MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

This chapter describes the methods used for the preparation of cocoa mucilage wine, assessment of various characteristics of prepared wine, development and optimization of hydrodynamic cavitation reactor and characterization of the accelerated aged cocoa mucilage wine in comparison with fresh and aged wine. The experimental design of the study and plan were out lined under the following sections.

3.1 MATERIALS

The fresh cocoa pods were purchased various districts of Kerala, India and stored at 10˚C under cold storage. The chemicals used for the experiments were of analytical grade purchased from Chemind, Thrissur. The standard sugar and *Saccharomyces cerevisiae* were procured from Tavanur local market.

Plate 3.1 Fresh cocoa pods

3.2 PREPARATION OF COCOA MUCILAGE

Fresh cocoa fruits, collected from the local farmers were used for the study. The cocoa fruits were fed to the cocoa pod breaker, developed under ICAR AICRP on PHET, to break the cocoa pod. The cocoa beans were collected from the cocoa pod breaker for further extraction of cocoa mucilage. A cocoa bean pulp extractor developed under ICAR AICRP on PHET was used to remove the required percentage of pulp from the cocoa bean and hence create a healthy environment for the fermentation process. The

cocoa pulp extractor consists of two concentric cylinders. The inner cylinder composed of uniform holes in the body which is rotating at fixed rpm using a variable frequency drive (VFD) and motor. The percentage of pulp extraction varies with the variation in the rpm of inner cylinder. In cocoa pulp extractor, a 20% extraction of pulp was obtained at 25 rpm of inner cylinder. The physicochemical characteristics such as pH, TSS, titrable acidity, ascorbic acid content, colour, antioxidant scavenging activity content (DPPH), reducing sugar content and TPC of cocoa mucilage were determined using standard procedures.

3.3 QUALITY ANALYSIS OF COCOA MUCILAGE

3.3.1 Total phenolic content

The total phenolic content was determined using the colorimetric method described by Gao *et al.,* (2019), with the Folin-Ciocalteu reagent and gallic acid as the standard. To 1 ml of appropriately diluted sample, 5 ml of Folin-Ciocalteu reagent solution was added, mixed thoroughly and incubated for 5 minutes. Subsequently, 4 ml of 7.5% (m/v) Na_2CO_3 solution was added, mixed well and the mixture was kept in the dark at room temperature for 1 hour. The absorbance was measured at 760 nm and the total phenolic content was calculated and expressed as milligrams of gallic acid equivalents per millilitre of sample.

3.3.2 Antioxidant activity

The antioxidant activity was evaluated using the DPPH free radical scavenging assay as described by Hayat *et al.,* (2011). Various concentrations of sample were placed in clean test tubes and 2 ml of DPPH solution was added to each. The tubes were incubated in the dark for 30 minutes and the absorbance was measured at 517 nm using spectrophotometer. Control absorbance was also measured alongside the samples. The scavenging activity was calculated using the formula provided in equation.

$$DPPH\ Scavenging\ activity\ (\%) = \left(\frac{A-B}{A}\right) \times 100 \qquad (3.1)$$

where A is control absorbance, and B is the absorbance of DPPH and substrate (Ur Rehman *et al.,* 2019).

3.3.3 Vitamin C

The volumetric titration method was used to determine ascorbic acid content. In a conical flask, 5 ml of standard ascorbic acid solution (100 µg/ml) containing 10 ml of 4% oxalic acid was titrated against 2,6-dichlorophenol indophenol dye. The endpoint was indicated by the appearance and persistence of a pink color. The volume of dye consumed (V_1 ml) was equivalent to the amount of ascorbic acid. Similarly, 5 ml of the sample (prepared by dissolving 5 g of sample in 100 ml of 4% oxalic acid) was added to a conical flask along with 10 ml of 4% oxalic acid and titrated against the dye (V_2 ml). The ascorbic acid content was calculated using the formula provided by Rekha *et al*., (2012).

