Requirements of mixed tangerine (*Citrus tangerine*) and pineapple (*Ananas comosus*) powdered peel wastes fermentation for citric acid production

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Abstract: In this study, mixed substrate submerged fermentation of fruit peel wastes for the production of citric acid is reported. Powders of the peel wastes of tangerine and pineapple were used as substrates. Methanol (1%-3% w/w) and sucrose (5%-15% w/w) were supplemented in the fermentation media (pH on the range of 2.0-6.0) with moisture content of 90% (wet basis). Effect of substrate mix, pH of the media, methanol and sucrose concentrations on the yield of citric acid was studied, and their optimal levels for the maximum yield of citric acid were identified based on Taguchi method. Methanol as stimulant in the medium and ratio of substrates in the mix had the highest influence on the yield of citric acid. Fermentation media with tangerine and pineapple peel wastes mixed in the ratio of 1:1, initial pH of 4, and supplemented with methanol and sucrose to the extent of 5% and 15% (w/w), respectively was found to be optimal, and that resulted in the maximum citric acid yield of 137.2±0.4 g kg^{-1} of dry matter after 12 days of fermentation. The paper and thin layer chromatography, and spectrophotometric absorption spectra of the sample confirmed the production of citric acid. The fermentation kinetics of the citric acid revealed that the production of citric acid is a two stage process. In the first stage, slow production of citric acid at rapid biomass growth takes place, whereas in the second stage, very rapid citric acid production at declined biomass growth rate takes place. Valorisation of fruit peel waste for the production of citric acid reduces the environmental pollution, carbon emissions and greenhouse gas effects.

Key words: citric acid, mixed substrate fermentation, Taguchi method, tangerine and pineapple

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1 Introduction

Citric acid, a tricarboxylic acid, is a common metabolite of plants and animals. Citric acid is in high demand, and it is widely used in food processing and pharmaceutical industries (Angumeenal and Venkappayya, 2013).The most common method of production of citric acid is by submerged fermentation or liquid surface fermentation of sugarcane molasses. The process organism *Aspergillus niger* is very sensitive to trace metal ions. Despite pre-treatment with chelating agents such as sodium or potassium ferro-cyanide and ethylene diamine tetracetic acid, molasses gives low yields of citric acid (Max et al., 2010; Sawant et al., 2018). Low cost substrates, such as agro-industrial waste have been tried (Karthikeyan and

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Sivakumar, 2010; Kareem et al., 2010; Dhillon et al., 2011; Adeoye et al., 2015; Morgunov et al., 2018), and optimum process parameters have been identified with a view to commercialise the process to industrial scale.

Peels are the major solid waste of fruit processing industries. Recycling and valorisation of peel wastes to useful products through the action of microorganisms has recently gained increasing interest (Torrado et al., 2011; Patel, 2012; Wu, 2016). Tangerine (*Citrus tangerina*) and pineapple (*Ananas comosus*) are available in huge quantities during late winter and summer in the north eastern region of India, sometimes leading to glut in the market due to the transportation problems. Peels of both fruits are rich in sugars, cellulose, hemicelluloses and insoluble fibre-rich fractions (Torrado et al., 2011; Tropea et al., 2014; Ahmadi et al., 2015). Composition and physico-chemistry of tangerine and pineapple peels indicate the potentiality of peel wastes to produce various useful and valuable added products.

Researchers have focused on the utilization of tangerine and pineapple peel wastes for the production of citric acid. Kumagai et al.(1981) found that the fermentation of dried peels of tangerine required the addition of molasses juice to overcome the negative effect of large amounts of impurities. The maximum yield of citric acid (55%-65%) was achieved in a medium consisting of 6 g of tangerine peel and 11 mL molasses juice (14% sugar content) at 30ºC after three days. Kang et al. (1989) reported citric acid yield of 80.4% based on total sugar added by semi-solid fermentation of tangerine peels. The addition of 0.2% NH_4NO_3, 0.1% of $MgSO_4.7H_2O$, 2.5% methanol or 1.5% of ethanol was found to be the optimum for the maximum yield of citric acid. Tran and Mitchell (1995) reported citric acid yield of 161 g kg^{-1} of dried pineapple peel waste with culture broth moisture content of 70% in presence of 3% methanol. Kareem et al. (2010) found that fermentation of pineapple peel waste supplemented with sucrose (15%, w/v), ammonium nitrate (0.25%, w/v) and methanol (2%, v/v) produced 66.6 g citric acid per kg of waste in 5 days at 30ºC with initial moisture content of 65%. These works reveal that tangerine and pineapple peel wastes are the potential substrates for the citric acid production.

