Comparison of Alcohol Free GSH Production by Ultrasonic and Homogenizing Method

Mohamad Ramlan Mohamed Salleh*[+]; Parveen Jamal; Abd. Elaziem Farouk Gad; Yumi Zuhanis Has-Yun Hashim; Rohani Salleh; Haslinda Hasim

Department of Biotechnology Engineering, Faculty of Engineering, International Islamic University Malaysia, Jalan Gombak 53100, Kuala Lumpur, MALAYSIA

ABSTRACT: *Homogenizer and ultrasonicator that we call as mechanical methods were used in the lab scale production of alcohol free reduced glutathione (alcohol free GSH) from baker's yeast. The study was done to determine the effects of specific parameters of the methods and the pH of the medium on the production of the GSH. The cell disruptions were run at a wide range of those parameters at the suitable common parameters of the methods. The disrupted cells suspension was then centrifuged at 12,000 rpm for 20 min and the GSH content in the supernatant was analyzed by taking the absorbance (A) value of the solution by spectrophotometer at 412 nm. The results obtained showed that the production of GSH was affected by the adjusted specific parameters of each method and the yield of production by the homogenizing and ultrasonic method was found 3.3 and 4.1 times, respectively, better than the yield of production by the unadjusted parameters. Ultrasonic method which produced 23.2 mg/L product was found 1.3 times better than the homogenizing method (18.5 mg/L) in producing the GSH. The result obtained from the lab scale study of the above mentioned mechanical methods showed that the implementation of them in the large scale production of the alcohol free GSH is possible.*

KEY WORDS: *Alcohol free GSH, Ultrasonic method, Homogenizing method, Baker's yeast, Cells disruption, Specific parameters.*

INTRODUCTION

GSH is a promising material that can be used as a protein flavor, antibiotic, and antioxidant [1] in the development of healthy/functional food which is currently becoming so popular. Accordingly, large amount of GSH must be isolated from various sources of it. Among the raw materials that contain GSH, yeast was found superior to others [2] and many studies had been performed to utilize it in producing the GSH [3-6]. In those studies the GSH were produced by the fermentation of yeast in the bioreactors. The production cost of this process is considerably high so that it is unaffordable by some of the producers/consumers. Furthermore, in those processes ethanol was not only being formed during the fermentation process but it was also being used as a solvent in the

* *To whom correspondence should be addressed.*
+ *E-mail: ramlan@iiu.edu.my*
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Table 1: Comparison between the mechanical methods run at their suitable common process parameters and the autolysis method.

Method	Suitable Values of the Common Parameters	GSH Concentration (mg/L)	Comparison With Autolysis
Autolysis	Concentration : 9 % (m/V) Temperature : 28 °C Duration time: 1 hour	15.1	–
Homogenizer	Concentration : 9 % (m/V) Temperature : 22 °C Duration time: 30 seconds	5.6	2.7 times less
Ultrasonic	Concentration : 6 % (m/V) Temperature : 22 °C Duration time: 15 seconds	5.6	2.7 times less

isolation/extraction of the GSH [7-12]. These discourage the producers who have restriction in dealing with capital/financial source and ethanol from implementing the methods. Accordingly, for the above mentioned reasons, it is in need to develop an alternatively simple and inexpensive method where no alcohol is accumulated or used during the production. In other words, there is a need to develop alcohol free method for the production of alcohol free GSH.

Autolysis method used in the isolation process of GSH can also be used as the production method of GSH if the amount of the GSH is not the major concern. It is actually the direct way of GSH production which can reduce the operational cost and shorten the production time. To show this, in our preliminary lab scale study, we had implemented the autolysis method which used water as a solvent (to avoid the use of alcohol). The process was done at the common process parameters and it was found that GSH could be produced in a short period of time (1 h) (table 1). Since the yield of GSH production was low, homogenizer and ultrasonicator (mechanical methods) which were reported as effective methods for the disruption of yeast and other microbial cells [13-16] were then used to produce the GSH at their common process parameters. As compared in table 1, the autolysis method was found to be better than the mechanical one. However, this method was considered inappropriate because unlike the mechanical methods, there were no more parameters that can be considered in enhancing the yield of GSH production by using it.