$$Ascorbic\ acid\ \left(\frac{mg}{100\ ml}\right) = \left(0.5\ \frac{mg}{V_1 ml}\right) \times \left(\frac{V_2}{15\ ml}\right) \times \left(\frac{100\ ml}{Weight\ of\ sample}\right) \times 100 \quad (3.2)$$

3.3.4 Reducing sugar

The reducing sugar content was determined using a modified DNS method (Khatri, 2020). To prepare the DNSA reagent, 1 g of 3,5-dinitrosalicylic acid (DNSA) was dissolved in 80 ml of 0.5 N NaOH at 45°C, along with 30 g of sodium-potassium tartrate. The solution was brought to a final volume of 100 ml with distilled water after cooling to room temperature. A 0.1 ml sample was diluted with 10 ml of distilled water and 0.5 ml of this diluted sample was transferred to a test tube containing 2.5 ml of distilled water. Then 3 ml of DNSA reagent was added and the mixture was heated in a boiling water bath for 5 minutes. The absorbance was measured at 540 nm using a spectrophotometer. The reducing sugar content was calculated from a standard calibration curve of D-glucose, and the results were expressed in terms of percentage of reducing sugar.

3.3.5 pH

The pH of wine samples was measured using a digital pH meter of model (M/s. Systronics; Model MK VI). Initially, the pH meter was standardized with distilled water of pH 7.0 and standards of pH 4.0, 7.0 and 9.0. Sample was taken in a beaker and the electrode of pH meter was immersed in the sample. The reading was directly recorded

and displayed in pH meter. This procedure was repeated three times for accuracy and the average value was noted with standard deviation. The electrode should be wiped with tissue paper and immersed in distilled water in between each repetition (AOAC, 1990).

Plate 3.2 Digital pH meter

3.3.6 Titrable acidity

A 10% sample was prepared by diluting 10 ml of juice to 100 ml with distilled water in a volumetric flask. Using phenolphthalein as an indicator, 10 ml of this 10% sample was titrated against 0.1 N NaOH, which had been standardized using normal oxalic acid. The endpoint was identified by a color change from colorless to pale pink (Rekha *et al*., 2012). The total acidity, expressed as a percentage of tartaric acid was calculated using the formula provided in the equation.

$$Acidity\ (\%) = \frac{Normality\ of\ alkali \times Titre\ volume \times Equivalent\ weight\ of\ tartaric\ acid \times 100}{Weight\ of\ sample \times volume\ made\ up}$$

(3.3)

3.3.7 TSS

Plate 3.3 Refractometer

A hand refractometer (Lead series, ERMA, Japan) displayed in Plate 3.3 was used to assess the total soluble solid (TSS) of freshly extracted jamun fruit juice. The TSS ranged from 0% to 32% ºBrix was employed in this study. The device operates on the basis of the sample's refractive index. Using a cleaned dropper, two to three drops of the sample were put onto a prism plate. The sample's °Brix reading was then acquired, and the amount of TSS was expressed appropriately (Joy *et al.,* 2015).

3.3.8 Colour

Colour is a critical visual quality factor for most food products. The color of sample was assessed using a Lovibond tintometer. The optical density value of the sample was measured to determine its colour. The tintometer operates on the principle of focusing light on the sample and measuring the energy reflected across the visible spectrum. The standard observer curves of the colorimeter include blue, green and red. Colour was expressed using the three-dimensional L*, a*, and b* scale, where L* represents brightness (black/white coordinate) ranging from 0 to 100, a* represents the red/green coordinate ranging from 100 to -100, and b* represents the yellow/blue coordinate ranging from 60 to -60.