In general, fruits are region and season specific horticultural crops in India. Hence, the specific fruit peel wastes may not be available in sufficient quantities for perennial production of citric acid. Therefore, a mixed substrate fermentation using tangerine and pineapple peel wastes has been attempted in the present study for the production of citric acid. Synergistic and supplementary effect of the combined substrate is also expected in the fermentation process. Further, kinetic behaviour of the fermentation media in terms variation in physico-chemical and biological attributes throughout the course of fermentation was studied. It has the potentials to understand the fermentation pattern and inter-relationships among the factors, and shed light on the performance, optimisation and control of the fermentation process.

The present work consists of the following aspects:

(i) Identification of the optimum set of mixed substrate fermentation process parameters for obtaining maximum yield of citric acid;

(ii) Analysis of variation in physico-chemical and biological attributes of fermentation media during the process of fermentation.

2Materials and methods

2.1Substrates and pre-treatment

Good quality, fresh and uniformly ripe fruits of tangerine and pineapples ready for selling in the consumer market were collected from the organic garden of a renowned grower in the north eastern region of India. The tangerines were peeled and the loose pulp fibres were separated. The pineapple fruits were peeled using a knife carefully such that the fruit pulp adherence with peel is minimised. The peels were cut into pieces of size approximately 5 cm × 3 cm. The peels were separately air dried, and then oven dried at 60ºC for 24 h until a constant weight was achieved (Massini et al., 2013). The dried peels were ground in a grinder. The powder was passed through a standard 212 μm sieve. The powder was stored in a freezer at 4ºC for further use.

2.2 Physico-chemical analysis of substrates

Standard methods of AOAC (2016) were used to determine moisture content, protein, total carbohydrate and ash content of the tangerine and pineapple peel powders. Volatile solid was determined by subtracting ash content from total solid. Total sugar was determined by phenol sulphuric acid method (Masuko et al., 2005). Reducing sugar was estimated by dinitro salicylic acid method (McCleary and McGeough, 2015). Cellulose content was determined by Updegraff method (Yu et al., 2014).

2.3 Microbial culture and inoculum preparation

Aspergillus niger MTCC 282 was procured in the freeze-dried form from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The microorganism was maintained on Czapek yeast extract agar slants at 4ºC, and sub-cultured at interval of 15 days. The spores were suspended by using glass beads to collect the spores in distilled water with tween-80 (2%). The spores were counted using a haemocytometer to maintain a density of 1–2×10^8 spores per mL (Gershater, 2010).

2.4 Experiment

Yield of citric acid in terms of quantity of citric acid produced per unit weight of substrate is a vital parameter to judge the potential of the substrate for citric acid production. Yield of citric acid is affected by various parameters of the fermentation process like, substrate, type and concentration of carbon sources, pH, aeration, oligoelements concentration, type of producing microorganism etc. An experiment was therefore conducted to study the effect of various parameters of the fermentation process on the yield of citric acid (dependent parameter).

In the past research studies, citric acid was produced using only one substrate. Mixed substrate submerged fermentation was employed for the first time in the present study for the production of citric acid. Synergistic effect of the mixed substrates in the fermentation media has increased the production of lovastatin (Subhagar et al., 2009), *n*-butanol (Sabra et al., 2014) and lactic acid (Kazakos et al., 2016). In the present study, pineapple peel powder and tangerine peel powder were mixed in three proportions, and the selected mix proportions were 1:3, 1:1 and 3:1 (tangerine peel :pineapplepeel).

Initial pH of the fermentation media (in the range of 2–6) and percent supplementation of methanol (1%–5% w/w of solid component) and sucrose (5%–15% w/w of solid component) to the fermentation media were the other independent parameters taken up for the experiment based on the past research (Dhillon et al., 2011; Auta et al., 2014; Yu et al., 2017). Three levels of initial pH of the media (2.0, 4.0 and 6.0),and methanol (1.0%, 3.0%, 5.0% w/w of solid component) and sucrose concentration (5%, 10% and 15% w/w of solid component)were selected for the experiment.

