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Optimization of Fermentation Factors for Vinegar Preparation from Sugarcane *Jaggery* using Response Surface Methodology

Vishal Lohan[1], Kanika Pawar[2], Anju Kumari[1], Rakesh Gehlot[1]

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ABSTRACT

Background: Vinegar, an acidic functional fermentation product and known for its diverse health benefits was developed using liquid jaggery. A study was conducted to standardize the method of vinegar production from liquid *jaggery* using two different sugarcane varieties *viz.,* CoH160 and Co89003.

Methods: The submerged fermentation of liquid *jaggery* was carried out using yeast *Saccharomyces cerevisiae* and vinegar was produced using *Acetobacter aceti.* For the computation analysis of an optimized solution, a central composite rotatable design was applied which constituted three variables temperature (°C), inoculum concentration (%) and incubation time (days) and its influence on acetic acid percent as an important response was studied using Response Surface Methodology. Overall, twenty experimental trials were conducted as well as assessed on the vinegar quality indexes.

Result: Vinegar prepared with 7.5 per cent inoculum concentration, incubated for a fermentation period of 12.5 days at 28.5°C was selected due to maximum acetic acid concentration achieved (> 3.5% (w/v). The optimized vinegar contained total soluble solids (4.55-4.75°Brix), reducing sugar (0.26-0.29%), total sugars (2.7-2.8%), titratable acidity (4.72-5.10%), pH (4.28-4.52), ascorbic acid (17.64-19.97%), alcohol content (0.68-0.79%), acetic acid (3.52-3.83%) and total phenols (36.69-38.22 mg/100 ml). The liquid *jaggery* vinegar had a bright yellow- brown color, cane flavor and vinegar aroma. It was found organoleptically acceptable.

Key words: Liquid jaggery, Optimization, Response surface methodology, Sugarcane, Vinegar, Wine.

INTRODUCTION

Vinegar a fermented aqueous product, with unique sour and tart flavor and is used as an excellent ingredient for variety of food preparations, salad dressings, ketchup, hot sauce. It is also prepared from diverse substrates such as fruits, malted barley or other cereals, hydrolyzed cereals, starches, sugar solution, molasses, jaggery, sugarcane juice *etc.* with or without added spices or caramel (Adebayo-Oyetoro *et al*., 2017). It is an age long preservative and condiment used since centuries. Food cooked in natural vinegar has a good shelf life because it inhibits the growth of micro-organisms by reducing the pH of food products to a level. It has a huge demand for its industrial production using cheap substrate material. Vinegar has multiple health benefits which is mainly due to its bioactive compounds (melanoidins, fructooligosaccharides, phenolic compounds, minerals, vitamins and α-glucan), antioxidant and antimicrobial potential, affecting physiological activities like improving digestive system, stimulating appetite, preventing inflammation and hypertension, lowering serum cholesterol, triacylglycerols and lessen glycemic index (Chen *et al*., 2015).

Sugarcane is a prime yield in Northern India and its extract is a substrate of choice for natural vinegar as a result of its high sugar content and accessibility (Kocher *et al*., 2006). Vinegar is being produced from sugarcane juice in Philippines, some parts of India like Punjab and Southern parts of India, but its production from liquid jaggery have not been reported so far. Liquid jaggery is semi-liquid syrup, natural sweetener obtained from sugarcane juice. It is having 40 to 60 per cent sucrose, 30 to 36 per cent water, 15 to 25 per cent invert sugar and also rich in important vitamins and minerals (Nath *et al*., 2015). The micronutrients which are present in jaggery have many nutritional and medicinal aspects such as it is anti-carcinogenic and antitoxic activity, produce heat and give instant energy to a human body (Kumar and Singh, 2020). Now-a-days, brewed vinegars are gaining popularity as compared to synthetic vinegar which is derived from petroleum residues that are harmful to human health. Presently vinegar is being produced from a lot of substrates, using sugarcane as a source of jaggery production is also prevalent, but it often faces fermentation during storage and mass production, thus suffers losses. Hence vinegar preparation from liquid jaggery will provide

[1]Centre of Food Science and Technology, Chaudhary Charan Singh Haryana Agricultural University, Hisar-125 004, Haryana, India.
[2]Chaudhary Charan Singh Haryana Agricultural University, Regional Research Station, Karnal-132 001, Haryana, India.