Plate 3.4 Lovibond tintometer

3.4 SETUP FOR ANAEOROBIC FERMENTATION

The procedure developed by the ICAR-AICRP on PHET, KCAEFT Tavanur, was employed in the preparation of cocoa mucilage wine. Approximately 1 litre of filtered cocoa mucilage drippings was mixed with 1.5 litres of water and about 1 litre of sucrose solution. Saccharomyces cerevisiae strains from a commercial yeast culture were then added to the mixture. A 2% concentration of commercially dried yeast was

used as the starter culture. The Fermentation experiments were carried out in ceramic vats of 5 L capacity. The ceramic vats were tied with muslin cloth and stored in a dark room. The fermentation temperature for cocoa wine production was approximately 25-30°C and stirring was performed during the initial seven days of fermentation. The vat was stirred once in a day at regular time intervals in clockwise and anticlockwise direction for seven days. After seven days, the vats were tied tightly and kept without agitation up to 21st day of storage. At 21st day, the suspended solid particles were removed with filter under aseptic conditions. The wine was stored for 21 days more and then tested for quality parameter. The physico-chemical properties such as pH, TSS, titrable acidity, ascorbic acid content, colour, antioxidant scavenging activity content (DPPH), reducing sugar content, TPC and alcoholic content values were evaluated for the wine produced from cocoa mucilage using standard procedures. The wine was transferred to bottles after the maturing period and kept for conventional aging and optimization of the HC reactor.

3.5 QUALITY ANLYSIS OF COCOA MUCILAGE WINE

3.5.1 Alcohol content

The alcohol percentage was determined using the dichromate-spectrophotometric method (Miller, 1959). A 3 ml sample was transferred to a 100 ml distillation flask, diluted with 30 ml of distilled water and heated in a distillation unit at 70–80°C for 20 minutes. The distillate was collected in a 50 ml flask containing 25 ml of potassium dichromate solution. This mixture was then placed in a water bath maintained at 60°C for 20 minutes. After cooling to room temperature, the volume was adjusted to 50 ml with distilled water and the absorbance was measured at 600 nm using a spectrophotometer. The total alcohol content (%) was calculated using a calibration curve with ethanol as the standard.

3.5.2 Mineral analysis

Calcium, potassium, magnesium, and phosphorus were analysed using the atomic spectroscopic method as per the procedures specified in the AOAC method (Poitevin *et al.,* 2009).

All the other wine parameters were analysed following the procedure outlined in Section 3.3.

3.6 FABRICATION OF HYDRODYNAMIC CAVITATION REACTOR

3.6.1 Design

The components used in the hydrodynamic cavitation reactor are as follows. This setup contains three different hydrodynamic cavitation reactors. The Orifice, slit venturi and elliptical venturi were the three different cavitation elements. Theses reactors are attached to the reactor one at a time for the treatment, elements are replaced for the subsequent treatments.

3.6.1.1 Slit venturi

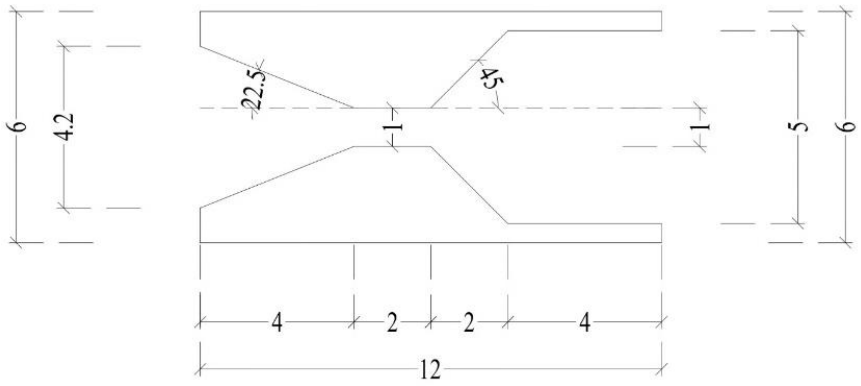

Fig. 3.1 Schematic diagram of slit venturi

This component is one of the three elements used for hydrodynamic cavitation. It was crafted from a stainless steel block measuring 12 cm in length and 6 cm in width using a lathe machine.

The design includes a cylindrical inlet section with an inner diameter of 5 cm, followed by a conical convergent section with a 45˚ angle. After the convergent section, a cylindrical throat was created with an inner diameter of 1 cm and a length of 2 cm by punching a 1 cm diameter rod into the block. The conical divergent section was formed as a continuous extension of the cylindrical throat, with an angle of 22.5˚.

