Analytical Methods for the Selected Properties
Determination of 3-hydroxypropionaldehyde (HPA)

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3-hydroxypropionaldehyde (3-HPA) is an intermediate metabolite of glycerol metabolism towards the formation of 1,3-propanediol. Voisenet (1910) in fact has observed this substance and its formation from glycerol during bacterial spoilage of wine. Being one of the antibiotics and precursors for high quality plastics, unfortunately there are still no complete set of analytical data available for this substance. From the experimental data from this paper, NMR analysis using 2-H showed that –CHO bond of aldehyde observed at δ 9.5, RCH3 at δ 1.37, R2CH at δ 3.68, R3CH at δ 1.9 and so on. The carbon-13 NMR spectrum showed six signals, at chemical shifts of 40.1, 46.3, 56.2, 58.7, 89.7, and 207.7 ppm. In FTIR, spectral contained a broad O-H stretch band at 3,450 cm⁻¹ and a large peak due to C-O stretching at 1,050 to 1,150 cm⁻¹, indicating hydroxyl functional group. A C-H stretches at 2,880 and 1,380 along with a C=O stretch typical of aldehydes was present at 1,730 cm⁻¹. All the analytical information above is similar to the previous record (Talarico, 1989). GPC data, although not fully accurate, will help to estimate the molecular weight of 3-HPA as 257 with 63% errors. The HPLC data with three peaks may reveal the three components of 3-HPA available. SEM analysis did not reveal any important solid data but revealing its solid state as homogenous particle. By applying Circle plot, quantitative data on the concentration of 3-HPA synthesized chemically is estimated to be 19.62 µmol/ml at absorbance of 600 nm.

Keywords : 3-hydroxypropionaldehyde, NMR, acrolein, propanediol, glycerol fermentation

3-hydroxypropionaldehyde (3-HPA, β-hydroxypropionaldehyde, reuterin, CAS number 2134-29-4) is an intermediate metabolite of glycerol metabolism towards the formation of 1,3-propanediol, the precursor for high quality plastics. Reuterin or its synonym is known as one of the antibacterial agents that is normally produced by the Lactobacillus as growth inhibitor during the fermentation of milk. In glycerol reduction to 3-HPA, increasing glycerol content and uptake rate cause the accumulation of intermediate metabolite, 3-HPA, causing further the cessation of product formation and growth (Barbirato, 1996). Evidence has proved that reuterin is at the equilibrium state with β-hydroxypropionaldehyde as the mixture of monomeric, hydrated monomeric and cyclic monomeric forms as shown below, proven by the molecular weight of 148 analyzed by NMR data (Talarico, 1989). In the anaerobic conditions with semicarbazide hydrochloride, 3-HPA in accumulated in the fermentation broth, similar to that investigated by Abeles (1960) in the fermentation process involving Aerobacter aerogenes, but in aerobic conditions the accumulation of 3-HPA in glycerol by Klebsiella oxytoca has been observed too.

3-HPA is readily reduced to alcohol when NADH is available. Reduction of 3-HPA is physiologically important in order to detoxify cells, thus permitting the reoxidation of coenzyme for hexose fermentation, leaving more acetyl from sugar fermentation to generate ATP as energy source when the reduction of fructose to mannitol exists in the reoxidation of the coenzyme (Claisse, 2000). 3-HPA is used to function as oxidation agent for NADH to NAD⁺ in cell free extract during the accumulation of 1,3-PDO in cell culture (Toraya, 1980). In the presence of heat and hydronium ions in acidic conditions, the reactions tend towards
the formation of acrolein during distillation process (Pressman, 1942). It serves as one of the hydrogen acceptors during the fermentation of glycerol as: \( \text{CH}_2\text{OHCHOHCH}_2\text{OH} \rightarrow \text{CH}_2\text{OHCH}_2\text{CHO} \).

Despite various chemical and biological studies on 3-HPA, unfortunately up to now there is still no complete set of experimental data available. The main objective of this paper is to outline a few properties available for this peculiar substance based on the instrumentation analysis like NMR, FTIR, HPLC, GPC and SEM in addition to traditional colorimetric method. It is hopefully too that complete analytical data of this substance could be available in various handbook in future. There is still no complete set of data available for such material. The importance of the findings may assist the exploration of new forms of biomaterial available in nature.

MATERIALS AND METHODS

CHEMICAL SYNTHESIS OF 3-HPA: 100 mL Acrolein (90%, Aldrich) was first distilled. 10 mL was stored at 4°C for use latter. 100 ml 1.5 M sulphuric acid (3N H\₂O) was mixed with 75 ml acrolein into a round bottom flask containing RO water 325 mL. The mixture was contained in a 50°C water bath for 3 hr before being placed on ice. Calcium carbonate (CaCO\(_3\), BDH / Merck Chemicals) in the solid form was added through neutrality, shown by universal pH indicator strips. The solution was then filtered in a sintered funnel with residues washed with RO funnel in vacuum. The solution was latter transferred to a separating funnel, washed with 3 times volumes of dichloromethane (DCM). The water layer was collected and evaporated at 50°C using a high vacuum rotary evaporator.