Corresponding Author: Kanika Pawar, Chaudhary Charan Singh Haryana Agricultural University, Regional Research Station, Karnal-132 001, Haryana, India. Email: kanikapawar@gmail.com

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cheaper, healthier and nutritious option with fresh taste, unique flavor and aroma. Moreover, high sugar content and lower shelf life which is due to its high moisture content makes it a good medium for vinegar production. More than 70 per cent of world jaggery is being produced in India, but suffers from business losses (Singh *et al*., 2011). An attempt was therefore, made to standardize the method of vinegar production from liquid jaggery using two different sugarcane varieties through Central composite rotatable design (CCRD) so as to optimize the process parameters in lieu of maximum acetic acid content and thereafter, the good sensory acceptability.

MATERIALS AND METHODS

The research work was carried out in Centre of Food Science and Technology, CCS Haryana Agricultural University, Hisar and Regional Research Station, Karnal during the year 2019-2020. Two early sugarcane varieties, namely CoH 160 and Co89003 were selected as source materials from Regional Research Station, Karnal, Haryana. To extract fresh sugarcane juice (18-20°Brix) from sugarcane stalks, mechanical crusher was used. All media and chemical solutions used in the analysis of jaggery, wine and vinegar were of good quality and analytical grade.

Liquid *jaggery*

The clear extracted juice was clarified and filtered using okra mucilage. The preheating (75-80°C) of sugarcane juice is followed by concentration of juice done by boiling at 103-106°C. Finally, semi-liquid syrup (liquid *jaggery*) was cooled and stored in bottles for its further processing into the vinegar. Total soluble solids (TSS) of the liquid *jaggery* was measured with hand refractometer (using 58-90°Brix) expressed as per cent soluble solids.

Micro-organisms used

The fermentation yeast *Saccharomyces cerevisiae* (MTCC 4787) and *Acetobacter aceti* (MTCC 2945) were collected from the Institute of Microbial Technology, Chandigarh, Punjab, India. In this study, both microorganisms were used to carry out the submerged fermentation (Chen *et al*., 2015) method for preparation of vinegar from liquid *jaggery*. Thus, double fermentation (alcoholic and acetous fermentation) was carried out during the vinegar production from liquid *jaggery*. The ethanol was produced by yeast (*Saccharomyces cerevisiae*), during fermentation under anaerobic conditions, while it is further converted to acetic acid (acetification) by acetic acid bacteria (*Acetobacter aceti*) under aerobic conditions. The detailed process is explained below.

Wine (Alcoholic fermentation)

Liquid jaggery (68°Brix) was pasteurized and cooled. It was further diluted to desired total soluble solids (20°Brix) as suggested by Ribeiro *et al.* (2015). Potassium metabisulphite @ 100 ppm was added and left for 48 h. To carry out the alcoholic fermentation the pitching of the liquid jaggery was done by the addition of 10 per cent (v/v) yeast *Saccharomyces cerevisiae* inoculum maintained on yeast extract peptone dextrose (YEPD) medium and then incubated at 28°C for 14 days. Observations were carried out till steady state total soluble solids were obtained. After that, decanting in bottles was done and agar bentonite (2:1) @ 200 ppm was added in wine at 4-5°C for 3-4 days. Thereafter, siphoning and bottling was done.