3.6.1.2 Elliptical venturi

This component is also utilized for the hydrodynamic cavitation treatment of wine samples. It was made by carving a stainless steel block measuring 12 cm in length and 6 cm in width using a lathe machine. The design features a cylindrical inlet section with an inner diameter of 5 cm, followed by a conical convergent section at a 45˚ angle. Beyond the convergent section, an elliptical throat was created with a major dimension of 1.416 cm and a minor dimension of 0.708 cm. The conical divergent section, angled at 22.5˚, was seamlessly integrated as a continuation of the elliptical throat.

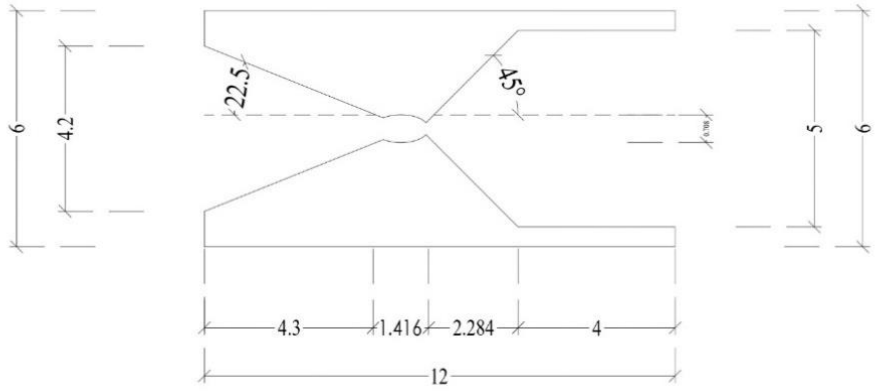

Fig. 3.2 Schematic diagram of elliptical venturi

3.6.1.3 Orifice

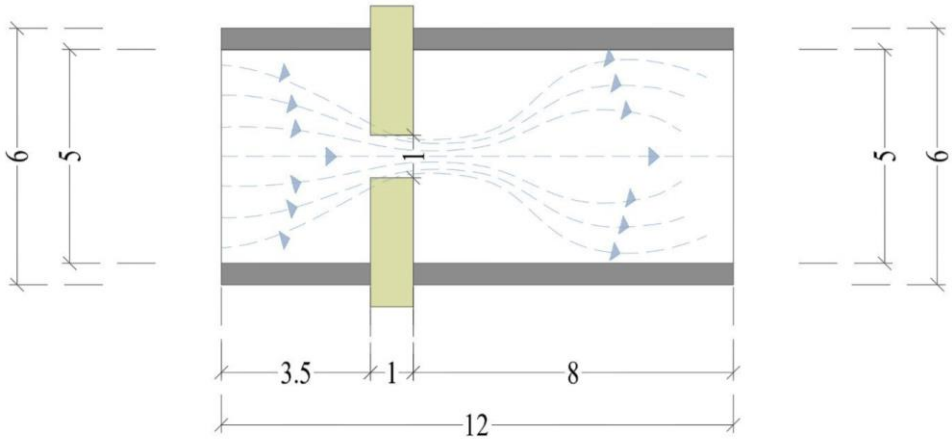

Fig. 3.3 Schematic diagram of orifice plate

It was manufactured from a stainless steel block measuring 12 cm in length and 6 cm in width using a lathe machine. A single orifice plate was positioned 3.5 cm from

the cylindrical inlet section, which had an inner diameter of 5 cm. The plate featured a single orifice with a diameter and thickness of 1 cm, serving as the site for the cavitation reaction. The divergent section of the orifice plate extended 8 cm towards the outlet.

