In silico optimization of enzyme mediated debittering of Assam lemon: biochemical and sensory evaluation studies

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Abstract Commercialization of citrus fruit juice is always hindered by the bitterness development in juice when stored for a significant period of time. In order to debitter citrus juice, an attempt has been taken up by treating the juice with tannase. Central Composite Design (CCD) based Response Surface Methodology (RSM) has been implemented to evaluate and optimize the effect of underlying process parameters viz., enzyme volume, temperature, incubation time and enzyme titre on debittering effect of Assam lemon juice. The significance of parameters and their interaction were assessed by analysis of variance at 95% level of confidence. Optimization study reveals that the maximum debittering ($40.12 \pm 0.02\%$) of Assam lemon juice takes place at ambient temperature (37°C) within an incubation time of 2 h and 1.12% (v/v) enzyme volume while 30 IU/ml enzyme activity. Moreover, percentage contribution of the underlying process parameters demonstrate that the enzyme volume and enzyme titre as first and second most significant contributors in process of debittering. As part of validating the above results, experimental debittering has been performed and compared with predicted debittering percentage which showed a high coefficient value (0.971) which ensures the effectiveness of the proposed model. Biochemical analysis of the treated

juice reveals improved antioxidant property after enzymatic treatment by 15.30%. Total sugar and reducing sugar content has also been enhanced by 1.38 and 1.49 folds, respectively, after enzymatic treatment of juice. Furthermore, no alteration in the elemental composition of the treated juice ensure that the quality of the final juice is retained with the enzyme applications. Sensory analysis based on nine-point Hedonic scale advocates the best organoleptic property in 1% (v/v) enzyme treated juice.

Keywords Assam lemon · Debittering · Response surface methodology · Antioxidant property · Elemental composition

Introduction

Tannase (tannin acyl hydrolase, EC 3.1.1.20), an inducible extra-cellular enzyme which can be produced by animals, plants and microbes, has wide applications in different sectors like tannery, alcohol, pharmaceutical and beverage industries. It plays the pivotal role in bioconversion of hydrolysable tannins especially gallo-tannins to glucose and gallic acid (Hadi et al. 1994; Purohit et al. 2006). Moreover, in food and beverage industries, tannase helps in removing undesirable effects of tannins (Aguilar et al. 2007).

On the other hand, citrus fruit juice has great popularity all over the world due to its essence, therapeutic value and numerous health benefits. Consumption of fruit juices has been associated with healthful diet. Apart from its nutritional properties, current interest lies in its antioxidant property which inhibits oxidation in our body. With respect to the world citrus production, India holds third rank (Patil and Dhake 2014). In 2016–2017, total production of fruit was 92846000 MT and total citrus fruit production was

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12746000 MT in which lime/lemon production accounted as 22.81% of the total (Horticultural Statistics at a Glance 2017). Assam, a state of North East India is the lead producer of lemon known as Assam Lemon (*Citrus Limon* L. Burm. f.). The processing of citrus juice and its commercial viability had a major setback on long shelf life due to bitterness development, predominantly caused by limonin, naringin and tannin compounds (Kundu et al. 2018; Premi et al. 1994). Immediate bitterness is caused by naringin where limonin is accountable for delayed bitterness in citrus fruit (Premi et al. 1994). Debitting of grapefruit juice is an important process for controlling quality and improving commercial value. Several physiochemical approaches have been attained to reduce the developed bitterness in citrus juices. Among the physicochemical approaches, resins (polystyrene divinyl benzene etc.), chemicals (ethylene, carbon dioxide etc.), adsorption processes (polyamides, cellulose acetate, nylon-based matrices, porous polymers, ion exchangers etc.) and synthetic adsorbents have been reported to be used for debittering purposes. Biological approaches for debittering of juices include application of bacterial cells (*Corynebacterium fascians*, *Rhodococcus fascians* etc.), free and immobilized enzymes. Other approaches that are used to reduce the bitterness include blending with non-bitter citrus juices and sugars, plant growth regulators to encumber the production of bitterness causing compounds, use of low pressure techniques in juice extraction, ultrafiltration and supercritical carbon dioxide extraction have been extensively employed (Ghanem 2012; Kundu et al. 2018). Unlike physiochemical processes, enzyme-mediated debittering process is gaining wide impetus because of its selective removal of bitterness without changing the nutritional property of the juice. Moreover, enzyme catalysis contributes in biotransformation of bitterness causing compounds with concomitant enhancement of antioxidant property. There are several advantages of enzymatic bioconversion processes over chemical processes like high yield, improvement of nutritional value and thereby facilitating a safe eco-friendly process. Enzymatic debittering of juices has immense advantage over conventional method for providing high quality of juice through lowering the haze and unaltered juice quality attributes (Dash et al. 2016). Presence of tannin in fruits resulted into haze, sediment formation, bitterness and astringency in extracted juices. Owing to several bottlenecks in the conventional juice debittering, alternative enzyme mediated processes emerge as a promising preferred pathway. Moreover, phenolics compounds present in fruits have a great potentiality to scavenge free radicals in the body and thus act as a potent antioxidant compounds. Thus, optimization of the process parameters is need of the hour for providing