Adjustment of alcohol in wine

Partially clarified wine prepared from liquid *jaggery* of both the sugarcane varieties (CoH160 and Co89003) were found to have 8.8 per cent (w/v) and 8.0 per cent (w/v) alcohol content, respectively and the results are in agreement with (Ribeiro *et al.,* 2015 and Oliveira *et al.,* 2018). Before proceeding for acetous fermentation, wine was diluted with water in 1:1 ratio so that the final ethanol content in wine would be around 4.4% (w/v) and 4.0% (w/v) as suggested by Chen *et al.* (2015) in the CoH160 and Co89003 sugarcane varieties, respectively. Raspor and Goranovic (2008) also reported that the alcohol content affects a performance of acetic acid bacteria both in the beginning and at the end of fermentation. Thus, an optimum amount of alcohol is recommended because high initial alcohol concentration decreased the bacterial vitality due to the antimicrobial effect of ethanol. In contrast, if the initial concentration of ethanol was too less (0.1-0.2%) it will enhance the risk for over-oxidation. The alcohol content in the wine was estimated as per Caputi *et al*. (1968) method.

Vinegar (Acetous fermentation)

Wine was subjected to acetic acid fermentation under submerged conditions by the addition of *Acetobacter aceti* inoculum. Loopful culture of *Acetobacter aceti* from slants were inoculated in sterilized yeast extract peptone mannitol (YPM) broth and kept for 24 h at 28°C on shaker at 150 rpm. Then broth was added to partially clarified wine to carry out acetous fermentation. Apart from *Acetobacter* inoculum mother vinegar from cider was also added at 5% level. Sterile wood shavings were also added at 1% level which served as platform for *Acetobacter* cells to grow and helped in reducing time for acetous fermentation. Acidity was periodically measured till constant value was obtained. The acetic acid (%) in the prepared vinegar was estimated firstly by distillation of a sample and then it was titrated against 0.1 N sodium hydroxide (Rangana, 2003). Finally, after the filtration, the vinegar was tested for the qualitative parameters. It was pasteurized and stored in ambered coloured bottles for further sensory and quantitative analytical studies (Fig 1). The physico-chemical composition of the vinegar produced is mentioned in the Table 4.

Statistical analysis

A statistical package design expert version 12 of Response Surface Methodology was applied on the experimental data and different response surface plots were studied. The statistical significance of terms in the regression equation

was examined by analysis of variance (ANOVA). To obtain statistical significance three replications of the different variables were taken for the computation study.

RESULTS AND DISCUSSION

Optimization using RSM design

Three process parameters (inoculum, temperature, time) were used for liquid *jaggery* vinegar production and were incorporated into optimization RSM software to establish their optimum levels. The CCRD design of three factors was adapted to study the optimum levels and to explicate the effect of these independent variables on acetic acid concentration of the liquid *jaggery* vinegar. Twenty experimental trials were carried out in randomized order as per the CCRD design. The results are presented in Table 1. The data generated was analyzed using Design Expert Software (12 version) and a present polynomial equation was obtained for the response. The second order equation (Eq. 1) was built to describe the response.

$$y = b_o + \sum_i b_i x_i + \sum_i b_{ii} x_i^2 + \sum_{ij} b_{ij} x x_j \quad \text{.....(1)}$$

In the above equation, y and x are the response and factor for the above design. On the right hand side x_i, x_i^2 and $x x_j$ are the regression coefficient for linear, quadratic and interaction effect, b is coefficient for each term which is being calculated by applying multiple regression analysis and a lack of fit was also calculated using RSM optimization tool. This model is accounted for by coefficient of determination (R^2) which shows the proportion of variability in data and a larger value of R^2 which suggests better fit of model data. An adequacy ratio of more than Table F value depicted that the model is adequate. A second order polynomial model was used to develop three dimensional plots and contour graphs which demonstrated the interaction between independent variables and their effect on the acetic acid content as response. The effects of different variables on acetic acid response of liquid *jaggery* vinegar, prepared as per experimental design are presented in Table 2.

Effect of temperature, time and inoculum on acetic acid concentration in liquid *jaggery* vinegar of CoH160 sugarcane variety

The average acetic acid concentration of liquid *jaggery* vinegar ranged from 0.53 to 3.99%. The lowest acetic acid

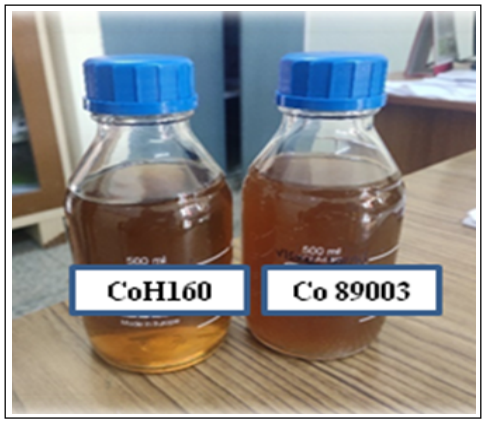

Fig 1: Picture of vinegar produced from liquid jaggery of CoH 160 and Co 89003.

