A new application of deep eutectic solvent (DES) in fish preservation

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Abstract. Technology in fish preservation had gone through various scope in food industry. Application of new green chemistry of deep eutectic solvent (DES) as preservative capable to help in the prevention of fish contamination by pathogenic bacteria. The aims of this study were including the synthesis of DES, the evaluation of antibacterial activity and sterility testing of the DES using fish samples. The synthesis of choline chloride-based DES was prepared by mixing with acetic acid and oxalic acid respectively. Further testing was done using disc diffusion method for the analysis of the solvent’s antibacterial activity towards E. coli, S. aureus, B. subtilis, S. Typhi and K. pneumonia. The sterility evaluation on the preservative solvent was carried out using fish samples in different temperature and time of storage. Results analysis showed potential of the DES in antibacterial activity even at low concentration of mixture. Sterility test showed promising result by maintaining pH of the solvent at 6-7. Reduction of bacteria concentration was found in the bacterial-spiked fish samples when preserved in the DES mixture. Thus, the study proved the potential of DES as a good preservative for fish compared with the controls from single component of choline chloride, acetic acid and oxalic acid respectively..

1. Introduction

The preservative plays a crucial role in reducing food spoilage by preventing from deterioration of food systems. For the last decade, people used various methods for fish preservation such as salting, drying and dehydration, chilling and freezing[1], fermentation[2], smoking and pickling. Commonly nitrate salts has been added as preservative for meat and fish. However, nitrate salts were found to be harmful to human[1].

Therefore, an initiative to explore new alternative medium that can replace those harmful preservative is needed. Numerous studies showed that the organic acids such as lactic acid, acetic acid and citric acid were used at high concentration on food as food preservatives[3]. Unfortunately, the antibacterial effectiveness of these acids is weak.

In this study, an alternative solution had been made by preparation of new green solvent known as deep eutectic solvent (DES). DES is the mixture of two compounds which are ammonium salt and hydrogen bond donor[4]. DES is well known for its unique properties such as low cost of components, easy to prepare, tunable physicochemical properties, negligible vapour pressure, non-toxicity, non-reactivity with water, non-flammability, conductivity, bio-renewability and biodegradability[5].
The aims of this study were to synthesis the DES, evaluated the antibacterial activity and sterility testing of the DES using fish samples. In order to achieve the objective of this study, the DES composed of choline chloride (ChCl): oxalic acid and ChCl: acetic acid were synthesized and characterized to inhibit the bacterial growth in fish. The new preservatives were expected to be beneficial for both environment and society.

2. Method

2.1 Synthesis of DES
Carboxylic acid based deep eutectic solvents were prepared by adding ChCl to different hydrogen bond donours: acetic acid and oxalic acid at 1:2 molar ratios. 5.7 ml of acetic acid was mixed with 7.0 g of ChCl while 7.2 g of oxalic acid was mixed with 5.6 g ChCl. The mixture of the two compounds were then heated up to 50°C for 15 minutes and then cooled down slowly to room temperature.

2.2 Antibacterial Activity
The antibacterial activities of DESs against the foodborne pathogen were assessed quantitatively by evaluating the presence of inhibition zones using disc diffusion method. This assessment was measured for 5 strains of bacteria, Escherichia coli (E. coli), Salmonella Typhi (S. Typhi) and Klebsiella pneumonia (K. pneumonia) which are gram negative bacteria while Staphylococcus aureus (S. aureus) and Bacillus subtilis (B. subtilis) as gram positive bacteria.

2.3 Sterility Testing
The fish blocks with range around 19-25 g were immersed with the carboxylic acids based DES in the containers for 30 minutes and quickly rinsed behind. The fish blocks were prepared by wrapping with plastic food wrapper in individual packaging and each of the fish block were placed at 10°C and room temperature (25°C).

At the 0 day, the sterile cotton-tipped swab was used to swab the entire part of the fish. The surface of a nutrient agar was swabbed with cotton swab from the fish and the agar was incubated at 37°C for 18 hours. The formation of the colonies was observed for the next day. The control samples was prepared without the use of any chemicals and tested using the same procedure.

The fish were evaluated at day 0, day 1, day 3, day 7 and day 10. For each subsequent day, the samples that observed to have formation of the bacteria colonies were further tested using gram staining method for identification and classification of bacteria. At day 3, serial dilution was done for each sample to determine the number of bacteria per unit volume in the original culture.