better quality of juice without alteration of its nutritional quality.

This study presents a predictive model of enzymatic debittering of Assam lemon juice where enzyme volume, time, temperature and enzyme titre have been considered as input variables and the predictive model is based on response surface methodology (RSM) and the optimization model is constructed using response optimizer. After successfully optimizing the underlying debittering process nutritional quality evaluation and sensory evaluation have been carried out to ensure the final quality of the treated juice.

Materials and methods

Substrate

Assam lemon is selected as a substrate in the present study. Fruits are collected from the North-Eastern part of India (25°45'24"N 93°50'26"E).

Preparation of juice and enzymatic treatment

After peeling and proper washing of fruits, juices have been extracted and stored in room temperature to determine the increase in concentration of bitterness causing compounds followed by mixing of the juice with enzymes and incubation in the range of 25–45 °C for 30–150 min.

Enzyme production

Terminilia chebula, collected from local market as substrate for tannase production has been mixed with Czapek-dox medium, by maintaining a suitable substrate to media ratio. Then this mixture has been autoclaved for 15 min at 15 psi. After proper cooling, the required amount of induced inoculums (*Rhizopus oryzae*, isolated from the soils of IIT Kharagpur campus) have been added aseptically and incubated at 35 °C, at specified humidity (83% RH) for 4 days (Kar and Banerjee 2000).

Enzyme assay

Spectrophotometric estimation of Tannase activity has been carried out by the method of Iibuchi et al. (1967) where 2% tannic acid has been used as substrate. One unit of enzyme activity is defined as the amount of enzyme required for hydrolyzing one μmol of ester in one min.

Estimation of bitterness removal

At each interval of 30 min, bitterness removal has been estimated by measuring the removal of tannin, up to a total duration of 150 min.

Tannin content has been measured by protein precipitation method (Haggerman and Butler 1978). Percentage of bitterness removal has been calculated by the following formula.

$$B_r = \frac{(B_i - B_f)}{B_i} \times 100$$

where B_r , percentage of bitterness removal; B_i , initial bitterness and B_f , final bitterness.

Enzymatic debittering of Assam lemon

Enzymatic debittering of Assam lemon juice has been carried out by incubating enzyme and juice in Erlenmeyer conical flask in various process conditions. At regular time interval, juice has been taken out for the estimation of debittering percentage. Debittering of juice has been monitored at different process conditions like enzyme volume, temperature, incubation time and enzyme titre. To find out the effect of individual parameter on debittering of lemon juice, different parameters such as enzyme volume (0.5–5%), temperature (25–45 °C), incubation time (0.5–3 h) and enzyme titre (15–35 IU/mL) have been chosen. Upon selection of the individual parameters, central composite design (CCD) based RSM optimization study has been carried out for optimization of debittering process.

Experimental design for the optimization of enzymatic debittering of Assam lemon juice

Optimization and evaluation of enzymatic debittering process have been carried out employing a three-level, 2^4 , full factorial CCD with 4 process parameters at three coded levels i.e. -1 , 0 , $+1$. Four process parameters like enzyme volume (0.5–5%, v/v), temperature (30–40 °C), incubation time (h) and enzyme titre (IU/mL) have been selected for enzyme mediated debittering of citrus fruit juice.