The remaining layer was subjected to proton NMR analysis along with thin-layer chromatography (TLC) using a 1:1 acetone : ethyl acetate separate solvent mixture. TLC plates were visualized by using Goofy’s Dip (Table 1). A sintered funnel was then filled halfway up with silica gel (about 100 ml funnel). Synthesized material was then dissolved in equal volumes of acetone-acetate mixture. Vacuum was applied then until the material was immersed into the silica. About 20-30 mL of solvent was poured on top then. Vacuum was applied until the solvent had run through. The process was repeated for three times and each volume solvent latter was collected in separate round bottom flask. Solvent then was evaporated off using the rotary evaporator. The remaining compound was analysed by H-NMR using D\(_2\)O as solvent (Bruker, AV 400, 400 MHz).

<table>
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<th>Table 1 : Formulation for “Goofy’s Dip”</th>
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<td><strong>Stock Solution</strong></td>
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ANALYSIS OF THE PURIFIED PRODUCT

NUCLEAR MAGNETIC RESONANCE (NMR) : Proton NMR studies were carried out with both deuterium oxide and deuterated methanol (Cambridge Isotope Laboratory, www.isotope.com) and carbon-13 NMR studies were carried out with deuterium oxide. Proton NMR analyses (including decoupling studies) were conducted on an FTNMR (no. WM 250; Bruker Instruments Incorporation, Billerica, Mass) operated at 250 MHz. Carbon-13 spectra were generated on an FTNMR (NR-100 AF; IBM) operated at 25 MHz with a superconducting magnet.

FOURIER-TRANSFORM INFRARED SPECTROSCOPY (FTIR) : FTIR analysis was carried out on purified 3-hydroxypropionaldehyde (3-HPA). 3-HPA was dissolved in HPLC-grade methanol and evaporated onto sodium chloride absorption cells (Sargent-Welch Scientific Company, Skokie III). Analysis was done on an FTIR spectrometer (no. 1550; the Perkin-Elmer Corp., Norwalk, Conn), and data were manipulated on a data station (no. 7500, Perkin-Elmer). Samples were scanned from 600 to 4,000 cm⁻¹, and the curves were smoothed by using the auto program available with the Perkin-Elmer software.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) : The 3-HPA sample was purified for the detection of the components of the 3-hydroxypropionaldehyde (3-HPA) system. About 50 µL of the sample was injected into the HPLC column of 150 x 4.6 mm Alltima C18 with channel 410. The mobile phase applied is 10% methanol and 0.1 % trifluoroacetic acid (TFA) and the aqueous method set consists of 100% A PDA R1.

GAS PHASE CHROMATOGRAPHY (GPC) : Molecular weights of the predicted polymer were characterized by gel permeation chromatography (GPC) performed in tetrahydrofuran (THF, 1.0 mL/min) at 25°C using a Waters GPC instrument, with a Waters 2414 Refractive Index Detector, a series of four Polymer Laboratories PLGel columns (3 * 5mm Mixed-C and 1 * 3mm Mixed-E), and Millennium Software. The GPC was calibrated with narrow polydispersity polystyrene standards (Polymer Laboratories EasiCal, MW from 264 to 256000), and molecular weights are reported as polystyrene equivalents.

SCANNING ELECTRON MICROSCOPE (SEM) : Sample of 3-HPA is initially placed in the vials and quickly cooled below the solidification point of 3-HPA. The SEM used is a Philips XL30 FESEM (Field Emission Scanning Electron Microscope). The cryogenic system used is a Polaron LT7400 Cryoprep, manufactured by Fisons Instruments. The accelerating voltage used is 2KV. Photo of the structure was then taken by Mark Greaves as shown in Figure 6.

3-HPA COLORIMETRIC QUANTIFICATION : To obtain standard curve, 0 – 6 µmol of acrolein was added to 6 ml of RO water in 50 ml disposable polypropylene Eppendorf centrifugation tubes. Then 4.5 ml of DL-tryptophan solution, consisting of 0.01 M of solution in 0.05 M HCl, stabilized with a few drops of toluene) and the 18 ml volume of HCl 37% were added immediately. In the 3-HPA quantification process, a 1-ml sample was mixed with 3 ml of HCl 37% and 0.75 ml of DL-tryptophan solution. For 20 minutes, mixtures containing the standards and samples were incubated in a 37°C water bath and optical density was measured at 600 nm and 1100 nm. Prior to the test of 3-HPA concentration
determination, 3-HPA samples were diluted with distilled water before mixing with reagent to ensure a final OD is less than 1.0. According to Lüthi-Peng (2002), this method allowed a precise quantification of 3-HPA using acrolein as a standard.