2.3.1 Measurement of pH samples
The pH paper was used to determine the pH of fish samples at all tested conditions from the day 0 to the day 10.

2.3.2 Colonies Observation
The colonies formation was observed in all the fish samples from the day 0 - 10. The colonies were counted for each agar plates.

2.3.3 Serial dilution method
The fish sample at day 3 was immersed in 0.9% NaCl solution (saline water) in the beaker. Eight test tubes with 9 ml of saline water were prepared in the test tube rack and were labeled as 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸. An amount of 1 ml solution from the beaker containing fish was transferred into the test tube labeled 10⁻¹ containing 9 mL of saline water and the solution was mixed thoroughly. For second dilution, 1 ml of solution from test tube 10⁻¹ was transferred into the test tube labeled 10⁻². Thoroughly mixed the test tube 10⁻² before added to the next test tube. The procedure was repeated until reached test tube 10⁻⁸. For each dilution tubes of 10⁻⁴,10⁻⁷ and 10⁻⁸, an amount of 100 μl was draw out.
onto the nutrient agar plate. The solution was spread for the entire surface of the agar and incubated at 37°C for 18 hours. Any formation of bacteria colonies was observed and count for determination of bacteria concentration in CFU/ml. The formula as followed below:

$$\text{CFU/ml} = \frac{\text{Number of colonies}}{\text{volume of culture plate x dilution factor}}$$ (1)

3. Results and Discussions

3.1 Antibacterial Activity

The potential of prepared DESs as antibacterial activity was assessed by using Kirby Bauer disc diffusion method. The inhibition zone were represented the efficacy of antibacterial properties in the materials. From the result portrayed in table 1, the ChCl: acetic acid had shown high antibacterial activity at different concentration against the tested bacteria strains. The bacteria strains showed susceptible towards the DES. While for the ChCl: oxalic acid, its showed low activity in antibacterial tests. The oxalic acid incapable to function without the mixture of ChCl. The tested DESs have shown a great potential as antibacterial agent than its individual components.

The study showed that the chemical interaction of each antibacterial agents was increased when the concentration was increased. The components in DES capable to disturb and damage the bacteria cells walls and retard the growth. This study has indicated that the combination of carboxylic acid and quaternary ammonium salt offers significant potential for the development of novel antibacterial cause by food-borne pathogen in the fish.

Table 1. The inhibition zone of bacteria using different concentration of 10%, 30%, 50% and 100% of ChCl, acetic acid, oxalic acid, ChCl: Oxalic acid and ChCl: Acetic acid.

| Bacteria | Samples       | Zone of Inhibition (mm) |
|----------|---------------|-------------------------|
|          |               | 10%  | 30%  | 50%  | 100% |
| E. coli  | ChCl          | 0    | 0    | 0    | 6    |
|          | Acetic acid   | 26   | 29   | 30   | 39   |
|          | ChCl: Acetic acid | 31  | 31   | 33   | 40   |
|          | Oxalic acid   | 0    | 0    | 0    | 0    |
|          | ChCl: Oxalic acid | 6    | 6    | 7    | 10   |
| S. aureus| ChCl          | 6    | 6    | 6    | 6    |
|          | Acetic acid   | 28   | 29   | 31   | 35   |
|          | ChCl: Acetic acid | 29  | 34   | 36   | 37   |
|          | Oxalic acid   | 0    | 0    | 0    | 15   |
|          | ChCl: Oxalic acid | 6    | 6    | 6    | 8    |
| K. pneumonia| ChCl           | 0    | 0    | 0    | 0    |
|          | Acetic acid   | 27   | 30   | 34   | 36   |
|          | ChCl: Acetic acid | 28  | 30   | 35   | 37   |
|          | Oxalic acid   | 0    | 0    | 0    | 0    |
|          | ChCl: Oxalic acid | 6    | 6    | 6    | 7    |

From table 2, the antibacterial potential of ChCl: acetic acid was further tested at lower concentrations (< 20%) in both bacteria strains of B. subtilis and S. Typhi. The DES showed antibacterial activity in all the low range of concentrations. Both strains of bacteria showed susceptible effect towards the DES. Hence, the result showed the effect of increasing the concentration of DES would increase the performance of DES.
Table 2. The inhibition zone of bacteria using different concentration of 2%, 5%, 10%, 15% and 20% of ChCl, acetic acid and ChCl: acetic acid.