Response surface methodology for process optimization

RSM is an empirical statistical model which is employed to predict, analyze, evaluate and optimize a response model which depends on some independent variables. RSM helps in determination of regression model and computing the optimal condition of the process from the result of design of experiment (DOE). DOE is an experimental plan which

can establish a relation between process response and factors affecting the underlying process. From the optimization process, coefficients of the model, predicted response value are obtained and model adequacy is evaluated. Response surface is a curved surface using which the relationship between the factors or design variables (x_i) and response (y) can be established (Eq. 1) (Bhaumik and Mondal 2016). Also, it can be used to identify a point where a minimum and maximum value of the response occurs. β_0 is the intercept term (constant) and $\beta_1, \beta_2 \dots \beta_n$ are the slopes or coefficients of the linear terms containing design variables.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \dots + \beta_n x_n + e \quad (1)$$

Here e is the model error.

DOE has been carried out using Minitab 17. Along with the empirical second order polynomial quadratic (i.e. full quadric model) model, an interaction impact can be imposed for fitting the data in the following way (Eq. 2).

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i < j}^k \beta_{ij} x_i x_j + e \quad (2)$$

In the above equation (Eq. 2), Y is the response vector (debittering %), $\beta_0, \beta_i, \beta_{ij}$ are coefficients for the linear, intercept and interactions among all the factors. The response include the linear terms $x_1, x_2, \dots, x_k$, square terms $x_1^2, x_2^2, \dots, x_k^2$, and interaction terms $x_1 x_2, x_1 x_3, \dots, x_{k-1} x_k$.

Significance of the input factors in the relationship between input and output factor also determined by analysis of variance (ANOVA) which further ensures the adequacy of the model. Coefficient of determination (R^2) facilitates the determination of accuracy and desirability of second order polynomial regression equation, whereas, P values indicate the significance of the individual model coefficients.

Optimization

The optimal conditions of design variables for debittering process derived from RSM have further been verified for validation of the regression model by conducting experiments in triplicates.

HPLC analysis

Estimation of gallic acid in raw and enzyme treated Assam lemon juice has been carried out using HPLC to ensure the biotransformation of hydrolysable tannin. Chromatographic analysis has been carried out with Agilent technologies 1100 series. Separation is done by using a C18 (250 mm $\times$ 4.6 mm I.D., 5 μ m) reverse phase column using acetonitrile and water (80:20) containing 0.01% (v/v)

ortho phosphoric acid as mobile phase. Run time of each sample has been fixed for 2–3 min at a flow rate of 1 mL/min. Injection volume is 20 µL and ultra-violet spectra monitored at 270 nm (Ganesan et al. 2010).

Biochemical analysis

Analysis of effects of different parameters like pH (Ranganna 1986), ascorbic acid, titrable acidity (Ranganna 1986), reducing sugar (Somogyi 1944), total sugar (Hedge and Hofreiter 1962), total phenol (Gamez-Meza et al. 1999), protein and anti-oxidant property (Shimada et al. 1992) has been carried out before and after enzymatic treatment of citrus fruit juice.

Elemental analysis

Elemental analysis of treated and untreated juices have been carried out using Inductive Coupled Plasma-Mass Spectroscopy (Thermo Scientific™ iCAP™ RQ). For the estimation, samples have been filtered through 0.22 µm Millipore PES membrane filter. The instrument has been equipped with an autosampler, quadrupole and a detector. In the instrument, Argon acted as a carrier gas. The data from the analysis of the sample is derived from Qtegra™ Intelligent Scientific Data Solution™ (ISDS) software.

Sensory evaluation

To carry out the sensory evaluation, a group of 12 trained judges were chosen for evaluation of the treated juice as mentioned by Chakraborty et al. (2015). During the evaluation process, the following parameters such as color, taste, aroma and mouth feel have been considered. Consumer acceptance for the juice has been evaluated on a 9 point Hedonic scale (Lawless et al. 2010) to find out the best product for different sensory properties.