Table 1: Experimental design for the optimization of experiment as computed using RSM (CoH 160 and Co89003)

Std. no.	Coded values			Actual values		
	Factor 1A: Temperature (°C)	Factor 2B: Time (days)	Factor 3C: Inoculum (%)	Factor 1A: Temperature (°C)	Factor 2B: Time (days)	Factor 3C: Inoculum (%)
1	-1	-1	-1	32.0	10.0	5.0
2	-1	-1	-1	25.0	10.0	5.0
3	-1	1	-1	28.5	12.5	3.29
4	1	1	-1	25.0	15.0	5.0
5	-1	-1	1	32.0	15.0	5.0
6	1	-1	1	28.5	12.5	7.5
7	-1	1	1	25.0	10.0	10.0
8	1	1	1	32.0	15.0	10.0
9	-1.684	0	0	28.5	12.5	7.5
10	1.684	0	0	25.0	15.0	10.0
11	0	-1.684	0	28.5	12.5	7.5
12	0	1.684	0	28.5	12.5	7.5
13	0	0	-1.684	28.5	16.0	7.5
14	0	0	1.684	28.5	8.00	7.5
15	0	0	0	32.0	10.0	10.0
16	0	0	0	28.5	12.5	7.5
17	0	0	0	22.6	12.5	7.5
18	0	0	0	28.5	12.5	11.7
19	0	0	0	28.5	12.5	7.5
20	0	0	0	34.3	12.5	7.5

concentration (0.5%) in vinegar was found in the experimental trial 17, whereas a highest acetic acid (3.99%) was achieved in the experimental trial 16 as shown in the Table 2. The maximum acetic acid (3.99%) was observed at a temperature of 28.5°C, fermentation period of 12.5 days and 7.5% inoculum concentration (CoH160), while minimum acetic acid (0.53%) was observed at 22.61°C temperature, 12.5 days fermentation period and 7.5% inoculum concentration (Table 2). The regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation. Thus, to predict acetic acid per cent as affected by different factors which are represented in terms of their actual factors, a multiple regression equation was generated as shown below (Eq 2):

Acetic acid (%) =

+3.92 + 0.44 *Temperature + 0.03 *Time + 0.51 *Inoculum-0.13 *Temperature *Time + 0.10 *Temperature* Inoculum + 0.45 *Time *Inoculum -0.94 *Temperature^2- 0.49* Time^2-0.63 *Inoculum^2 ....(2)

The analysis of variance (ANOVA) depicted the acetic acid as a function of initial values of parameters. Table 3 shows the regression analysis of the data, in which the coefficient of determination (R^2) was found to be 0.985 indicating that the statistical model was significant and can explain the variability in the response. The model F value was found to be 77.75, which is more than the tabulated F value as indicated in the ANOVA of quadratic model. A minimum desirable adequate precision value (APV) of 4.00 is required for high prediction ability, where in the study it was found to be 24.59 which are higher than the minimum value required. Therefore, the statistical analysis demonstrates that this model can be efficiently applied to explain the effect of independent variables on acetic acid content of the developed vinegar (Table 3).