Experiment was designed based on orthogonal array technique. Orthogonal arrays are 2-dimensional arrays of numbers which possess the interesting quality that by choosing any two columns in the array, an even distribution of all the pair-wise combinations of values in the array can be received (Lee and Kuo, 2013). An orthogonal array with 9 experiment runs for 4 parameters (substrate mix, pH, concentration of methanol and sucrose) each with 3 levels, L_9 (3^4) was selected from the standard orthogonal test array table. The parameters and their levels were mapped onto the array. Set of combination of the parameters for the orthogonal test is shown in Table 1. Mono-substrate fermentation medium containing tangerine peel powder and pineapple peel powder alone without the addition of anysupplement were taken as a control.

Table 1 Set of combination of the parameters for the orthogonal array test

Experiment No.	Set of combination	Substrate mix	pH	Methanol (% w/w)	Sucrose (% w/w)
1	1 1 1 1	T:P = 1:3	2.0	1.0	5.0
2	1 2 2 2	T:P = 1:3	4.0	3.0	10.0
3	1 3 3 3	T:P = 1:3	6.0	5.0	15.0

4	2 1 2 3	T:P = 1:1	2.0	3.0	15.0
5	2 2 3 1	T:P = 1:1	4.0	5.0	5.0
6	2 3 1 2	T:P = 1:1	6.0	1.0	10.0
7	3 1 3 2	T:P = 3:1	2.0	5.0	10.0
8	3 2 1 3	T:P = 3:1	4.0	1.0	15.0
9	3 3 2 1	T:P = 3:1	6.0	3.0	5.0
10	Control	T:P = 1:0	5.15	-	-
11	Control	T:P = 0:1	5.01	-	-

Note: Substrate mix is expressed as Tangerine peel powder: pineapple peel powder - T: P

2.5 Preparation of media and fermentation

Ten grams of powder was taken in 500 mL Erlenmeyer flasks. Methanol and sucrose were added to the flasks in the required quantities to obtain the necessary proportion in the fermentation media. The moisture content of the media was increased to 90% (wet basis) by adding distilled water. The pH of the media was adjusted by adding 0.1N HCl to achieve pH of 2.0 and 4.0, and 0.1N NaOH to achieve pH of 6.0. The content of the fermentation media was thoroughly mixed and autoclaved at 121ºC for 30 minutes. After sterilization, standard inoculum (1 mL of 10^8 spores mL^{-1}) was used to inoculate the flasks. The flasks were then incubated at 30ºC under shaking conditions at 200 rpm. Fermentation process was decided to be completed when TSS of the fermenting product and the citric acid produced were found to be constant for consecutive 3days (Paul et al., 2016). After the completion of fermentation process, the slurry was filtered through Whatmann filter paper No. 42, and the filtrate was subjected for further analysis. Three replications were conducted for each set of combination of parameters.

2.6 Determination of citric acid yield

Citric acid was estimated gravimetrically, using pyridine-acetic anhydride method (Adeoye et al., 2015; Vidya et al., 2018). One mL of the diluted culture filtrate along with 1.30 mL of pyridine was added in the test tube and swirled briskly. Then 5.70 mL of acetic anhydride was added in the test tube. The test tube was placed in a water bath at 32ºC for 30 min. The optical density was measured on a UV-VIS spectrophotometer (Cecil-7400, 7000 Series Aquarious) at 405 nm and citric acid contents of the sample were estimated with reference (run parallel, replacing 1.0 mL of the culture filtrate with distilled water) to the standard.

2.7 Identification of optimum process parameters and data analysis

In Taguchi method, signal to noise ratios (S/N ratio) are the objective functions for the optimization. They are used in data analysis and prediction of the optimum results. Goal of the present study is to maximize the conversion of substrate mixture into citric acid yield. It is a 'larger the better' type of quality characteristic. The standard formula for computing S/N ratio for this type of response is,

$$(S/N)_i = -10\, log\left[\frac{1}{n}\sum_{j=1}^{n}\frac{1}{y_{ij}^2}\right] \quad (1)$$

where, i= experiment number; y_{ij} = measured value of quality characteristic for the j^{th} replication of the i^{th} experiment

n= number of replications for the experimental combination

S/N ratio was computed for each of the 9 experimental runs. The effect of independent parameters was separated out in terms of S/N ratio. The average values of S/N ratios of the 4 fermentation process parameters at each of the levels were calculated. The levels corresponding to the highest S/N ratio values were chosen for each fermentation process parameter representing the optimum condition for maximising the citric acid yield. The difference (Δ) between the maximum and minimum values of the average S/N ratio for each fermentation process parameter was determined. The parameters were ranked in terms of contribution to citric acid yield according to the descending values of Δ.