After acquiring all the preference data given by all judges on organoleptic properties, specific value of each properties and order of consumer preference have been obtained through Eqs. 3 and 4.

Specific value

$$= \frac{(9 \times \text{number of judges preferred the juice 'like extremely'} + 8 \times \text{number of judges preferred the juice 'like very much'} + \dots + 1 \times \text{number of judges preferred the cake 'dislike extremely'})}{\text{Total number of judges}} \quad (3)$$

Overall consumer preference value

$$= \frac{(\text{Specific color value} + \text{Specific taste value} + \text{Specific aroma value} + \text{Specific mouth feel value})}{4} \quad (4)$$

Statistical analysis

Analysis of Variance (ANOVA) and Fisher's Least Significant Difference (LSD) have been carried out by Minitab 17 software.

Results and discussion

Effect of single parameter study on enzymatic debittering process

Assam lemon (*Citrus limon* Burm. f.) is abundantly produced in North Eastern part of India but the major hindrance in commercial acceptance of this juice lies in production of the bitterness producing compounds such as tannin, naringin and limonin. To popularize this variety of lemon, an attempt has been taken to study the enzymatic debittering process by adopting single variables such as enzyme volume, temperature, contact time of enzyme with the bitter compounds and the enzyme titre on bitterness removal and its debittering efficiency have been studied and documented in the following sections.

Effect of enzyme volume on debittering process

Concentration of enzyme in substrate is the most crucial factor for an enzymatic reaction. Moreover, the ratio between substrate and enzyme is of prime importance, because variations of enzyme volume change the yield of desired final product. Thus, identification of optimum enzyme volume leads to maximum yield (Rajak and Banerjee 2015).

In the present study, 0.5–5% (v/v) enzyme volume has been used for the purpose of debittering process while other parameters like temperature, time and enzyme titre are kept constant at 35 °C, 2 h and 35 IU/mL, respectively. Results reveal that percentage of debittering significantly increases with the increase in enzyme volume from 14.92 to 29.90% ($F_{5,12} = 8.89$; $P < 0.001$) (Fig. 1a). As there is a feeble increment of bitterness removal from 1 to 5% (v/v) enzyme loading, then selection of 1% (v/v) enzyme volume has been found to be economically and industrially viable. No significant increase of debittering with the increase of enzyme volume may be due to the saturation of tannase active sites with tannin molecules present in the

Fig. 1 Effect of **a** enzyme volume, **b** temperature, **c** incubation time and **d** enzyme dose on debittering process (grouping information using the Fisher LSD method and 95% confidence. Means that do not share a letter are significantly different)

juice. Previous study reveals 57% tannin degradation in pomegranate juice using 1:2 tannase and juice ratio (Kapoor and Iqbal 2013).

Effect of temperature on debittering process

Rate of enzymatic reaction and distortion of enzyme structure mostly depend on the temperature of the system and thus, balance between these two responses lead to optimum temperature. While varying the temperature from 25 to 45 °C for debittering of fruit juice (Busto et al. 2007) and the hold values were enzyme volume (1%, v/v), time (2 h) and enzyme titre (30 IU/mL). The present study reveals that 30.25% debittering has been occurred at 35 °C significantly higher than other experimental temperature ($F_{4,10} = 11.28$; $P < 0.001$) (Fig. 1b). With the rise of temperature (from 25 to 35 °C) debittering percentage increases as tannase has more and more kinetic energy. This energy facilitates the successful collision with the substrate and thus rate of reaction increases. At temperature of 35 °C, tannase catalysis activity has been found to be the highest. Further increment of the temperature does not shown any increase in debittering percentage. It may be due to the initiation of the breakdown of intra- and inter-molecular bonds in enzymes because of attaining more kinetic energy due to temperature rise. Study has shown the

increase of tannase activity with the increase of temperature till 40 °C and after that subsequent decrease has been observed. Basically, microbial tannase has temperature optimality in the range of 20–60 °C and the thermostability is between 30 and 60 °C (Mukherjee and Banerjee 2003, 2006; Yao et al. 2014).