Thus, in the current study, the effects of specific parameters of the homogenizing and ultrasonic methods and also the effect of their pH medium on the production of the mentioned product were determined by running a lab scale production of them in order to evaluate the possibility of using those methods in a large scale production of alcohol free GSH. These methods are considered inexpensive (approximately 50 % lower than the operational cost spent by fermentation process) because they provide the direct way for the production of GSH since there is no fermentation process involved and water is being used as the medium. Moreover, the large scale production can be run easier due to fewer number of parameters compared to fermentation process.

MATERIALS AND METHODS

Preparation of yeast suspensions

Unfermented fresh yeast used throughout this experiment was purchased at mini market in Balakong, Selangor Darul Ehsan, Malaysia. This non alcohol raw material was used to make sure that the product would contain no alcohol. The specific genus of yeast was not used because we just wanted to evaluate whether the mechanical methods used in this study were suitable to be used in a large scale production of the GSH. The yeast was stored in the frozen box (4 ºC) and was taken out just before running the experiment to avoid contamination and the reduction of its enzyme activities which can reduce the production of GSH. Yeast suspension with the concentration of 9 and 6 % (m/V) for the homogenizing and ultrasonic method was prepared by dissolving 9 and 6 g yeast, respectively, in the distilled water which was then adjusted to the volume of 100 mL. The pH of suspensions was adjusted to the desired value used in the production process.

Autolysis method

The yeast cells were disrupted by autolysis method based on heat as a way to disrupt the cells. The disruption process was done inside a 500 mL beaker under the suitable values of common parameters for the method (yeast concentration, process temperature, and the

production time with the values of 9 % (m/V), 28 °C, and 1 h, respectively) and during the process the temperature was maintained at 22 °C with ice bath.

Homogenizing method

Yeast suspension (100 mL) was put inside a 500 mL beaker and was disrupted by a homogenizer (IKA® WERKE GmbH & Co KG, Germany). In all experiments related to this method, the process was done at the suitable values of common parameters for the method which were already found in our previous study (yeast concentration, process temperature, and the production time with the values of 9 % (m/V), 22 °C, and 30 sec, respectively) and similarly during the process, temperature was maintained at 22 °C with ice bath.

Ultrasonic method

The sonication process for the 100 mL yeast suspension was done inside a 500 mL beaker by using a sonicator (Braun Biotech International, Sortorius AG, GőHingen) with the 100 % amplitude of 210 μm through probe diameter of 3 mm. Similar to what was done to the homogenizing method, the suitable common parameters of this method (yeast concentration, process temperature, and the production time with the values of 6 % (m/V), 22 °C, and 15 sec, respectively) were set constant along the process and the process temperature was maintained at 22 °C by ice bath.

Effects of the specific parameters of the methods

The specific parameter for the homogenizing method is the homogenizer speed and the one for the ultrasonic method is the ultrasound amplitude. The effect of those parameters on the GSH production was investigated by running the experiments with the homogenizer speeds of 11,000, 13,000, 16,000, 19,000, and 22,000 rpm, and the ultrasound amplitudes of 20, 40, 60, and 80 %. In each of the experiment, the pH medium was maintained at its original value (about 4.5 to 5).

Effect of pH medium

In our previous study, pH value was not so much concerned because we thought that it was better to produce the GSH at its original pH because at that pH, it is suitable to be used as food ingredients (not bitter or too sour). But, this parameter cannot be neglected for it might significantly affect the GSH production. Therefore, in this study, by using the suitable value determined in the previous section for the homogenizer speed and ultrasound amplitude, the effect of pH medium in both methods was investigated accordingly using the pH values of 1, 2, 3, 4, 5, 6, and 7.

GSH analysis

To evaluate the GSH content in the supernatant, the absorbance (A) value of the solution was taken by using a spectrophotometer (SECOMAM, France) at 412 nm.

Statistical analysis

Statistical difference between the GSH produced by the three replicated samples was determined by the Student's t-test. The concentrations of the product were considered significant when the probability value was less than 0.05 ($P < 0.05$).

