from the 'Mother of Vinegar' for this simulation test.

The reaction kinetic of 40% Vol of ethanol hard liquor can be simulated using the acetic acid bacteria generated form the 'Mother of Vinegar'. The whisky of 40% vol of alcohol was used in the test. The presence of the reaction product (acetic acid) by a given reaction time can be detected by analyzing the reduced pH and increased electrical conductivity of the reaction solution. The Gas Chromatographic (GC) method- micro analytical procedures (APHA 5560D, Standard method 23rd edition) were carried out to verify the presence of the acetic acid as the reaction product.

This is a laboratory-based simulation test for the sole purpose as explained in the abovementioned. It is not a clinical test in any capacity.

MATERIAL AND METHODS:

Reagents Used

The Singapore Public Utilities Board supply water was used in this study. The high purity water for dilution was obtained from a Milli-Q system (Milli-pore Corporation, Australia) with a resistivity of 18.2 MΩ-cm and less than 50 μg/L of organic carbon content. The chemicals/reagents used in this study include stabilized extra pure 60% Acetic acid (Resource, Singapore), 40% Vol Whisky (Johnnie Walker, United Kingdom), Alcohol 5.5%Vol GUINNESS Foreign Extra STOUT (ST James; Fate Dublin, Ireland) and Raw (unfiltered) Apple Cider Vinegar with the 'Mother' (Bragg Live Food Products, USA).

Liquid Solution Analysis

The various water and reaction solution quality parameters were determined using the standard methods (APHA, 1998). The ORP measurement was carried by using Lutron (Lutron Electronic Enterprise, Tiwan) YK-23RP ORP meter and the ORP-14 electrode. A Thermo Scientific (Thermo Fisher Scientific Inc. Waltham, Massachusetts, U.S.) EUTEH pH 150 meters with a pH probe was used for water/reaction solution pH value measurements. Another Themo Scientific EUPEH CON 150 meter equipped with conductivity electrode (K = 1.0) was used to measure the conductivity. The Digital Themo sensor probe (for *in-situ* temperature measurement) of the Themo Scientific meter was immersed in the untreated and treated alcoholic drink after the Entropy treatment process. For the electrical conductivity measurement for the micro volume sample, the HORIBA (Horiba, Japan) Compact conductivity meter EC 22 was used.

Entropy Treatment Process

The Entropy unit (Ecospec NovelTech, 480 W, liquor chamber platinum emitter with 1 liter capacity, as shown in Figure 2) was turned on and whisky was filled in the liquor tank. Liquor chamber at the touch screen panel was selected and activated the Entropy treatment by pressing the start button. Treated whisky was collected in the beaker by pressing the dispense button. This treated whisky was then used for the various testing purposes.

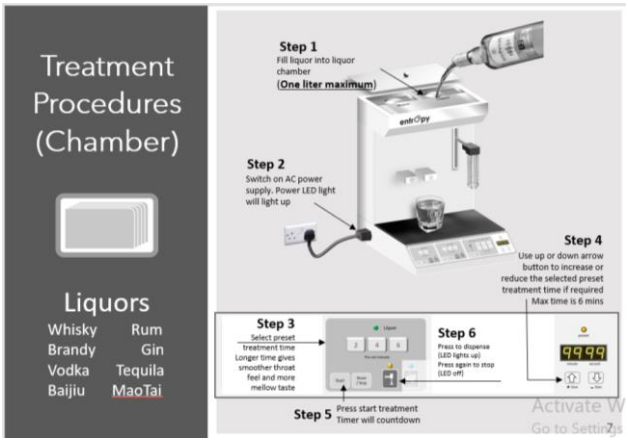


Figure 2 Entropy Treatment Unit

Simulated Test Using AAB as Liver Enzyme in Alcohol to Acetic Acid Conversion Process

The method to compare the rate of Alcohol Dehydrogenase conversion to Acetic acid between Entropy treated and untreated whisky is detailed as below.

The ADH enzyme was obtained from the acetobacter. These enzyme producing bacteria were cultured using the vinegar production method. The following is the details in producing the required 'Mother of Vinegar' used in the simulated test.

- Add 100ml unpasteurised Vinegar "with the mother" into 400ml of Guinness Beer (5.5% Alcohol)
- Cover with paper towel to prevent any contaminants (dust and flies) from entering the glass jar. However, air must be able to pass through.

Pictures below show that thin layer of cloudy cellulose starting to form on sample surface after 3 days of culture.