Effect of incubation time on debittering process

Time plays a crucial role in any type of chemical or biochemical reaction. Efficiency of reaction depends on the contact time of substrate and reactant in that particular system. Yield from the reaction highly depends on incubation period. In any enzymatic reaction, rate of substrate consumption and product formation relies on incubation time (Rajak and Banerjee 2015).

Hence, to study the consequences of incubation time on debittering process, enzymatic action on targeted biomolecules has been carried out for a time period of 0.5–3 h at 1% (v/v) enzyme loading, 35 °C, 30 IU/mL enzyme titre. Highest debittering has been observed (31.30%) at 3 h but optimum significant debittering occurred at 2 h incubation period (30.79%) ($F_{5,12} = 8.89$; $P < 0.001$) (Fig. 1c). This indicates that the debittering efficiency of fruit juice depends on the incubation time in a noteworthy manner. Rout and Banerjee (2006) reported 25% decrease in the

tannin content of pomegranate fruit juice after 2 h of incubation with enzyme.

Effect of enzyme titre on debittering process

Enzyme titre plays an important role in any enzyme base reaction process. Activity of the enzyme ensures proper catalysis of the targeted substrate into a definite product. Low titre of enzymes also has potential to catalyze large amount of substrate into desired product. Thus, optimum enzyme titre plays a crucial role in enzymatic bioconversion process (Gujjala et al. 2016).

A range of enzyme titre (15–35 IU/mL) has been selected to observe the effect of different titre value on debittering process while other parameters viz., enzyme volume, temperature and time were kept constant at 1% (v/v), 35 °C and 2 h, respectively. From the obtained data, it is observed that 30 IU/mL enzyme activity gives maximum debittering, where further increment in activity resulted no increase in the debittering process (Fig. 1d).

Optimization of enzyme mediated debittering process

After analyzing the effects of single parameters on response, further optimization has been carried out employing RSM, a multiple regression analysis technique.

Prediction of debittering study using RSM

RSM study has been carried out by coupling it with ANOVA, its precursor, to determine the debittering efficiency of juice by the significant effects of enzyme volume, temperature, incubation time and enzyme titre as well as the effects of their interaction on debittering. ANOVA for debittering study included percentage contribution (PC) of the factors, degree of freedom (*df*), *F* value, *P* value and level of significance as mentioned in supplementary Table 1 which has been carried out at 95% confidence level (5% significance level). The standard ANOVA confirms the fitting of the quadratic polynomial model. The model *F* value (54.48) of the regression model for enzymatic debittering of citrus fruit juice is higher than the tabular value ($P < 0.001$), implies that the regression model rejects the null hypothesis. The probability greater than *F* value is the probability of *F* statistics value which is used for null hypothesis test. The factors which have an *F*-statistics probability value less than 0.05 are denoted as significant (Sadhukhan et al. 2014).

In ANOVA, enzyme volume (*X*1), temperature (*X*2), incubation time (*X*3) and enzyme titre (*X*4) exhibit the statistical significance at 5% along with their square terms and interaction terms. While studying the impact of

individual or squared term of variables on percentage debittering it was found that the maximum percentage of debittering was recorded when enzyme volume was one of the important variables. In terms of percent contribution on overall debittering of juice, the individual and squared term had 7.68% and 9.19%, respectively. While finding out the impact of other parameters on percent debittering of juice it was observed that enzyme titre and temperature were found to be most significant. A 4.62% and 1.34% impact were observed on both individual and square term, respectively. While analyzing the impact of independent variables interaction of parameters on juice debittering, interaction of enzyme volume and enzyme titre; enzyme volume and temperature were the most significant (1.35%) and second most significant (0.98%) contributors.

Relationship between debittering percentages and process parameters are figured out by a full quadratic regression equation. 31 data points from the experiment have been used in order to develop the regression model. Second-order regression equation for percentage bitterness removal with different factors such as temperature, time and enzyme loading factors are quadratic as computed by the software is given below:

$$\begin{aligned} \text{Debittering (\%)} = & -294.3 + 55.3(X_1) + 11.07(X_2) \\ & + 15.61(X_3) + 4.67(X_4) \\ & - 48.48(X_1^2) - 0.1848(X_2^2) - 3.04(X_3^2) \\ & - 0.1086(X_4^2) + 0.636(X_1 \times X_2) + 2.65(X_1 \\ & \times X_3) + 0.749(X_1 \times X_4) \\ & + 0.006(X_2 \times X_3) + 0.0577(X_2 \times X_4) \\ & - 0.133(X_3 \times X_4) \end{aligned} \quad (5)$$

where *X*1, Enzyme Volume; *X*2, Temperature; *X*3, Time and *X*4, Enzyme titre.