Effect of temperature, time and inoculum per cent on acetic acid concentration in liquid *jaggery* vinegar of Co 89003 sugarcane variety

The average acetic acid concentration of liquid jaggery vinegar ranged from 0.41 to 3.73%. The lowest acetic acid concentration (0.41%) in vinegar was found in the experimental trial 17, whereas highest acetic acid (3.73%) was achieved in experimental trial 6 as shown in Table 2. The maximum acetic acid (3.73%) was observed at a temperature of 28.5°C, fermentation period of 12.5 days and 7.5% inoculum concentration (Co 89003), while minimum acetic acid (0.41%) was observed at 22.61°C temperature, 12.5 days fermentation period and 7.5% inoculum concentration (Table 2). The regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation. Thus, to predict acetic acid per cent as affected by different factors which are represented in terms of their actual factors, a

Table 2: Acetic acid percent of vinegar prepared with different levels of temperature, time and inoculum percent from liquid jaggery of sugarcane varieties CoH 160 and Co89003.

Std. no.	Factor 1A: temperature (°C)	Factor 2B: time (days)	Factor 3C: inoculum (%)	Response acetic acid (%) CoH 160	Response acetic acid (%) Co 89003
1	32	10	5	2.28	2.14
2	25	10	5	1.00	1.02
3	28.5	12.5	3.29	1.41	1.39
4	25	15	5	0.87	0.81
5	32	15	5	1.05	0.99
6	28.5	12.5	7.5	3.98	3.73
7	25	10	10	1.34	1.27
8	32	15	10	3.58	3.49
9	28.5	12.5	7.5	3.89	3.72
10	25	15	10	2.42	2.35
11	28.5	12.5	7.5	3.91	3.70
12	28.5	12.5	7.5	3.88	3.69
13	28.5	16	7.5	2.35	2.29
14	28.5	8	7.5	2.63	2.57
15	32	10	10	2.44	2.39
16	28.5	12.5	7.5	3.99	3.67
17	22.6	12.5	7.5	0.53	0.41
18	28.5	12.5	11.7	2.84	2.77
19	28.5	12.5	7.5	3.87	3.71
20	34.3	12.5	7.5	1.97	1.89

Response values are average of three replications.

multiple regression equation was generated as shown below (Eq 3):

Acetic acid (%) =

$$+3.70 + 0.44 *\text{Temperature} + 0.02 *\text{Time} + 0.50 *\text{Inoculum} - 0.11 *\text{Temperature}* \text{Time} + 0.12 *\text{Temperature}* \text{Inoculum} + 0.44 *\text{Time} *\text{Inoculum} - 0.89 *\text{Temperature}^2 - 0.44* \text{Time}^2 - 0.56* \text{Inoculum}^2 \quad \ldots(3)$$

The analysis of variance (ANOVA) depicted the acetic acid as a function of initial values of parameters. Table 3 shows the regression analysis of the data, in which the coefficient of determination (R^2) was found to be 0.987 indicating that the statistical model was significant and can explain the variability in the response. The model F value was found to be 85.02, which is more than the tabulated F value as indicated in the ANOVA of quadratic model. A minimum desirable adequate precision value (APV) of 4.00 is required for high prediction ability, where in the study it was found to be 26.05 which are higher than the minimum value required. Therefore, the statistical analysis demonstrates that this model can be efficiently applied to explain the effect of independent variables on acetic acid content of the developed vinegar.

A similar trend was observed for the regression coefficients and ANOVA of fitted quadratic model for acetic acid percent in liquid *jaggery* vinegar of both the sugarcane varieties (CoH 160 and Co 89003). The coefficient estimate of the acetic acid model (Table 3) shows that the level of temperature and inoculum at linear terms had highly significant ($p \leq 0.01$) effect and an increase in temperature and inoculum percent enhanced the acetic acid production in vinegar due to the greater bioconversion of the sugars into alcohol and acetic acid. Similar results were also

Table 3: Regression coefficients and ANOVA of fitted quadratic model for acetic acid percent of liquid jaggery vinegar of sugarcane varieties CoH 160 and Co 89003.

Partial coefficients	Sugarcane varieties	
	CoH 160 acetic acid	Co 89003 acetic acid
Intercept	3.92	3.70
A-Temperature	0.44**	0.44**
B-Time	0.03^{NS}	0.02^{NS}
C-Inoculum	0.51**	0.50**
A^2	-0.94**	-0.89**
B^2	-0.49**	-0.44**
C^2	-0.63**	-0.56**
AB	-0.13^{NS}	-0.11^{NS}
AC	0.10^{NS}	0.12^{NS}
BC	0.45**	0.44**
Lack of fit	S	S
Model F value	77.75**	85.02**
R^2	0.985	0.987
Adequate precision value	24.59	26.05

**p≤0.01 and NS non-significant; S- Significant

Table 4: The physico-chemical composition of the vinegar.