Day1

Day2

Day3

Day6

Mother of Vinegar (MOV)

Below table shows the parameters of Guinness Beer, Apple cider Vinegar and Mother of Vinegar:

	pH	Conductivity, mS/cm	ORP
Guinness Stout	3.73 / 23.8°C	2.14	+205
Apple Cider Vinegar "with mother"	3.10 / 23.8°C	2.54	NA
Mother of Vinegar / MOV (after 6days)	3.04 / 23.4°C	2.75	NA

Upon successfully producing the "floats" which is the acetobacter containing mother of vinegar, it was sampled and applied in the simulated test for the alcohol to acetic acid reaction process.

To simulate the conversion of alcohol to acetic acid in the liver cell, the Red Label Whisky is used as alcohol in the simulated alcohol to acetic acid conversion test. Its initial pH was 3.91 at 23.7°C and ORP +216mV prior to the test.

About 50mL of whisky poured from the bottle and treated with Entropy treatment process for 20 minutes. Each batch of whisky sample (untreated whisky and Entropy treated whisky) was divided

into 3 replicates with a volume of 10mL each. A portion of MOV (± 2.3gr) was then added into each sample. The A&D analytical balance model FX-300i with d=0.001 g (A&D company, UK) is used for this weighing purpose.

To simulate the similar process time in human digestion system, the sample reaction period is set at 2 hours. Another batch of 10mL untreated whisky sample without the addition of MOV was prepared as the blank test. After 2 hours, the pH, conductivity and ORP of the resulting whisky were then measured. These samples together with the blank sample were then subjected to the Gas Chromatographic test to determine the presence or absence of the acetic acid compound in them.

Acetic Acid analysis by Gas Chromatographic Method

The Agilent Technology model 7890B GC system which included the Gas chromatograph with a flame ionization detector, programmable column oven, Chromatographic columns and Guard column is used for the acetic acid analysis. The test procedure is in accordance with APHA 5560D, Standard method 23rd edition (2017). This method requires that acetic acid containing solution must be converted to protonated (volatile) form before injection into the gas chromatograph for vaporization to occur. The sample is analyzed by direct injection into a gas chromatograph equipped with a flame ionization detector after both centrifugation and filtration. Prior to the sample analysis, 1 µL of acetic acid calibration standard mixture concentrations is injected into the GC for calibration. The gas chromatograph for each sample analysis was printed by the chromatographic data system software for evaluation.

The simulation test and their respective GC analysis were conducted by the Marchwood Laboratory Service Singapore, affiliated with Marchwood Scientific Service (MSS) United Kingdom, Southampton.

RESULTS AND DISCUSSION

Validation Test of Electrical Conductivity as Evaluation Parameter for Alcohol to Acetic Acid Conversion Process

Six acetic acid samples of 10 ml each with different acetic acid concentration using mixtures solutions of DI water and 60% acetic acid were prepared and listed in the following table. All samples were then added with 1.6158 to 1.6199 gram of close similar weight of mother of vinegar.

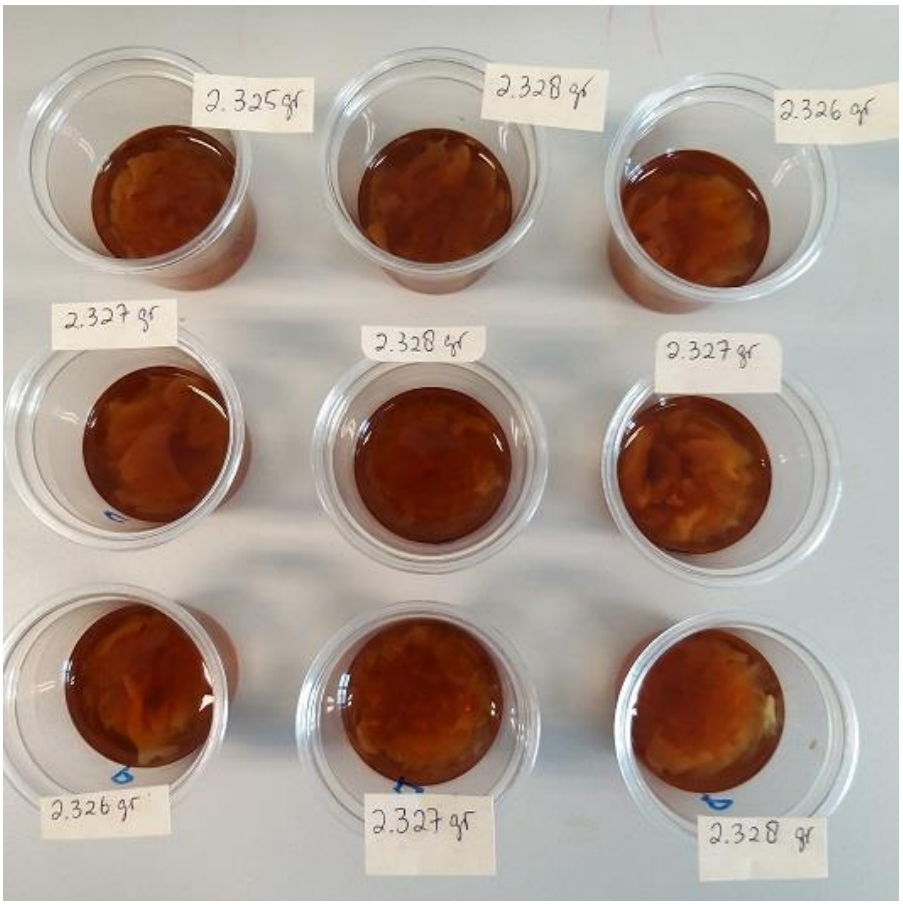
Solution	DI water volume, mL	Acetic acid volume, mL	Mother of vinegar, g
A	10.0	0.0	1.6159
B	9.9	0.1	1.6158
C	9.7	0.3	1.6191
D	9.4	0.6	1.6194
E	9.0	1.0	1.6161
F	8.0	2.0	1.6199