The mean citric acid yield for each fermentation process parameter at different levels was determined. The citric acid yield was analysed to extract the main effects of the process parameters. The analysis of variance (ANOVA) technique was then applied to determine the statistically significant parameters at 5% level of significance.

The predicted S/N ratio at optimal process conditions can be computed by the following mathematical equation:

$$S/N_{predicted} = \overline{S/N} + \sum_{k=1}^{m}(S/N_k - \overline{S/N}) \tag{2}$$

where, $\overline{S/N}$ = mean of all S/N ratios

S/Nk= S/N ratio at the optimal level for kth parameter

m = number of process parameters that significantly affect the process

Theoretical maximum citric acid yield at the optimal fermentation process parameters was determined using the following equation:

$$y_{predicted} = \sqrt{\frac{1}{10^{\frac{S/N_{predicted}}{-10}}}} \tag{3}$$

2.8 Confirmation experiment

Confirmation experiment (with 3 replications) was conducted at the optimal fermentation process parameters to validate the predicted results. The procedure as mentioned above was followed. Homogeneously shaken 10 g of sample was drawn and analysed for the citric acid yieldevery day since fermentation was initiated. The process of fermentation was continued till there was no significant improvement in the yield of citric acid. $Y_{predicted} \pm CI$ give the 95% confidence interval for the predicted value of citric acid yield (Cumming and Calin-Jageman, 2016). The Confidence interval(*CI*) was estimated using the following two Equations:

$$CI = \sqrt{F_{(0.05,1,df_{error})} MSS_{error}\left[\frac{1}{N_{eff}} + \frac{1}{R}\right]} \tag{4}$$

$$N_{eff} = \frac{N}{1 + df_{total}} \tag{5}$$

where, $F_{(0.05,\ 1,\ df_{error})}$ = F-ratio required for 95% confidence interval.

df_{error}= degrees of freedom of error

df_{total} = degrees of freedom of total associated with estimate of mean optimum

MSS_{error}= mean sum of squares of the error

R= number of trials for the confirmation experiment

N= total number of experiments

2.9 Analysis of variation in physico-chemical and biological attributes of fermentation media during the process of fermentation

Fermentation kinetic behaviour at the optimised process parameters was studied by analysing the variation in citric acid yield, pH, residual total sugar, reducing sugar, cellulose and biomass content in the fermentation media with time. Ten grams of sample was drawn from each fermentation flask aseptically, and these attributes were analysed daily till the fermentation was completed. Citric acid yield, residual total sugar, reducing sugar and cellulose content wereestimated by the method as mentioned above. The pH of the sample was measured using a digital pH-meter (Sartorius PB-11, 67 Germany). Weight of *A. niger* (biomass) was estimated by overall mass balance (Behera and Varma, 2017). Results were expressed in g kg^{-1} of initial dry matter. Specific kinetic rate of changes of all the parameters were calculated and analysed for each day, and expressed on the basis of change over 24 hours with respect to the dry substrate (g kg^{-1}24 h^{-1}). Rate of change towards decreasing or increasing trend was expressed as negative or positive value, respectively.

Variation in physico-chemical and biological attributes, and their kinetic rate throughout the fermentation period were plotted. Inter-relationship among the attributes wasstudied based on the Pearson correlation coefficient for variation in physico-chemical and biological attributes, and their kinetic rate throughout the fermentation period.

2.10 Characterisation of citric acid

Citric acid produced was partially characterised by analysing its behaviour in paper and thin layer chromatography, and compared with the standard citric acid. Spectrophotometric absorption spectra of the sample at ultraviolet and visible wavelength were also studied.

2.10.1Chromatographic separation

Concentrated citric acid was subjected to paper chromatography with 1:1 butanol water system. After the completion of chromatographic run, spray of bromocresol green was used for the development of chromatograph.