RESULTS AND DISCUSSION

The mechanical methods

For the feasibility study of the so called mechanical methods, in our preliminary study, we had compared the performance of those methods with that of the autolysis by water. The lab scale production of the GSH was run under the suitable common parameters of the methods at their original pH values (about 4.5 to 5). The results indicated that the produced GSH concentration for both methods which was about 5.6 mg/L was 2.7 times less than that of produced by autolysis method as compared in table 1.

At a glance, it seems that the implementation of these methods was impossible but due to the shorter period of the production time, the methods were considered worth to be used. In contrast to the time spent by the autolysis (1 h), the homogenizing and ultrasonic methods were able to produce 5.6 mg/L of product in a short period of 30 and 15 sec, respectively (table 1). Thus, these evidences have invited us to explore more about the methods in upgrading the production of GSH. Moreover, recently, *Lin et al.* reported that the formation of ethanol during the fermentation process had prevented the yeast from reaching high cell density and had decreased the yield of GSH on glucose [10]. Their findings had supported the future direction of our attempt in developing the GSH production methods within the alcohol free environment.

For that purpose, the methods were further developed

by running the experiments, first by using the various values of specific parameters of the two methods followed by the various values of the pH medium in order to determine the effects of those parameters on the GSH production. Finally, the suitable values for both common and specific parameters (the process parameters) of the methods in upgrading the production of the said product were determined.

Effect of the homogenizer speed

Fig. 1 shows the relation between the produced GSH concentration and the homogenizer speed for the process running by using various values of homogenizer speed at the suitable common parameters of the homogenizing method at the original value of pH medium. It is observed that the concentration of the product was significantly affected by the homogenizer speed. The concentration was found to increase up to the suitable value of the speed (13,000 rpm) where 8.8 mg/L GSH ($P < 0.05$) was being able to be produced. Then the concentration of the product was found reduced by the increase of the speed.

Up to 13,000 rpm, the higher speed has stimulated the higher concentration of the product. This was exactly in line with the finding of *Sauer et al.,* who had investigated that the increase in homogenizer pressure had increased the cells disruption of the native and recombinant *E. coli* [17]. It was also reported that increase of pressure had increased the shear force to the cells wall, resulting in an effective cells disruption [18]. As a result, in both cases, the production/isolation of the target products (GSH, in case of this study) was found more significant when the cells were effectively disrupted at the optimum homogenizer pressure (homogenizer speed, in case of this study).

Beyond 13,000 rpm, it was observed that the higher value of speed has stimulated lower concentration of product. In general, the increase of shear force/pressure on the cells wall by the increase of homogenizer speed is supposed to increase the production. However, it was not evident probably due to the deactivation of the product by the heat gained from the highly rotating homogenizer. Increasing the speed to more than 13,000 rpm had increased the process temperature to the higher value than that of the suitable one (22 °C). This high temperature caused the reduction of the GSH activity as also reported by *John* and *Alan* [19]. They found that the increase in agitation speed of the equipment used, had increased the temperature of the suspension resulting in loss of enzyme activity which resulted in less yield of the enzyme. In this study the temperature was able to be maintained at 22 °C only at the initial stage of the experiment but then was not so much cared because we just wanted to evaluate the possibility of implementing the methods rather than optimizing the process.

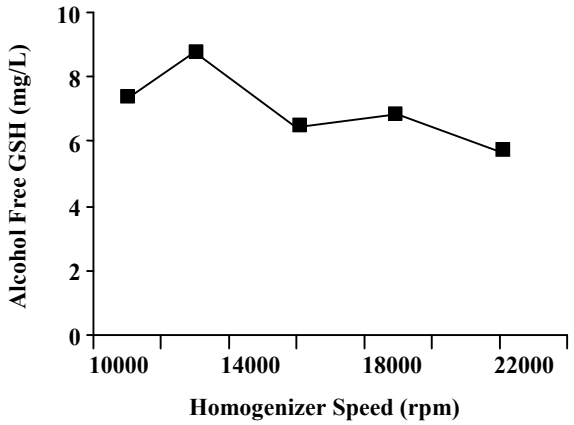

Fig. 1: Effect of the homogenizer speed on the production of GSH by homogenizer method. The process was run with the various values of homogenizer speed at the suitable common parameters for the homogenizer method at the original value of the pH medium. The plotted data were the average values taken from 4 experiments ($P < 0.05$).