A model summary is given in supplementary Table 1. From the developed model of debittering, the coefficient of determination R^2 (97.95%), adjusted R^2 (96.15%) and predicted R^2 (96.69%) indicated that the regression model is reliable for predicting the responses. No significant difference between R^2 and adj R^2 values signifies the adequacy of the regression model for enzymatic debittering.

This above mentioned equation reveals the individual variables or double interaction affected bitterness removal in citrus fruit juice (Sadhukhan et al. 2014). Regression coefficient value of actual and predicted debittering percentage from RSM prediction model is 0.971 which proves the competence of the model which is compatible to the experiments.

Table 1 Biochemical analysis and elemental comparison of treated and untreated juice

Parameters	Juice	
	Control	Enzyme treatment*
pH	3.74–3.76	3.78–3.79
Ascorbic acid (mg/100 g)	43.67 ± 0.02 ^a	41.63 ± 0.01 ^b
TSS (Brix)	4.98 ± 0.01 ^a	5.01 ± 0.03 ^a
Ash (%)	0.55 ± 0.01 ^a	0.55 ± 0.01 ^a
Titrate acidity (% citric acid)	0.58 ± 0.01 ^a	0.56 ± 0.01 ^b
Reducing sugar (mg/ml)	9.90 ± 0.20 ^a	13.61 ± 0.02 ^b
Total sugar (mg/ml)	77.32 ± 0.01 ^a	114.82 ± 0.01 ^b
Antioxidant property	70.57 ± 0.01 ^a	81.37 ± 0.03 ^b
<i>DPPH</i> scavenging activity (%)		
Total phenol (µg GAE/ml)	425.33 ± 0.58 ^a	576.33 ± 0.58 ^b
Protein (mg/ml)	0.72 ± 0.01 ^a	0.61 ± 0.01 ^b
Mg	7.38 ± 0.01 ^a	7.41 ± 0.02 ^a
Ca	23.71 ± 0.01 ^a	23.70 ± 0.02 ^a
Fe	1.11 ± 0.02 ^a	1.10 ± 0.02 ^a
Cu	0.41 ± 0.02 ^a	0.40 ± 0.01 ^a
Zn	1.03 ± 0.03 ^a	1.03 ± 0.02 ^a

All values are in ± SD, n = 3; Grouping Information Using the Fisher LSD Method and 95% Confidence. Means that do not share a letter are significantly different

*Optimum conditions of enzyme treatment

Fig. 2 Contour response plots depicting the interaction of **a** temperature and enzyme volume, **b** time and enzyme volume, **c** enzyme titre and enzyme volume, **d** time and temperature, **e** enzyme titre and temperature and **f** enzyme titre and time on debittering process

Contour response plot

Each contour response plot from the predicted model represents the effect of two parameters. In Fig. 2a–c describes the interaction effect between temperature, enzyme volume, time and enzyme titre on debittering. Whereas, (D–F) shows the interaction of time, temperature, enzyme titre and temperature on debittering process. Interaction of temperature and enzyme volume, time and enzyme volume, enzyme titre and enzyme volume, and enzyme titre and temperature is significant at the level of 5%, while interaction of time and temperature, and enzyme titre and temperature is not found significant.

Graphical representation shows the maximum debittering in 35 °C at 2 h. Though, there is a feeble increase in debittering with 1.5% than 1% but significant debittering occurred at 1%. ($38.95 \pm 3.27\%$) where the predicted debitter percentage by model is 37.87%. From the graphical representation of the quadratic equation, it is observed that at 1% enzyme loading, 35 °C and 2 h incubation time gives the best maximum debittering of citrus fruit juice by hydrolyzing the ester bonds of the tannin molecule (Rout and Banerjee 2006). de Lima et al. (2014) reported tannin degradation from 5.19 to 2.39 mg/g of grape juice after 2 h of incubation, using 20% tannase, produced from *Penicillium montanense* URM 6286.