R_f value of the citric acid sample was determined from the chromatograph and compared with standard citric acid. Thin layer chromatography (TLC) of citric acid sample was also performed using readymade plates of silica gel GF254 (20×20cm) of 0.25mm thickness (Merck, Germany).

2.10.2Spectrophotometric absorption spectra

Filtered citric acid sample was analysed to assess its uv-visible absorption phenomena by scanning the absorbance in the wavelength region of 200-1100 nm using UV-VIS spectrophotometer (Cecil-7400, 7000 Series Aquarious). Water was used as a solvent and the spectrum was compared with standard citric acid (Krukowski et al., 2017; Patil and Patil, 2014).

3 Results and discussion

3.1 Physico-chemical properties of tangerine and pineapple peel powders

Physico-chemical properties of tangerine and pineapple peel powders are shown in Table 2.

Table 2 Physico-chemical properties of tangerine and pineapple peel powders

Parameter	Tangerine peel powder (g kg^{-1} of dry matter)	Pineapple peel powder (g kg^{-1} of dry matter)
Moisture	33.52 ± 0.36	41.40 ± 0.64
Protein	41.60 ± 0.64	9.34 ± 0.38
Total carbohydrate	696.50 ± 1.35	863.50 ± 1.73
Ash	36.54 ± 0.82	16.65 ± 0.47
Volatile solid	963.83 ± 2.44	983.90 ± 3.87
Total sugar	294.55 ± 1.65	123.41 ± 1.44
Reducing sugar	263.68 ± 2.41	103.26 ± 1.75
Cellulose	163.25 ± 1.34	464.45 ± 3.56

Note: All values are mean of three replicates ± standard deviation

High fraction of total carbohydrate (69.6% and 86.3% for tangerine and pineapple peel, respectively) and low fraction of protein (4.2% and 9.3% for tangerine and pineapple peel, respectively) indicate the suitability of the substrate for microbial fermentation. Sugar content of tangerine peel powder (29.5% total sugar and 26.3% reducing sugar) was higher than that of pineapple peel powder (12.3% total sugar and 10.3% reducing sugar). Fibre content including cellulose of pineapple peel powder was almost 3 times higher than that in tangerine peel powder. Sugar is rapidly utilised during early fermentation periods. At the hypoglycaemic stage, cellulose becomes the primary substrate for the production of citric acid (Ali et al., 2012). Therefore, combination of tangerine and pineapple peel is expected to have complementary synergistic effect for the production of citric acid.

3.2 Effect of fermentation process parameters

Type and concentration of carbon source, pH, and addition of methanol and sucrose as supplements to the fermentation medium played crucial roles in citric acid production. The average yield of citric acid (average of 3 replications) for the 9 experimental runs of the orthogonal array test and the control is presented in Table 3. The highest citric acid yield of 109.9 gkg^{-1} of dry matter was obtained in the 5th experiment run that correspond to the substrate mix of T:P (Tangerine peel powder : pineapple peel powder) = 1:1, fermentation medium pH of 4, and addition of 5% methanol and 5% sucrose to the medium. The 9th experiment run corresponding to the substrate mix of T:P = 3:1, fermentation medium pH of 6, and addition of 3% methanol and 5% sucrose to the medium, resulted in the lowest citric acid yield of 45.4 g kg^{-1} of dry matter. Mono-substrate fermentation medium containing tangerine peel and pineapple peel alone without the addition of any supplement produced 29.4 and 77.7 g kg^{-1} of dry matter, respectively. The variation in data suggests that the combination of independent parameters at optimum level strongly support the production of citric acid from mixed substrates. S/N ratio for each experiment run was calculated using Equation 1 and it is also given in Table 3.

Table 3 Average yield of citric acid and S/N ratio

Experiment No.	Set of combination	Average citric acid yield (g kg^{-1} of dry matter)	S/N ratio
1	1 1 1 1	64.50	36.18
2	1 2 2 2	70.10	36.91
3	1 3 3 3	100.10	40.01
4	2 1 2 3	97.30	39.76
5	2 2 3 1	109.90	40.82
6	2 3 1 2	62.10	35.86
7	3 1 3 2	81.90	38.26
8	3 2 1 3	61.10	35.72
9	3 3 2 1	45.40	33.13
10	T:P = 1:0	29.40	-
11	T:P = 0:1	77.70	-

In order to determine the effect of fermentation process parameters on the citric acid yield, factor effects were separated out in terms of S/N ratio. The average values of the S/N ratio for the four fermentation process parameters at each level are shown in Figure 1, and the corresponding citric acid yield (g kg^{-1} of dry peel) is given in Table 4.