| Bacteria    | Samples          | 2%   | 5%   | 10%  | 15%  | 20%  |
|-------------|------------------|------|------|------|------|------|
| B. subtilis | ChCl             | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 6.0±0.0 |
|             | Acetic acid      | 0.0±0.0 | 6.0±0.0 | 7.5±0.7 | 8.8±0.4 | 10.5±0.7 |
|             | ChCl: Acetic acid| 6.0±0.0 | 8.0±1.4 | 9.5±0.7 | 10.5±0.7 | 11.8±1.1 |
| S. Typhi    | ChCl             | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 6.0±0.0 |
|             | Acetic acid      | 0.0±0.0 | 6.3±0.4 | 7.5±0.7 | 9.0±0.0 | 10.5±0.7 |
|             | ChCl: Acetic acid| 6.0±0.0 | 7.3±0.4 | 8.0±0.7 | 9.8±0.4 | 12.0±1.4 |

3.2 Sterility Testing

3.2.1 pH of the samples

The pH for all samples were increased as the incubation time increases. As for the mixture of ChCl: acetic acid, the initial pH recorded at both room temperature and 10°C was similar which is at pH 4 at 0 day. The pH increased to 5 constantly until day 10 at 10°C and increased to 6 until day 7 to day 10 at room temperature. It shows that 30 fish samples had become less acidic as the day increased. The similar graph pattern was observed for ChCl: acetic acid. Since the oxalic acid was found to be more acidic than acetic acid, the initial recorded for ChCl: oxalic acid at day 0 was at pH 1. The pH of the fish at 10°C remain at pH 1 until day 7 and increased to pH 2 at day 10. Meanwhile the pH at room temperature started increased to pH 2 and pH 3 at day 7 and day 10 respectively. The control samples which were consist of raw fish showed similar pattern for both condition room temperature and at 10. The initial pH taken at day 0 was at 6 and change at pH 7 on the day 10 which was at neutral condition. This is because there is no chemical and acids were involved in these samples.

Figure 1. The pH of the fish in different types of DES against incubation day for ChCl: acetic acid and ChCl: oxalic acid at two different environment temperature of 25°C and 10°C.
The pH of the samples was also increased and become less acidic day by day. The longer the samples were kept in both conditions, the pH of samples become less acidic as compared to initial condition. The reason was because when the carboxylic acid was reacted with amine that already exists in the fish and formed ammonium carboxylic salt. The direct reaction of carboxylic acid with amine to produce amide which is harmful to human was not likely to occurred because the basic amine would deprotonate the carboxylic acid to form a highly unreactive carboxylate. Moreover, the ammonium carboxylate salt can be identified as weak acid or high base. As the pH increase, the acidity will decrease. In addition, the fish were kept until day 10 caused the chemical interaction between acid and fish become weaker. Overall, the test showed that as the incubation day increased, the acidity of fish will be decreased.

3.2.2 Observation of the colonies formation

The tests were done at the room temperature and 10°C. The result for ChCl: acetic acid at day 0 showed no colony formation on the agar plates after the washing with DES. The colony of bacteria started to form after day 1, but with low number of colonies of 3 and 4 colonies formation per plate at room temperature and 10°C respectively, it was considered as almost none. From these results, it showed that the DES has the potential ability to prevent the bacteria growth in the fish even at room temperature.

As the day increase, the colony was formed. The numbers of colonies were started to form when the fish were kept until day 3. Large colonies were observed on plate when the fish was kept at room temperature but when compared to at 10°C, small colonies were formed. Until day 10, the sizes of the colonies for the room temperature were decrease as only small colonies were formed on the plates. The results indicate that the DES was effective by reducing the sizes of the colonies formed.

Meanwhile for ChCl: oxalic acid, at day 0, there was no colony formed at both conditions. But when tested on day 1, the fish located at room temperature already produced large colonies while at 10°C maintained with no colony formed. It showed that the ChCl: oxalic acid more effective to inhibit bacteria growth when the sample was stored at 10°C compared to 25°C. The size of colonies formed at room temperature increased in longer incubation periods and vice versa when the sample kept at 10°C as the size were reduced when the sample incubation achieved at day 10.