However, the report on enzyme mediated tannin removal of Assam lemon is feeble in scientific domain. In the present study, enzymatic hydrolysis of tannin facilitates removal of 38.95% tannin from the Assam lemon juice.

Optimization of debittering process

The maximum debittering percentage from composite desirability optimization as shown in Fig. 3, is 40.30%. The suggested optimal conditions for maximum debittering are 1.12% (v/v) enzyme volume, 37 °C and 2 h incubation time. As per the proposed optimization model, experiment has been conducted in triplicates. In situ experiment shows $40.12 \pm 0.02\%$ debittering as per recommended experimental conditions. Composite desirability and individual desirability in the optimization of Assam lemon debittering are 0.9255 and 0.92551, respectively which demonstrates how well a combination of variables fulfill the defined goals. Both the desirability values are close to 1, implying favorable debittering for all responses.

HPLC analysis

HPLC study has been carried out for the quantification of gallic acid which is the hydrolysis product of hydrolysable tannin. This hydrolysis is catalyzed by tannase which acts upon ester and depside bond present within the hydrolysable tannins such as tannic acid, methyl gallate, ethyl gallate, npropylgallate, and isoamyl gallate, gallotannins and complex tannins, resulting in the production of gallic acid and glucose (Kar and Banerjee 2000; Aguilar et al. 2007). HPLC study reveals that the gallic acid concentration in debittered citrus juice is 55% higher than untreated juice which is an indication of the biotransformation of tannin to gallic acid. Total 55% increment of gallic acid

Fig. 3 Response optimization using desirability function

resulted from the removal of $40.12 \pm 0.02\%$ bitterness with 1.12% (v/v) enzyme treatment at 2 h of incubation time. Optimum experimental condition facilitates the biotransformation of tannin molecules into gallic acid through enzymatic intervention. According to Aguilar et al. (2007) acting upon tannic acid, tannase can produce gallic acid and glucose through 2,3,4,6,-tetragalloyl glucose and two other kinds of monogalloyl glucose. Gallic acid has strong antioxidant property among various polyphenol with extensive use in pharmaceutical industry. Thus, enrichment of gallic acid in enzyme treated Assam lemon juice not only established the fact of debittering through biotransformation of tannin molecules but also simultaneously assists in enrichment of antioxidant property in treated juice. Hence, enzyme treated juice can act as a scavenger of free radicals inside the body (Malinda et al. 2017).

Biochemical analysis

Among all the fruits, citrus varieties play an important role by providing different nutritious components like minerals, vitamins, phenolic compounds, carbohydrate, etc. Thus, consumption of fruit juice is the best and easy way to fulfill the nutritional deficiency.

In order to determine the change in quality due to the enzyme mediated debittering, the juice was subjected to the analysis of pH, ascorbic acid, TSS, titrable acidity, protein content, total and reducing sugar, antioxidant property and total phenol content. In this aspect treated juice and untreated juice have not shown any significant variations (Table 1).

No significant changes has been found in juice pH, TSS and ash content when compared to treated and raw juices while ascorbic acid is decreased by 4.69% in treated juice. Decrease in bitterness with the raised pH has also been observed in kinnow juice and Thai-tangerine juice processing (Ranote and Bains 1982; Chaisawadi et al. 1998). However, treated juice (13.61 mg/ml) shows 37.51% higher reducing sugar content than untreated juice ($F_{1,4} = 7.71$; $P < 0.05$). There is an increase of 48.50% in total sugar content than untreated juice. This depicts that as the enzyme system is effective in hydrolyzing the ester bond in the tannin molecules, the sugar content increased to a significant extent (Rout and Banerjee 2006). The antioxidant activity of the juice has also been evaluated by DPPH radical scavenging activity. It has been observed that the antioxidant activity has increased by 15.30% in enzymatic treated juice than untreated one ($F_{1,4} = 7.71$; $P < 0.05$). As increment of gallic acid in the treated juice is the prime element for antioxidant property as gallic acid itself has been employed in food materials as antioxidant (Aguilar et al. 2007). According to several studies, it is well accepted that gallic acid, low molecular weight