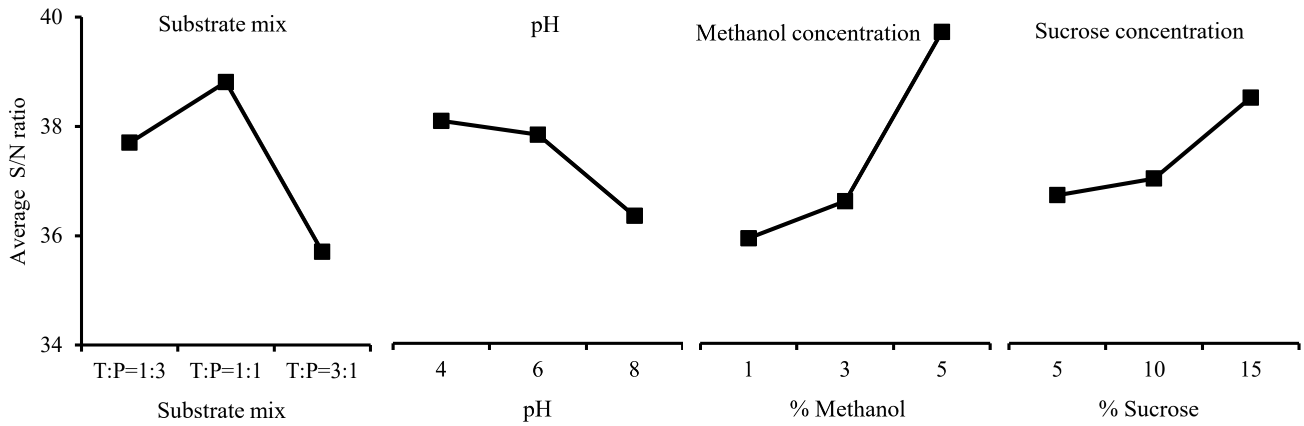


Figure 1 Effect of various fermentation process parameters on citric acid yield (S/N ratio)

Table 4 Average yield of citric acid at various levels of fermentation process parameters

Process parameters	Average yield of citric acid (g kg^{-1} of dry peel) at		
	Level # 1	Level # 2	Level # 3
Substrate mix	78.23	89.77	62.80
pH	81.23	80.37	69.20
Methanol concentration	62.57	70.93	97.30
Sucrose concentration	73.27	71.37	86.17

3.2.1 Effect of substrate mix

Table 3 indicates that the fermentation of pineapple peel as a mono-substrate using *A. niger* produced higher quantities of citric acid (77.7 g kg^{-1} of dry matter) as compared to the fermentation of tangerine peel (29.4 gkg^{-1} of dry matter). Presence of tangerine peel powder in the substrate mix of pineapple peel powder and tangerine peel powder up to 50% of the mix produced 78.23 to 89.77g of citric acid per kg of dry matter (Table 4). This indicates that the addition of tangerine peel up to 50% of the substrate mix enhanced the production of citric acid, and hence, had the synergetic effect on the fermentation process. However, further increase in the proportion of tangerine peel in the mix decreased the production of citric acid to a great extent (Figure 1 and Table 4). Yield of citric acid is strongly dependent on the type, concentration of carbon source and nutrient availability (Show et al., 2015; Cavallo et al., 2017). Physico-chemical analysis of the raw materials (Table 1) has revealed that there are remarkable differences in the content of total sugar, reducing sugar, protein and ash (mineral) percentage in pineapple peel and tangerine peel. Variation in the content of fermentation substrate(s) in the media at constant moisture, sucrose and methanol concentration will lead to the significant difference in concentration of carbon source, type and nutrient availability. It will ultimately influence the final production of citric acid. Considering these facts and experimental outcomes, substrate mix containing tangerine peel and pineapple peel in the proportion of 1:1 was taken as the best mix for the production of citric acid.