Parameter	Solution A	Solution B	Solution C	Solution D	Solution E	Solution F
pH / temperature	4.70 / 24.0°C	2.93 / 24.4°C	2.68 / 24.1°C	2.46 / 24.1°C	2.36 / 24.0°C	2.15 / 24.3°C
Conductivity, µS/cm	591	681	1040	1224	1482	1721

The above test result showed that with the presence of acetic acid, the conductivity of the DI water + acetic acid + MOV mixture increased, and pH reduced. Hence by measuring the conductivity of the solution, it provides a good qualitative indicator to the changes in acetic acid concentration.

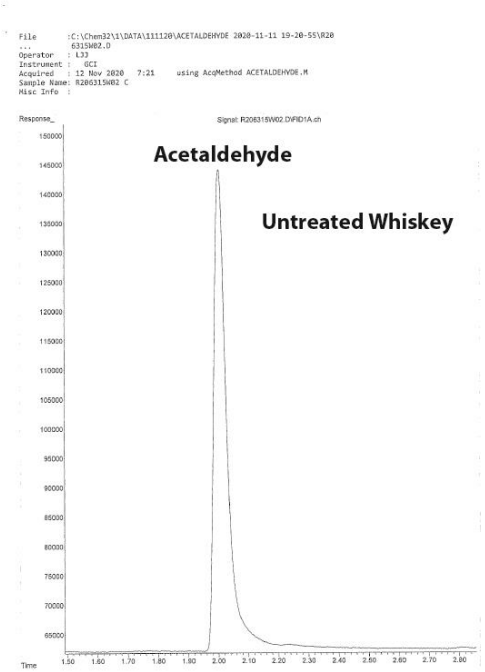
Effect of Entropy Treatment on Liver Enzyme Conversion of Alcohol to Acetic Acid Test

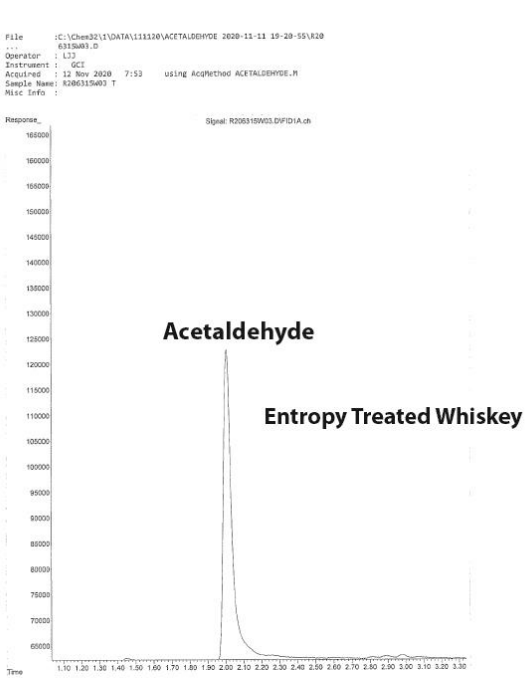
The cultured mother of vinegar MOV was used as hydrogenase enzyme for converting alcohol to vinegar. The triplicate samples for untreated and Entropy treated whisky, and their respective tests measurement are shown in the figure and table below.