triphenolic compound, possess strong antioxidant property (Badhani et al. 2015; Hu et al. 2016; Malinda et al. 2017). In this study, production of gallic acid in treated juice may facilitate the enhancement of antioxidant property. Total phenol content also significantly increases ($F_{1,4} = 7.71$; $P < 0.05$) in treated juice by 35.50% may be due to the release of polyphenols in enzymatic catalysis process. The results reveal that enzymatic treatment not only helps to sustain its quality attributes but also helps to increase some nutritional properties.

Fruit juices also contain some amount of essential trace elements those have great impact on dietary intake, and thus the concentration of those elements need to be monitored. Though some of those elements are the basic need for proper metabolic function of the body but excess amount possess several harmful impacts (Eneji et al. 2015). For the present study, several essential trace elements like Magnesium (Mg), Calcium (Ca), Manganese (Mn), Iron (Fe), Copper (Cu) and Zinc (Zn) have been monitored after treatment process to ensure the quality attributes of the final product. No significant alteration of elemental composition has been observed after enzymatic debittering of Assam lemon juice. It represents no impact of enzyme mediated debittering on the elemental constituents present in the juice (Table 1).

Evaluation of sensory attributes of bitter and debitter juice

Sensory attributes of any food material can be observed by a person with different sensory organs. Different types of sensory properties like colour, taste, aroma and mouth feel are considered for the evaluation. For the evaluation, different concentration enzyme (0.5–5%, v/v) treated juice has taken along with the untreated one. Juices are distributed among 12 panels of judges for the test and their performance have been recorded. Table 2 represents scores of each sensory trait along with the overall score value. By interpreting the specific value of each attribute, it can be inferred that though overall acceptability of 1% (v/v) enzyme treated juice is highest but in color and taste, 0.50% (v/v) enzyme treated juice has gained highest acceptability. More specific value of color and taste in 0.50% (v/v) enzyme treated juice may be due to the presence of low concentration of enzyme. While high concentration of enzyme treatment (5%, v/v) ranked lowest in acceptability in comparison to other concentration of enzyme added treatments. 1% (v/v) enzyme treated Assam lemon juice makes a distinctive difference in comparison to higher concentration of enzyme treated juice, which has gained the utmost importance (rank 1) among all other treatments with different concentrations of enzyme.

Table 2 Comparison of organoleptic property of debittered Assam lemon juices

	Untreated juice	Treated juice					
		0.50% (v/v)	1% (v/v)	2% (v/v)	3% (v/v)	4% (v/v)	5% (v/v)
Sensory properties (specific value)							
Color	7.92	7.67	7.50	5.75	3.17	2.50	2.67
Taste	1.17	6.75	6.67	4.58	2.75	3.08	2.42
Aroma	5.83	6.08	6.33	5.58	5.00	4.92	5.08
Mouth feel	1.83	6.75	7.83	7.75	6.33	5.33	5.42
Overall preference value	4.19	6.81	7.08	5.92	4.31	3.96	3.90
Overall rank	7th	2nd	1st	3rd	4th	5th	6th

Conclusion

Tannase mediated debittering of citrus fruit juices proves to be an effective approach for bitterness removal. According to the best knowledge of the authors, this is the first report of tannase mediated debittering of Assam lemon juice without compromising its nutritional quality. Prediction model of debittering shows high level of regression model adequacy value (R^2) which is almost similar to the predicted one (R^2). Furthermore, insignificant lack of fit ensures the model accuracy. Maximum debittering efficiency of $40.12 \pm 0.02\%$ is achieved at 37°C in 2 h. Percentage contribution in ANOVA denotes enzyme volume and enzyme titre as first and second significant contributor in debittering process. Comparison of experimental and predicted debittering percentage showed a high coefficient value (0.971) which ensure the soundness of the model. The process improves nutritional quality without affecting its natural characteristics. In addition, the enzymatic process has also enhanced antioxidant property of the juice.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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