3.2.2 Effect of initial pH of fermentation medium

Initial pH of the mono-substrate medium (control) of tangerine and pineapple peel powder was 5.15 to 5.01, respectively (Table 1). Fermentation medium with initial pH of 4 produced the highest citric acid yield of 81.23 g kg^{-1} of dry matter. Hence, fermentation medium with initial pH of 4 was considered as the best for the citric acid production. Decreasing trend of citric acid production with the increase in initial pH (4 to 8) was observed (Table 4). At pH greater than 4, citric acid might have converted to gluconic acid by the reaction catalyzed by glucose oxidase (Max et al., 2010; Torrado et al., 2011). Membrane transport of metabolites is also affected by the pH of the fermentation media, influencing many enzymatic reactions (Liang et al., 2010). Optimum initial pH of 4.0 and 4.5havebeen reported for the production of citric acid by submerged fermentation of oat bran (Rao and Reddy, 2013) and cassava peel-malted sorghum based medium (Adeoye et al., 2015), respectively.

3.2.3 Effect of methanol supplementation as stimulator

The production of citric acid was maximum when methanol was added as the supplement to the fermentation medium to the extent of 5% (Table 4). Past studies (Torrado et al., 2011) have reported that the addition of methanol reduced the toxicity of metal ions, altered the fungal morphology and stimulated the secondary metabolism leading to higher citric acid production Incorporation of sufficient methanol has increased the permeability of the cell membrane and increased the transfer of nutrients, which in turn increased the excretion of citric acid across the cell membrane (Dhillon et al., 2011). Various researchers have reported the stimulatory effect of methanol in the production of citric acid from various fruit wastes, like- sweet potato vine hydrolysate (Yu et al., 2017), pineapple waste (Kareem et al., 2010) and apple pomace solid waste (Dhillon et al., 2011).

3.2.4 Effect of concentration of sucrose

Addition of sucrose as supplement to the extent of 15% in the fermentation media increased the yield of citric acid upto 86.17 gkg^{-1} of dry peel from 73.27 and 71.37 g kg^{-1} of dry peel for the media having additional 5% and 10% sucrose,respectively (Table 4). Due to its relatively low molecular weight, sucrose is readily transported into microbial cells and is hydrolysed by intracellular enzymes. Incorporation of sucroseinto the fermentation media enhances the cellular and mycelial growth, and shortens the initial preparatory phase of citric acid production (Kareem et al., 2010).Hence, the addition of 15% sucrose to the fermentation medium was considered as the best for the production of citric acid.

3.3 Optimum process parameters for the production of citric acid

The levels of process parameter corresponding to the highest S/N ratio values were chosen for each parameter fromFigure 1 and it is listed in Table 5. The combination of levels of fermentation process parameters correspond to highest S/N ratio were, substrate mix of T:P = 1:1, fermentation medium pH of 4, and addition of 5% methanol and 15% sucrose to the medium. In Taguchi method, this represents the optimum set of fermentation process parameters for achieving the maximum yield of citric acid.

Table 5 Optimum fermentation process condition and contribution of factors

Process parameters	Highest S/N ratio	Value of the level corresponding to highest S/N ratio	Δ	Rank
Substrate mix	38.81	T:P = 1:1	3.11	2
pH	38.07	4.0	1.74	4
Methanol concentration	39.70	5% (w/w)	3.77	1
Sucrose concentration	38.49	15% (w/w)	1.78	3

The difference (Δ) between the maximum and minimum values of the S/N ratio for each control factor is presented in Table 5. It indicates that, the concentration of methanol in the fermentation medium has the highest contribution to the production of citric acid, followed by the substrate mix, sucrose concentration in the medium and initial pH of the fermentation medium, i.e., Concentration of methanol > substrate mix > concentration of sucrose >initial pH.

The analysis of variance (ANOVA) of the yield of citric acid is shown in Table 6. From the calculated F ratios, it can be inferred that the factors considered in the experimental design are statistically significant at 95% confidence limit. On the basis of F ratio (higher value of F ratio), methanol concentration and substrate mix are the most significant of all other factors, and showed the highest impact on the citric acid production by submerged fermentation. Sucrose concentration and initial pH of the fermentation medium showed the least impact on citric acid production.