Effect of the ultrasound amplitude

Fig. 2 shows the relation between GSH concentration and ultrasound amplitude for the sonication process run under various values of ultrasound amplitude at the suitable common parameters of the ultrasonic method at the original value of pH medium. The concentration of the product was increased up to the maximum amplitude of 60 % where the average value of 9.6 mg/L GSH ($P<0.05$) was produced. Then the concentration of the product was found reduced by the increase of the amplitude.

Up to 60 % amplitude, the higher value of amplitude has stimulated higher concentration of product. This phenomenon might happen because at the higher value of amplitude, the shear stress to the yeast cell wall was higher so that the cell walls were easily disrupted resulting in more production of GSH. This was in line with the findings of *Lyng et al.* [20] where in their study they had investigated that the increase in ultrasound frequency (amplitude in our study) had increased the breakage of the meat tissue.

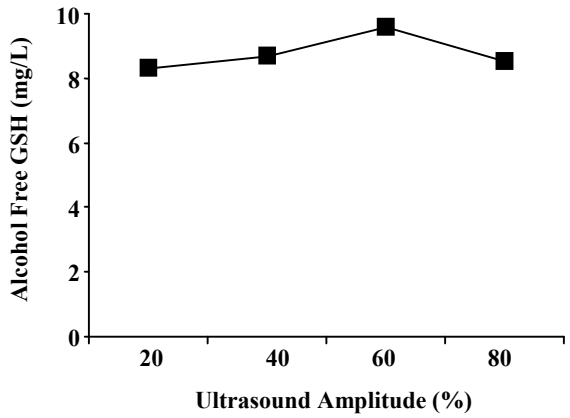

Fig. 2: Effect of the ultrasound amplitude on the production of GSH by ultrasonic method. The process was run with the various values of ultrasound amplitude at the suitable common parameters for the homogenizer method at the original value of the pH medium. The plotted data were the average values taken from 4 experiments (P < 0.05).

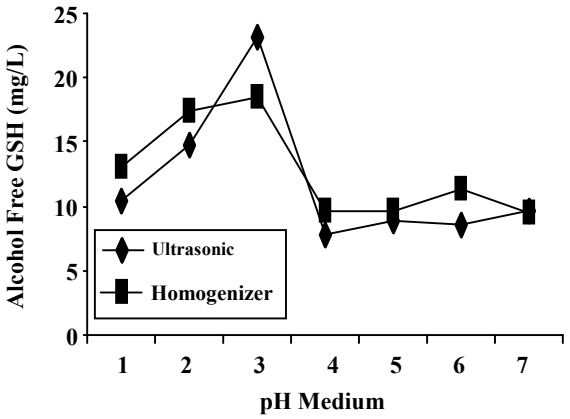

Fig. 3: Effect of the pH medium on the production of GSH by homogenizer and ultrasonic methods. The process was run with the various values of pH medium at the suitable process parameters for both of the methods. The plotted data were the average values taken from 4 experiments (P < 0.05)

Beyond 60 % amplitude, the higher value of the amplitude had increased the shear stress to the cell walls resulting in the increase of suspension temperature higher than that of the suitable process temperature for the sonication process as also observed by John and Alan in their experiments [19]. As a result, the activity of GSH was decreased and this was the acceptable reason why it was observed that at the amplitude higher than 60 %, the concentration of the product was found decreased.

Effect of pH medium

In fermentation, pH of the medium was controlled depending on the medium used and the values ranged between 5 and 7.2 [7-12]. In this study, since the medium used was distilled water, the suitable pH for maximizing the GSH production was determined using a large range of values from 1 to 7. Fig. 3 shows the GSH concentration produced from different values of pH medium by homogenizing and ultrasonic methods at their suitable process parameters.