Table 3. The morphology of colonies formation in ChCl: Acetic acid, ChCl: Oxalic acid and control at different condition of 25°C and 10°C.

| Incubation temperature | Samples          | D0 | D1     | D9     | D7     | D10    |
|------------------------|------------------|----|--------|--------|--------|--------|
| Room temperature 25°C   | ChCl: Acetic acid| -  | 3      | + (large) | + (small) | + (small) |
|                        | ChCl: Oxalic acid| -  | + (large) | + (small) | + (large) | + (large) |
|                        | Control          | + (small) | + (small) | + (small) | + (small) | + (large) |
| 10°C                   | ChCl: Acetic acid| -  | 4      | + (small) | + (small) | + (small) |
|                        | ChCl: Oxalic acid| -  | -      | + (large) | + (large) | + (small) |
|                        | Control          | + (small) | + (small) | + (small) | + (small) | + (small) |

The raw fish was used as a control without addition of any chemicals, the colonies were already formed at the day 0. The small colonies were observed for both samples at room temperature and 10°C. At day 10, colonies from sample of room temperature showed increasing of size while at the 10°C, the size of the colony remain small. Hence, the formation of colonies can be reduced compared to control when DES was used.
3.2.3 Serial dilution method

The serial dilution method was carried out at day 3 to determine the CFU/ml for each contaminated sample. The purpose of this method is to determine the number of bacteria per unit volume in the original culture as it reduces a dense culture of cells to a more usable concentration. From the result, the dilution factor for all the samples are at concentration of $10^{-5}$. The lowest colonies counting produced are from ChCl: oxalic acid plate in both room temperature and 10°C were $2.1 \times 10^6$ and $3.3 \times 10^6$ CFU/ml, respectively. The bacteria dose of standard value that can be ingested by human is less than $5.0 \times 10^6$ CFU/ml as recommended by public health which has been adopted by many countries\[8\].

From the calculated result, control test showed the highest colony formed unit (CFU/ml) on the day 3 with range from $7.0 \times 10^8$ to $8.0 \times 10^8$ CFU/ml while ChCl: oxalic acid recorded the lowest value which is $2.1 \times 10^6$ to $3.3 \times 10^6$ CFU/ml. Hence, it showed that control test is heavily contaminated and exceed the limit of bacteria dose that can be ingest by human. Thus, raw fish is not recommended to be consumed directly by human. When compared the value of CFU/ml of both DES, ChCl: oxalic acid showed less abundance of bacteria compared to ChCl: acetic acid. From the value, it can be concluded that the CFU/ml of ChCl: oxalic acid is less than the standard value of bacteria dosage, which means the numbers of bacteria in the fish treated with ChCl: oxalic acid are less than prohibited levels.

**Table 4.** The bacterial colonies formation (CFU/ml) from fish samples using ChCl: Acetic acid, ChCl: Oxalic acid at different condition of 25°C and 10°C.

| Samples and conditions | ChCl: AA 10°C | ChCl: OA 10°C | ChCl: AA RT°C | ChCl: OA RT°C | Control 10°C | Control RT°C |
|------------------------|---------------|---------------|---------------|---------------|--------------|--------------|
| Dilution factor        | $10^{-5}$     | $10^{-5}$     | $10^{-5}$     | $10^{-5}$     | $10^{-5}$    | $10^{-5}$    |
| Colonies counting (units) | 6800        | 5700          | 21            | 33            | 8000         | 7000         |
| Colony forming unit (CFU/ml) | $6.8 \times 10^8$ | $5.7 \times 10^8$ | $2.1 \times 10^6$ | $3.3 \times 10^6$ | $8.0 \times 10^6$ | $7.0 \times 10^6$ |

*RT: Room Temperature, AA: acetic acid, OA: oxalic acid

4. Conclusions

As conclusion, the results of analysis showed potential of the DES in antibacterial activity even at low concentration of mixture. Sterility test showed promising result by maintaining pH of the solvent at 6-7. Reduction of bacteria concentration was found in the bacterial-spiked fish samples when preserved in the DES mixture. Thus, the study proved the potential of DES as a good preservative for fish compared with the controls from single component of choline chloride, acetic acid and oxalic acid.

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