3.6.2 Equipment framework

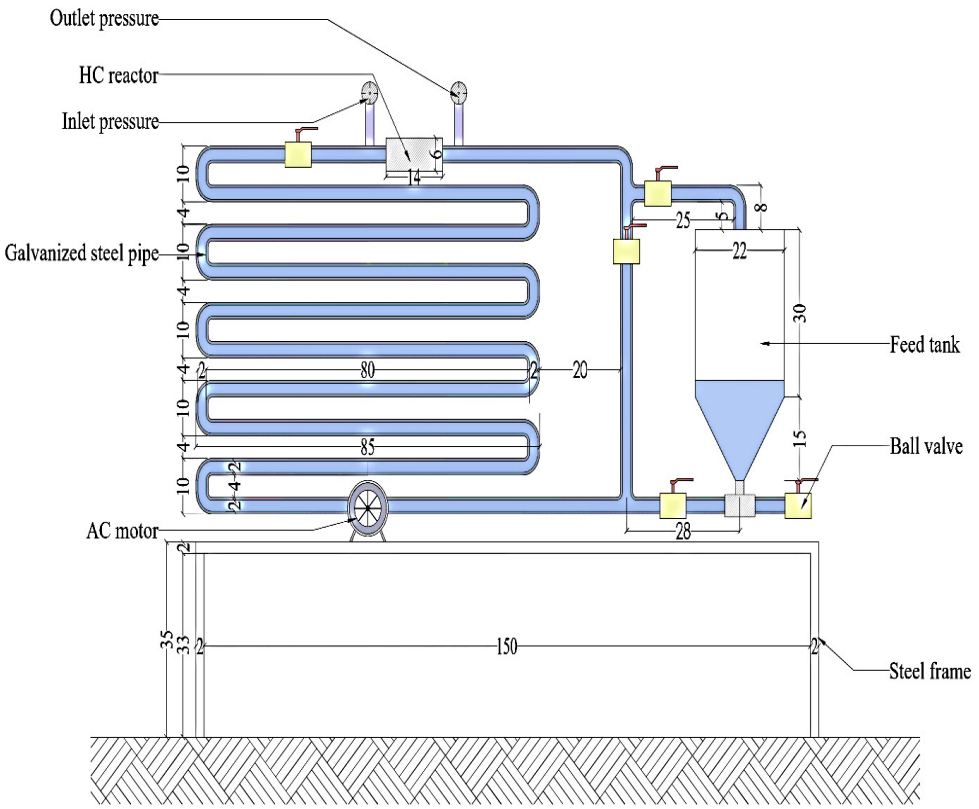

Fig. 3.4 Schematic diagram of hydrodynamic cavitation reactor system

The frame was constructed using mild steel (MS). The base of the frame measured 40 cm in width and 150 cm in length, supported by four legs, each 33 cm long and 2 cm wide. All components were securely mounted onto this frame structure with appropriate arrangements.

Chlorinated Polyvinyl Chloride (CPVC) pipes were used in the reactor to facilitate the circulation of wine throughout the equipment. The pipes had an outer diameter of 3 cm and an inner diameter of 2 cm. These pipes transported wine to all three cavitation reactors. On the both ends of the cavitation reactor, two pressure gauges of 10 kg/cm^2 were kept as inlet and outlet pressure measuring devices. A digital flow

meter was used to measure the volumetric flow rate of wine through different cavitation reactor systems.

The total height of the equipment from the ground level was 150 cm. A 1.5 hp multistage monobloc pump was used to operate the entire system. Various accessories, including ball valves, union joints and elbow joints, were utilized in the system's fabrication. The sample holding tank with a capacity of 10 L was constructed from stainless steel (SS). It was a large, pyramid-shaped container with an outer diameter of 22 cm and an inner diameter of 21 cm.

The entire equipment system was categorized into four different sections for the easiness of operation. They are feeding and discharge section, pumping and circulation section, HC reaction section and flow diversion section. During the feeding and discharge period, the valve V_1 is kept open to ensure the flow of wine into the pump. At the time of pumping and circulation, the wine was circulated into the rector system through the CPVC pipes. CPVC pipes were placed on above the other with a sufficient space between them by making bends on each end in order ensure good mixing effect. The valve V_2 is kept opened during the complete cycle of treatment. Hydrodynamic cavitation section is the place where the cavitation phenomena occur and is considered as the heart of the system. Flow diversion section is the final stage of the HC reaction where the valves V_3, V_4 and V_5 are crucially operated. If valve V_3 is closed and V_4 kept opened, the wine is circulated through the system. If valve V_3 is opened and V_4 kept closed, the wine flows into the storage tank for discharge. Operating pressure of the system can be adjusted with valve V_4. The HC treated wine samples could be discharged through valve V_5. The valve V_5 should be closed during the entire period of circulation of wine through the HC reactor, except at the time of discharge.