RESULTS AND DISCUSSIONS

CHEMICAL SYNTHESIS OF 3-HPA: In the synthesis of 3-HPA, multiple steps purification has been conducted in order to achieve the product of high purity. The process above is modified from that of Hall (1950). The acrolein or propenal in fact is a type of lachrymose and highly toxic chemical and must be handled with proper care in fume cupboard and all the waste from the acrolein should only be discarded in special organic waste flasks.

Since 3-HPA is not available commercially, the best way to obtain it is through the chemical synthesis via acid catalysis path from acrolein to 3-HPA. According to the present literature the product 3-HPA formed in the synthesis process has the boiling point of approximately 53°C but once cooling down process occur the 3-HPA has been converted to a viscous yellow liquid which is believed to be 3-HPA hydrate.

By using the sample of Soenju (2004), 3-HPA has been proven a type of unstable sample based on the random NMR analysis of the similar sample exposed to the temperature of 25°C. There are total 8 peaks detected in 19 April 2005, 14 peaks observed in 28 April 2005 and 10 peaks observed in 5 May 2005, indicating that there are some form of structural changes in the mixture of 3-HPA with deuterium oxide, possibly caused by the instability of the 3-HPA systems where the equilibrium exist in the hydrate, dimer and standard conditions. Minor impurities exists and some 3-HPA may be disintegrate into other by-products permanently or transiently, as in acrolein and other aldehydes, causing the fluctuations of the peaks of 1H-NMR spectrum. Based on the 1H-NMR spectrum of 3-HPA in deuterium oxide of Soenju (2004) and Talarico (1989) it is concluded that the modified chemical synthesis above is suitable for the production of 3-HPA. From Figure 2, more purified forms of 3-HPA could be observed using the evaporation and solvent extraction proposed above.
Figure 2: NMR 1-H Analysis of 3-HPA
NUCLEAR MAGNETIC RESONANCE (NMR) : The 1H-NMR of 3-HPA in deuterium oxide shows no peaks in δ6.5 (C=C), indicating that almost all the acrolein has been converted into products mainly of 3-HPA. Detailed analysis of NMR spectrum of 3-HPA indicates that –CHO bond of aldehyde observed at δ9.5, RCH₃ at δ1.37, R₂CH₂ at δ3.68, R₃CH at δ1.9, RORH at approximately δ2.6 and CH₂O at approximately δ3.7, whereas the sharp peak at approximately δ4.6 indicating the solvent peak with the structure CH(OD)₂.

Homonuclear coupling experiments revealed that the signals at 2.6, 3.68 and 9.5 were linked to that signals at 1.9, 3.7 and 5.0. Signal area ratio and splitting patterns together with the homonuclear coupling results indicate that two molecules were present in the aqueous solutions : 3-HPA and 3-HPA dimer. Some weak signals are also produced, indicating the possibility of the formation of dimer of 3-HPA and other side impurities.

The carbon-13 NMR spectrum showed six signals, at chemical shifts of 40.1, 46.3, 56.2, 58.7, 89.7, and 207.7 ppm. Please be noted that the signal at 207.7 ppm is not shown due to limited space but was interpreted as aldehydic carbon. Carbon bound directly to oxygen produces the peaks at 56, 58 and 89 ppm, and those at 40 and 46 ppm were interpreted as aliphatic moieties (Figure 1).

The proton NMR spectrum for 3-HPA in deuterated methanol also had three groups of signals, splitting results, signal area ratio and homonuclear coupling (data not shown) were interpreted to represent β-hydroxypropionaldehyde existing in the methoxy form, unfortunately this peak is not able to be detected because it is hidden by the signals of methanol present in the solvent of the carbon-13 spectral.

FOURIER-TRANSFORM INFRARED SPECTROSCOPY (FTIR) : Spectral contained a broad O-H stretch band at 3,450 cm⁻¹ and a large peak due to C-O stretching at 1,050 to 1,150 cm⁻¹, indicating hydroxyl functional group. A C-H stretches at 2,880 and 1,380 along with a C=O stretch typical of aldehydes was present at 1,730 cm⁻¹ (Figure 3).

![Figure 3 : FT-IR Analysis of 3-HPA](image)
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) : Three peaks has been observed (Figure 4) where there is a possibility that 3-HPA could be partially separated but more experiments will be required to confirm this matter. 3-HPA in fact could be converted to hydrate and dimer forms in equilibrium and further conditions of equilibrium require confirmation and additional experimentations. Based on the predictions of Stuart Litter, the peak exists between 2.00 to 3.50 minutes is estimated to be 3-HPA hydrate as more hydroxyl groups exists in the hydrate forms, facilitating its attraction to the mobile phase methanol and trifluoroacetic acid. HPA hydrate is the major composition with X mole % based on the area calculations. The second peak exists between 4.00 to 5.50 minutes, estimated to be HPA carbonyl, whereas the last peak and the smallest exists between 6.50 to 8.00 minutes, estimated to be HPA dimer because its high molecular weight has slowed down its motion during the carriage by mobile phase although having the same number of hydroxyl group per molecule.