Parameters	CoH160	Co89003
Total soluble solids (°Brix)	4.75±0.01	4.55±0.1
Reducing sugars (%)	0.26±0.05	0.29±0.04
Total sugars (%)	2.7±0.26	2.8±0.36
Titratable acidity (%)	5.10±0.17	4.72±0.05
pH	4.28±0.06	4.52±0.03
Total phenols (mg/100 mL)	38.22±0.38	36.69±0.32

*Values are mean±S.D of three replicates.

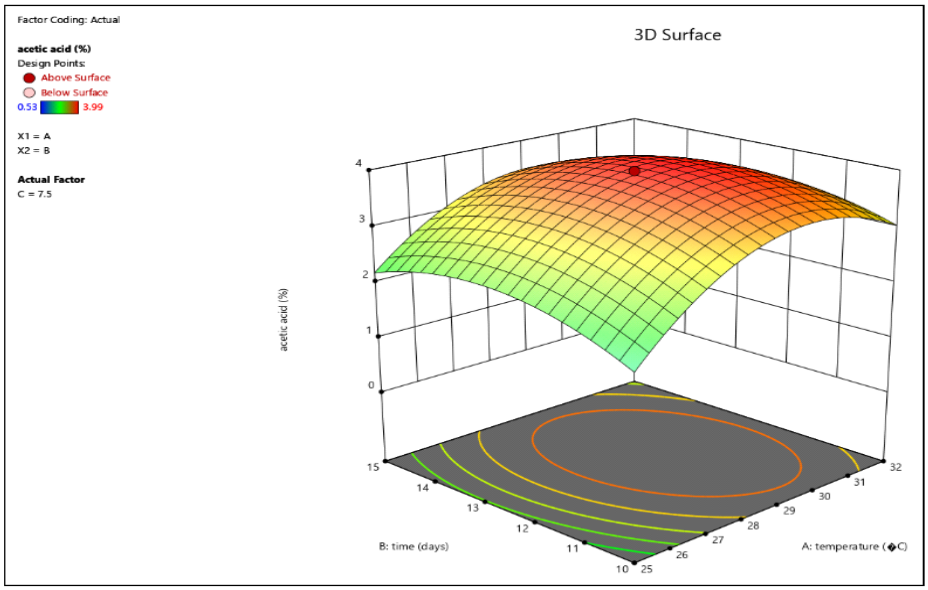

Fig 2: Effect of temperature, time and inoculum on an acetic acid content of the liquid jaggery vinegar prepared from CoH 160 sugarcane variety.