3.7 OPTIMISATION AND EVALUATION OF EFFECT OF HYDRODYNAMIC CAVITATION IN THE DEVELOPED WINE

3.7.1 Methodology

3.7.1.1. Experiment design by Response Surface Methodology

Response Surface Methodology (RSM) is a widely used tool for optimizing various processes, including blanching, drying, enzymatic hydrolysis, clarification,

microbial metabolite production, formulation, and extraction (Yolmeh and Jafari, 2017). RSM is particularly useful for optimizing processes where the response is influenced by the interaction of multiple independent variables (Mason, Gunst and Hess, 2003). This method efficiently constructs a design tailored to the input data, evaluates the responses in relation to the influencing factors and identifies the optimal solution. For this study, Design-Expert version 12 by Stat-Ease Inc. was employed. The objective was to optimize cavitation treatments conditions by identifying the optimal range of physicochemical parameters using the Central Composite Design (CCD).

3.7.2. Independent variables

Table 3.1 Independent parameters selected for HC treatment

Independent variables		
Type of cavitation element	**Inlet pressure (bar)**	**Treatment time (minutes)**
Slit venturi	3	30
Elliptical venturi	3.5	45
Orifice	4	60

The inlet pressure, treatment time and type of cavitation element were considered as independent variables. Inlet pressures of 3 bar, 3.5 bar and 4 bar were applied for durations of 30, 45 and 60 minutes for each element. The flow rate varied with pressure due to the system's construction and geometry.

Table 3.2 Experimental design with the actual values of process variables for the accelerated aging of cocoa mucilage wine through hydrodynamic cavitation

Trial No.	**Type of cavitation element**	**Inlet pressure (bar)**	**Treatment time (minutes)**
1	Orifice	3	30
2	Orifice	4	30
3	Orifice	3	60
4	Orifice	4	60

5	Orifice	2.7929	45
6	Orifice	4.20711	45
7	Orifice	3.5	23.7869
8	Orifice	3.5	66.2131
9	Orifice	3.5	45
10	Orifice	3.5	45
11	Orifice	3.5	45
12	Orifice	3.5	45
13	Orifice	3.5	45
14	Slit venturi	3	30
15	Slit venturi	4	30
16	Slit venturi	3	60
17	Slit venturi	4	60
18	Slit venturi	2.7929	45
19	Slit venturi	4.20711	45
20	Slit venturi	3.5	23.7869
21	Slit venturi	3.5	66.2131
22	Slit venturi	3.5	45
23	Slit venturi	3.5	45
24	Slit venturi	3.5	45
25	Slit venturi	3.5	45
26	Slit venturi	3.5	45
27	Elliptical venturi	3	30
28	Elliptical venturi	4	30
29	Elliptical venturi	3	60
30	Elliptical venturi	4	60
31	Elliptical venturi	2.7929	45
32	Elliptical venturi	4.20711	45
33	Elliptical venturi	3.5	23.7869
34	Elliptical venturi	3.5	66.2131
35	Elliptical venturi	3.5	45

36	Elliptical venturi	3.5	45
37	Elliptical venturi	3.5	45
38	Elliptical venturi	3.5	45
39	Elliptical venturi	3.5	45

Fresh cocoa mucilage wine was prepared for each treatment. After processing through the equipment, both machine and product parameters were evaluated for each treatment. The impact of the cavitation reaction on these parameters served as the basis for optimizing the treatments. Machine parameters, including cavitation number, volume flow rate and energy released along with the product parameter like total phenolic content were analysed for every treatment to assess the effect of hydrodynamic cavitation on cocoa mucilage wine.