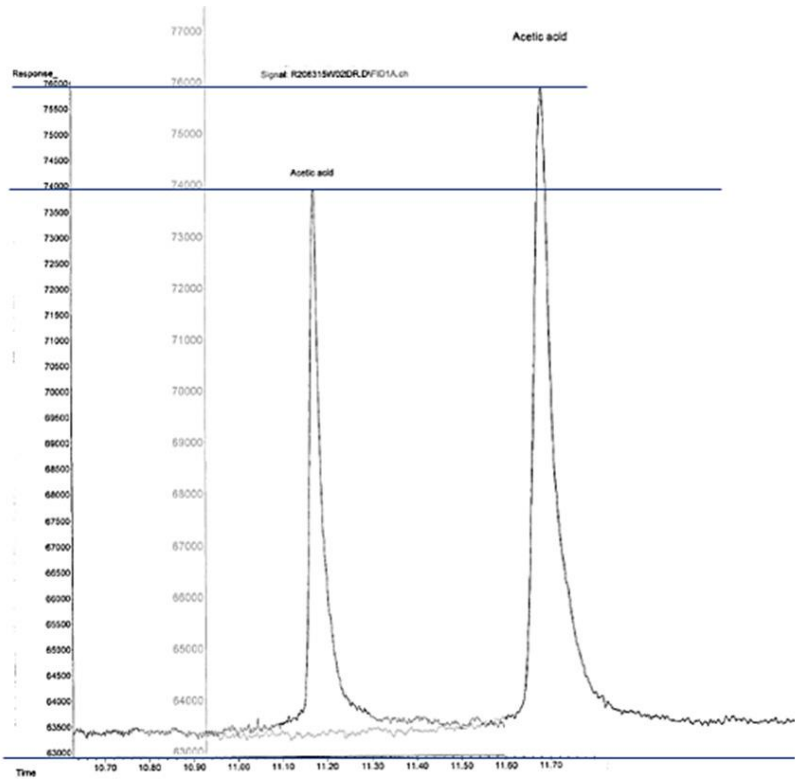


Solution Type	Volume of Whisky, mL	Weight of MOV, g	pH/Temperature °C	Conductivity, µS/cm	ORP, mV
Blank whisky without MOV sample	10	-	3.97/ 22.6°C	40.12	
	10	-	3.84 / 222.9°C	32.88	
	10	-	3.85 / 23.2°C	33.22	
Whisky with MOV at start of Test	10	1.5609	3.74 / 24.1°C	153.47	
	10	1.5621	3.74/ 24.1°C	154.76	
	10	1.5612	3.77 / 24.1°C	156.12	
Untreated whisky 2 hours after MOV addition	10	1.5636	3.40 / 23.2°C	170.7	+243
	10	1.5602	3.43 / 23.2°C	207.4	+232
	10	1.5625	3.43 / 23.3°C	179.3	+241
Entropy Treated whisky 2 hours after MOV addition	10	1.5683	3.44 / 23.3°C	173.1	+10
	10	1.5598	3.42 / 23.3°C	222.6	-15
	10	1.5697	3.44 / 23.4°C	201.5	-5

The acetic acid GC chromatogram for the untreated whisky and Entropy treated whisky are listed as follow:







The parameters for these alcohol to acetic acid conversion process was abstracted from Marchwood Laboratory Test report R206315 dated 16 November 2020 and summarized as follow:

Parameter	Blank / Whisky	Whisky with MOV at the start of the test	Untreated Whisky with MOV	Treated Whisky with MOV
pH	3.88	3.75	3.42	3.43
Conductivity, µS/cm	35.43	154.98	188.47	199.07
Acetaldehyde, ppm	174	-	185	150
Acetic Acid, ppm	74.4	1.617	2,028	2,090

The above test results show that conductivity of the Entropy treated whisky is higher than the untreated whisky. Since the acetic acid is the most conductive content in whisky, the higher conductivity result therefore indicates that acetic acid amount had increased, and GC acetic acid test result further confirmed this.

The nett increased of the acetic acid produced in the untreated whisky is 411ppm and the nett increase amount in the Entropy treated whisky is 472ppm. A 14.8% (61ppm/411ppm x 100%) nett increase in the alcohol to acetic acid conversion rate for the treated whisky as compared to the

untreated whisky. This is a clear evidence that the treated whisky accelerates the alcohol to acetic acid conversion.

In the human liver, the liver enzyme dehydrogenases can complete the conversion of 8 to 10 g of alcohol in one hour time.

After addition of mother of vinegar which contain AAB (to simulate liver enzyme dehydrogenases), the untreated whisky has a higher acetaldehyde content than the Entropy treated whisky (from 174 ppm to 185 ppm). Entropy treated whisky acetaldehyde content reduced significantly (from 174 ppm to 150ppm). This is another evidence showing Entropy treated whisky reduces the mildly toxic aldehyde quantity at much faster rate than the untreated whisky. Thus, the Entropy treatment to whisky can help reduce the negative toxic effect of alcohol.