It is observed that the concentration of the product was affected by the pH medium. The product was able to be produced at the highest value of 18.5 mg/L and 23.2 mg/L ($P < 0.05$) by the homogenizing and ultrasonic method, respectively, when the process was run at pH 3. Further increase in pH had reduced the concentration of the product. Similar tendency was also observed in the fermentation, where the lower pH value at the vicinity of 5 has stimulated higher yield of GSH than the yield obtained at the higher pH value at the vicinity of 7 [7-12].

General discussion

Table 1 presents the comparative data between the findings of our preliminary studies on the mechanical methods and autolysis method. Even though it was found that the yield of the two mechanical methods was 2.7 times less than that of autolysis method, it was believed that the methods were considered possible to be implemented since there were some more parameters that could be considered in improving the GSH production. Thus, we had tried to improve the production by running the experiments by determining the specific parameters of the methods. The yield of GSH was improved 1.7 and 1.6 times for the ultrasonic and homogenizing method, respectively. The study was further extended by determining the suitable pH value for both mechanical methods. As can be viewed from table 2, the yield of the GSH obtained by the homogenizing and ultrasonic method run at their suitable process parameters (common parameters, specific parameters, and pH medium) has been increased up to 3.3 and 4.1 times, respectively, better than that of obtained by the processes running at their common parameters (table 1).

The suitable values of the process parameters for the homogenizing method were found as yeast concentration of 9 % (m/V), process temperature of 22 °C, production

Table 2: Comparison between the mechanical methods run at their suitable process parameters with the one run at suitable common process parameters ([a]).

Method	Suitable Values of the Process Parameters	GSH Concentration (mg/L)	Comparison	
			Ultrasonic[a]	Homogenizer[a]
Homogenizer	Concentration : 9 % (m/V) Temperature : 22 °C Duration time: 30 seconds Homogenizer speed: 13,000 rpm pH medium: 3	18.5	–	3.3 times higher
Ultrasonic	Concentration : 6 % (m/V) Temperature : 22 °C Duration time: 15 seconds Ultrasound amplitude: 60 % amplitude pH medium: 3	23.2	4.1 times higher	–

time of 30 sec, pH 3 and the homogenizer speed of 13,000 rpm. The one for the ultrasonic method were yeast concentration of 6 % (m/V), process temperature of 22 °C, production time of 15 sec, pH 3 and the ultrasound amplitude of 60 %. Comparing between the two methods, it was found that the ultrasonic method was a little bit better (1.3 times) than the homogenizer one in producing the GSH. Similar observation was also reported by *Nandakumar et al.* [21] where in their study they had investigated that ultrasonic method was found superior than grinding (in case of this study, homogenizing) in disrupting the cell wall of the mucous producing cold-adapted bacteria. However, both the mechanical methods were found better than the autolysis one.

Even though the effectiveness of the production was less satisfied but the production time was found very short which was 30 and 15 sec for the homogenizing and ultrasonic method, respectively. In another words, repeating the simple procedures of these two methods will make it possible to enhance the GSH production. Thus, the lab scales study of those mechanical methods in the production of alcohol free GSH indicated that the use of them in a large scale production of the alcohol free GSH is possible. The product is considered free from alcohol because the raw material and the methods used were free from alcohol.

CONCLUSIONS

From the results of these studies, the conclusion that can be made is that the homogenizer speed, ultrasound amplitude, and the pH medium are the important parameters that affect the production of GSH by the mechanical methods used in this study. However, the effect of pH medium is more significant than that of the specific parameters of the methods. The yield of GSH increased 4.1 and 3.3 times better at the suitable pH value of the ultrasonic and homogenizing methods, respectively. While, the improvement is only 1.7 and 1.6 times at the suitable ultrasound amplitude and homogenizer speed, respectively. The ultrasonic method was found a little more efficient in producing the GSH than the homogenizing method. Even though those methods were only able to isolate very small amount of product at one time but their implementation is considerably possible because they will be able to isolate more by repeating the process. Therefore, it is fully suggested to use these methods in a large scale production of GSH. The used of these mechanical methods are not only giving the solution to the capital/financial source (inexpensive since no fermentation process is used) and alcohol restriction problem but they are also providing a large range of process parameters by which one can adjust their values depending on the alcohol free GSH concentration that they want to produce.

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