3.7.3 Dependant variables

3.7.3.1 Cavitation number

The intensity of cavitation at the point of interception is quantified using a dimensionless parameter known as the cavitation number (Cυ), which is determined using Equation (3.4). The cavitation number is a dimensionless quantity that helps assess the probability of cavitation occurring in a fluid flow, indicating the potential for vapor bubble formation caused by low pressure. On the other hand, cavitation intensity refers to the degree or severity of cavitation in a system. Generally, a lower cavitation number is associated with higher cavitation intensity.

$$C\upsilon = \frac{P_d - P_v}{\frac{1}{2}\rho v^2} \quad (3.4)$$

Here, Pd represents the downstream pressure (kPa), Pv denotes the vapor pressure of the fluid, ρ is the density of the solution and υ is the velocity at the orifice (m/s). Since wine consists of more than 95% water, the vapor pressure of wine was approximated as that of water (9585.878 Pa at 45°C) for calculating the cavitation number. The velocity at the orifice was determined using Equation (3.5).

$$v = \frac{VFR}{Area\ of\ constriction} \quad (3.5)$$

Where v is the velocity at the orifice and VFR is the volume flow rate (L/h).

3.7.3.2. Energy released

The total energy required for the process was estimated by calculating the total energy released by the system using Equation (3.6). This provides valuable insight into the scalability of the cavitation equipment.

$$E = \frac{(P_u - P_d) \times VFR \times t}{V} \quad (3.6)$$

Here, V represents the total volume (L), VFR denotes the volume flow rate, E is the energy released from the system (pump) in J/mL, Pu and Pd are the upstream and downstream pressures (Pa) respectively and t refers to the time.

3.8. CHARACTERIZATION OF ACCELERATED AGED COCOA MUCILAGE WINE IN COMPARISON WITH FRESH AND CONVENTIONALLY AGED WINE

3.8.1. Physicochemical analysis

After optimizing the HC treatment through the equipment, all wine parameters were analysed following the procedure outlined in Section 3.3. The results were compared with those of untreated fresh wine and 24-month-aged cocoa mucilage wine to evaluate the treatment's impact on wine aging and nutritional quality.

3.8.2. Sensory analysis

Sensory evaluation was carried out in five different samples viz., Conventionally aged, untreated and three different HC treated wine samples (Table 3.3). The panel consisted of 15 semi-trained members (9 men and 6 women), aged between 22 and 50 years, from KCAEFT, Tavanur, Kerala, India. These individuals were selected due to their familiarity with wine tasting. The sensory evaluation followed the methodology described by Patil *et al.* (2021), using a nine-point hedonic scale (Table 3.4) ranging from 1 (“dislike extremely”) to 9 (“like extremely”). The samples were presented in small transparent glasses labelled with coded identifiers. The evaluation was conducted two hours after a meal. Water was provided between samples to cleanse

the palate. The results were compared using one-way analysis of variance (ANOVA) performed in Minitab® software (Ver. 19.1). Significant differences ($p<0.05$) between the treatments were determined by Least Significant Difference (LSD) test and the data were expressed as mean and standard deviation for the triplicate values.

Table 3.3 Sample name and labelling selected for sensory analysis

Sample Labelling	Name of sample
T1	Conventionally Aged Wine
T2	Untreated (Fresh) Wine
T3	Optimised HC Treated Wine (Refrigerated)
T4	Optimised HC Treated Wine (Non -refrigerated)
T5	HC Treated (2^{nd} Optimized sample as per RSM)

Plate 3.5 Samples for sensory evaluation

Table 3.4 Nine Point Hedonic Scale

Grade	Score
Like extremely	9
Like very much	8
Like moderately	7
Like slightly	6
Neither like nor dislike	5
Dislike slightly	4
Dislike moderately	3
Dislike very much	2
Dislike extremely	1

3.8.3. Cost estimation of accelerated aged cocoa mucilage wine

The following expenses were taken into account while estimating the fixed, variable, and other associated costs for the manufacturing of accelerated aged cocoa mucilage wine using HC reactor system such as building costs, HC reactor system costs, raw material costs, manpower, electricity, processing, and other associated expenses. The expenses incurred for producing 10-year conventionally aged cocoa mucilage wine were also considered to calculate the cost-benefit ratio.