Figure 4 : HPLC Analysis of 3-HPA

GAS PHASE CHROMATOGRAPHY (GPC) : The GPC data analysis performed by Ming Chen shows that 3-HPA in fact has no ability to form long chain polymer. Unlike hydrocarbons, 3-HPA can only form dimer with the molecular weight of 157. The result shown by the GPC data in Figure 5 has depicted that the data obtained was just 257, showing 63% difference from the true molecular weight value of 3-HPA dimer. This could be said satisfactory because a least it is understood that HPA is not just has its original hydrate and carbonyl forms, with the molecular weight of 82 and 69 respectively.

In fact GPC is the method that could only be used for the molecular weight determination of long chain polymer. For the known compound that has low molecular weight, it is expected that this method could be used to analyse whether polymerization exist in the tested sample in order to further predict the structure of low molecular weight molecule. Normally GPC is used for polymers of molecular weight greater than 10,000. For aldehydes like 3-hydroxypropanal and acrolein, it is expected that this method could be used to analyse the polymerization conditions for short molecular weight molecules.
Figure 5: GPC Analysis of Liquid 3-HPA

SCANNING ELECTRON MICROSCOPES (SEM): Figure 6 shows that the crystal forms of 3-HPA when being cooled down below its freezing point would show homogeneous content. There are no signs showing the differences between 3-HPA dimer, hydrate and carbonyl forms. Most content of the crystal are transparent. If the melting point of the 3-HPA component is different, it is expected that at least certain indication of crack could be observed in this matter but Figure 6 could not observe such conditions, probably due to the image formed by SEM could not see properly the actual structure in the solidification condition due to the blurring of the image containing small black and white dots interfering with the observation of the researchers.

The homogeneous oily condition of the 3-HPA produced may not allow proper observation too as the light scattered through the crystal 3-HPA does not show any sign of the difference in the crystal composition. Not much difference has been observed when the crystal surfaces are being scratched, when the trend of sticky oily surface could still be observed at different scratching conditions.
3-HPA COLORIMETRIC QUANTIFICATION: The Circle (1945) method is based on the colour produced by the acrolein condensation with tryptophan induced by the concentrated hydrochloric acid, capable of detecting as little as 5 µg acrolein. This method is suitable only in the absence of other aldehydes or in the concentrations lower than that of acrolein.

Two graphs were plotted with optical density against the aldehyde concentration under the wavelength of 600 nm and 1100 nm using the Spectrophotometer UV-1601 Shimadzu (UV Visible). From the analysis it is observed that absorbance is favoured by the low wavelength. For the wavelength of 600 nm, the graph plotted has the equation of \( A = 0.0395 \, C + 0.106 \) and for 1100 nm, the slope of the graph is slightly lower, with the equation \( A = 0.0203 \, C + 0.0733 \), where \( A \) is the absorbance or optical density and \( C \) is the aldehyde concentration in the unit of µmol in 6 ml tubes. The 3-HPA produced has ABS-600 of 0.881, indicating the estimated concentration of the substance is \( C = (A-0.106) / 0.0395 = 19.62 \, \mu\text{mol} / \text{ml} \).
CONCLUSIONS

3-HPA in fact is not a new substance that has currently explored but rather it had first discovered biologically by Voisenet who observed its formation from glycerol during bacterial spoilage of wine by *Bacillus amaracrylus* (Voisenet, 1910). Abeles (1960) too has confirmed that 3-HPA is a product of dehydration of glycerol.

The data provided in this paper will briefly depict the structure of 3-HPA based on its bonding analysis applying NMR and FTIR. The molecular weight has been tentatively estimated although it is not fully accurate but will only be used as reference using GPC. No significant information could be viewed from SEM analysis of solid 3-HPA but fortunately little information has found on HPLC chart, of that 3 peaks may represent three components available in this substances.

From the initial experimental data available for 3-HPA, it is understood that the potential application of this substances, up to now, is still not fully explored, especially its application as the precursor of 1,3-propanediol and as a consumable preservative in food processing industries.

ACKNOWLEDGEMENTS

I would like to thank my colleagues working in various private laboratories in Victoria, Australia that has help me to design and set-up the experiment. I am looking further funding to continue to work out the research in this area, and hopefully those sponsors who have similar objectives as me in the HPA exploration could contact using the information at the front page for further discussion.

LITERATURE CITED

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