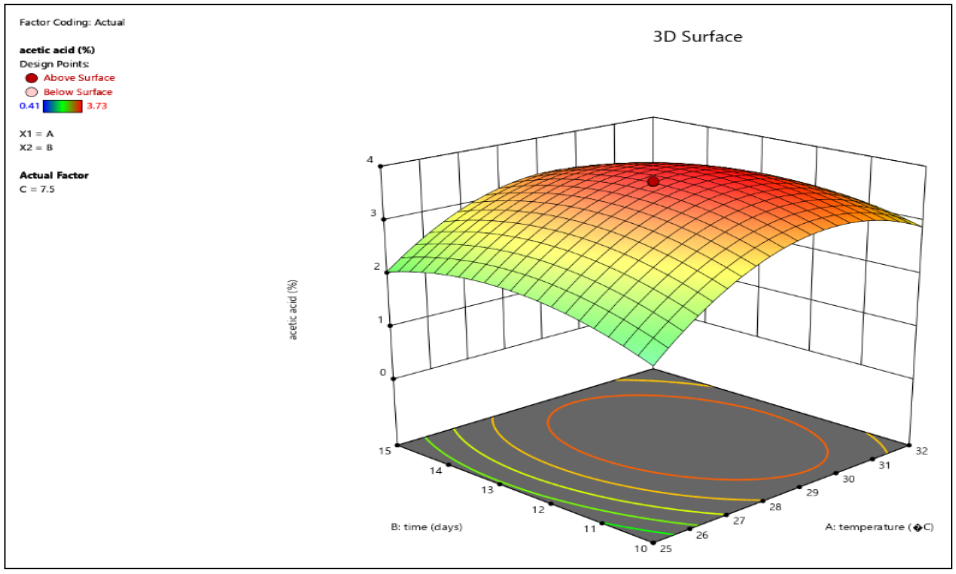

Fig 3: Effect of temperature, time and inoculum on acetic acid content of the liquid *jaggery* vinegar prepared from Co 89003 sugarcane variety.

depicted by Saha and Banerjee (2013) which have found that acetic acid concentration increased with an increase in an amount of inoculum per cent. At 15 and 10% inoculum concentration, acetic acid concentration of 4.67% and 4.62% was observed. Gullo *et al.* (2014) found that the optimum temperature for the wine vinegar production is 30°C whereas the growth of bacteria was inhibited if a temperature was above 35°C and below 8°C. In this study, an optimum temperature was observed at 28.5°C for the vinegar production at 7.5% inoculum level. However, non-significant effect of time was observed on the acetic acid response.

The squared terms of temperature, time and inoculum had negative highly significant effect ($p \leq 0.01$), thus increasing any of these independent factors, decreased the acetic acid concentration in liquid *jaggery* vinegar. Any of these factors alone, keeping others constant will affect the microbial metabolism in an inverse way. The interaction between the time and inoculum variables also had statistically significant effect ($p \leq 0.01$) on acetic acid, thus a higher acetic acid concentration was observed when the fermentation period (time) was increased from 10 to 15 days and inoculum per cent was increased from 5 to 10 per cent. A similar trend was observed by the Ghosh *et al.* (2012).

The interaction amongst variables and their effect on acetic acid per cent of vinegar is shown in the three dimensional graph (Fig 2). The acetic acid concentration achieved in the optimum liquid *jaggery* vinegar prepared from CoH 160 and Co 89003 sugarcane varieties was found to be 3.99 and 3.73 per cent which is in accordance with the acetic acid content of 3.04 per cent as found out by the Chen *et al.* (2015) in the sugarcane juice vinegar prepared from different yeasts and acetic acid bacteria. Also in both sugarcane varieties maximum acetic acid content in liquid *jaggery* vinegar was observed at a temperature around 28.5°C which is in agreement with (Arifuzzaman *et al.*, 2014) which found that all isolated *Acetobacter* strains were able to grow at 25°C and 37°C but the most favourable temperature in which a higher potency in terms of acetic acid content was observed at 30°C. Similar observation was also reported by Sharafi *et al.* (2010) where the acetic acid production was found to be optimum at temperature of 31°C. Optimisation of temperature is considered essential for any biotechnological process as the microbial inactivation and enzyme denaturation can occur above the optimum temperature.

The expected response of acetic acid and its correlation between the different independent variables are shown in three dimensional plots (Fig 2 and 3), with temperature on X-axis and time on Y-axis while inoculum percentage was kept (7.5%) constant at 'O' central level. With an increase in temperature, acetic acid concentration increases at first and then decreases. With an increase in the number of days, acetic acid concentration increases at first and then decreases.

CONCLUSION

Response Surface Methodology can be effectively employed to optimize the process parameters for the development of vinegar from liquid *jaggery*. The fermented natural vinegar prepared from liquid *jaggery* contained acetic acid >3.5% which is as per (FSSAI, 2011) requirements. The optimum levels of inoculum (7.5%), temperature (28.5°C) and time (12.5 days) used during the production of vinegar resulted in an overall acceptable product in context with acetic acid concentration, taste and aroma. The vinegar thus obtained was also adjusted sensorily acceptable.

Conflict of interest

All authors declare that they have no conflict of interest.

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