CONCLUSIONS:

Undesirable acetaldehyde toxic intermediate product of alcohol to acetic acid conversion is greatly reduced in the Entropy treated alcoholic drink as compared to the untreated alcoholic drink. This is demonstrated in the simulated alcohol to acetic acid conversion test. In this test, conductivity of the resulting reaction solution of the treated alcoholic drink had increased under the reaction, catalyzed by the acetoacetic bacteria from 'mother of vinegar'. This is an indication that acetic acid amount had increased, and further GC acetic acid test result confirmed the nett increase in acetic acid. Acetic acid nett increase in the untreated whisky is 411ppm and in Entropy treated whisky is 472ppm. This means a 14.8%. (61ppm/411ppm x 100%) nett improvement in alcohol to acetic acid conversion rate. This is another clear evidence that treated whisky can accelerate the alcohol to acetic acid conversion. In addition, acetaldehyde content in the untreated whisky increased (from 174 ppm to 185 ppm) after addition of hydrogenase enzyme. Whereas the acetaldehyde content in Entropy treated whisky reduced significantly (from 174 ppm to 150ppm). This minimises the undesirable acetaldehyde health impact of alcohol via the faster accelerating alcohol to non-toxic acetic acid conversion rate and the simultaneous reduction of the mildly toxic aldehyde. The above lab simulation test results showed Entropy alcoholic beverage treatment has good potential for application in liver health protection by reducing the acetaldehyde toxicity in liver alcoholic beverages metabolic process. Further full human clinical trial may be conducted to further confirm the result.

NOTATIONS

The following symbols are used in this paper:

NAD^+ = Nicotinamide adenine dinucleotide

ADH = Antidiuretic hormone

ALDH = Aldehyde dehydrogenases

NADH = Nicotinamide adenine dinucleotide hydride (coenzyme 1)

ATP = Adenosine triphosphate ($C_{10}H_{16}N_5O_{13}P_3$, 507.18 g/mol) is an organic compound and hydro trope that provides energy to drive many processes in living cells, e.g. muscle contraction, nerve impulse propagation, condensate dissolution, and chemical synthesis.

CS = specific conductivity (□S /cm);

ORP = oxidation/reduction potential (mV);

GI = Gastrointestinal

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Appendix

Marchwood Laboratory Test report R206315 dated 16 November 2020

MLS

TEST REPORT

Our Reference No.	:	**R206315**	Date Received	: 11/11/2020
Project Code / Ref.	:	-	Date Commenced	: 11/11/2020
			Date Reported	: 16/11/2020
Customer Ref. No.	:	-		
Customer Name	:	Ecospec NovelTech Pte Ltd		
Customer Address	:	8 Admiralty Street #05-11, Admirax (Lobby 2) Singapore 757438		
Attention To	:	Dr Chua and Ms Ningsih		
Sample Description	:	12 Liquid Samples		
RESULTS:		Refer to Page 2		

Tan Thuan Piang
Technical Manager

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Website: www.mls.sg

R206315

RESULTS:

Test Parameter	Unit	Test Method	Sampling Date: 11/11/2020			
			Sample 1	Sample 2	Sample 3	Sample 4
			O (A)	O (B)	O (C)	C (1.56355)
pH	-	APHA 4500-H^+ (B)	4.0	3.8	3.9	3.4
Conductivity	µS/cm	APHA 2510B	40.1	32.9	33.2	179

Test Parameter	Unit	Test Method	Sampling Date: 11/11/2020			
			Sample 5	Sample 6	Sample 7	Sample 8
			C (1.56016)	C (1.56253)	T (1.56825)	T (1.55979)
pH	-	APHA 4500-H^+ (B)	3.4	3.4	3.4	3.4
Conductivity	µS/cm	APHA 2510B	207	179	173	223

Test Parameter	Unit	Test Method	Sampling Date: 11/11/2020			
			Sample 9	Sample 10	Sample 11	Sample 12
			T (1.56966)	O	C	T
pH	-	APHA 4500-H^+ (B)	3.4			
Conductivity	µS/cm	APHA 2510B	202			
Ethanol	mg/L	GC-FID		331,533	285,197	293,863
Acetaldehyde	mg/L	GC-FID		174	185	150
Acetic Acid	mg/L	GC-FID		74.4	2,028	2,090

Note:

1. APHA is a standard method for Determination of Water and Waste Water (APHA 23rd Edition, 2017)
2. "<" = Less than. The data reported is less than Detection Limit of the test.