Table 6 Analysis of variance of the yield of citric acid

Source	df	SS	MSS	F ratio
Substrate mix	2	3295.19	1647.59	941.60[a]
pH	2	810.67	405.36	231.65[a]
Methanol concentration	2	5914.94	2957.47	1690.19[a]
Sucrose concentration	2	1167.21	583.66	333.55[a]
Error	18	31.49	1.75	
Total	26	11219.59		

Note: [a] Significant at p<0.01

3.4 Prediction of the yield of citric acid at the optimal process parameters

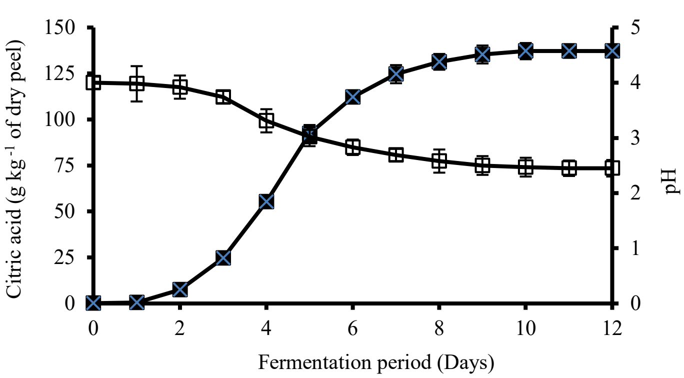
The S/N ratio of the yield of citric acid at the optimal process parameters(substrate mix of T:P = 1:1, fermentation medium pH of 4, and addition of 5% methanol and 15% sucrose to the medium) was predicted using Equation 2. The average of the S/N ratios ($\overline{S/N}$) of all the levels of process parameters (12 values shown in Figure 1) was 37.41. As all the process parameters had the significant effect on the yield of citric acid (Table 6), the values of the highest S/N ratio of all 4 process's parameters listed in Table 5 were considered for the prediction of citric acid yield. The predicted value of the S/N ratio was 42.86. The theoretical maximum value of the citric acid yield at the optimal process conditions was determined using Equation 3, and it was 138.92 g kg^{-1} of dry matter.

3.5 Validation of the Taguchi method of optimization

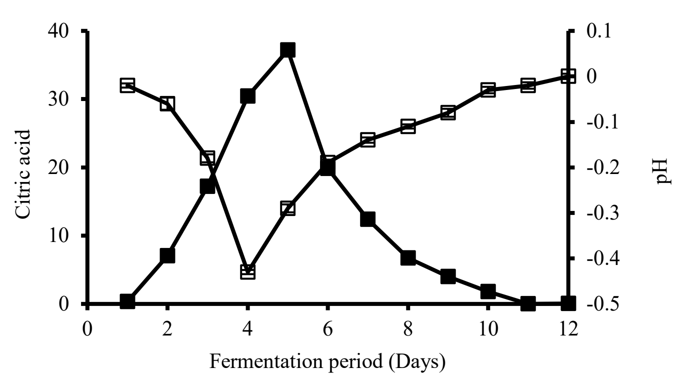
The average yield of citric acid in confirmation experiments conducted at the optimal settings of the process parameters (substrate mix of T:P = 1:1, fermentation medium pH of 4, and addition of 5% methanol and 15% sucrose to the medium) was 137.2±0.4 gkg^{-1} of dry matter after the completion of 12 days of fermentation. Though there was slight increase in the yield of citric acid between 10th and 12th day of fermentation, the difference was non-significant (data not shown). The 95% confidence interval for the predicted value was calculated using Equations 4 and 5. This value was within the range (136.88 to 140.95) of the 95% confidence interval. This validated the Taguchi optimized submerged fermentation process parameters. There was 1.77 and 4.67 -fold increase in citric acid yield with Taguchi optimised submerged process parameters as compared to the use of mono-substrate of pineapple peel and tangerine peel, respectively without any fortification of media.

3.6 Analysis of fermentation kinetics with variation in physico-chemical and biological attributes of fermentation media during the process of fermentation

Fermentation kinetics in terms of variation in yield of citric acid, pH, residual total sugar, reducing sugar, cellulose and biomass content throughout the fermentation period are shown in Figure 2. The inter-variable correlation for both variation and kinetic rate of variation are shown in Tables7 and 8.



(a)



(b)

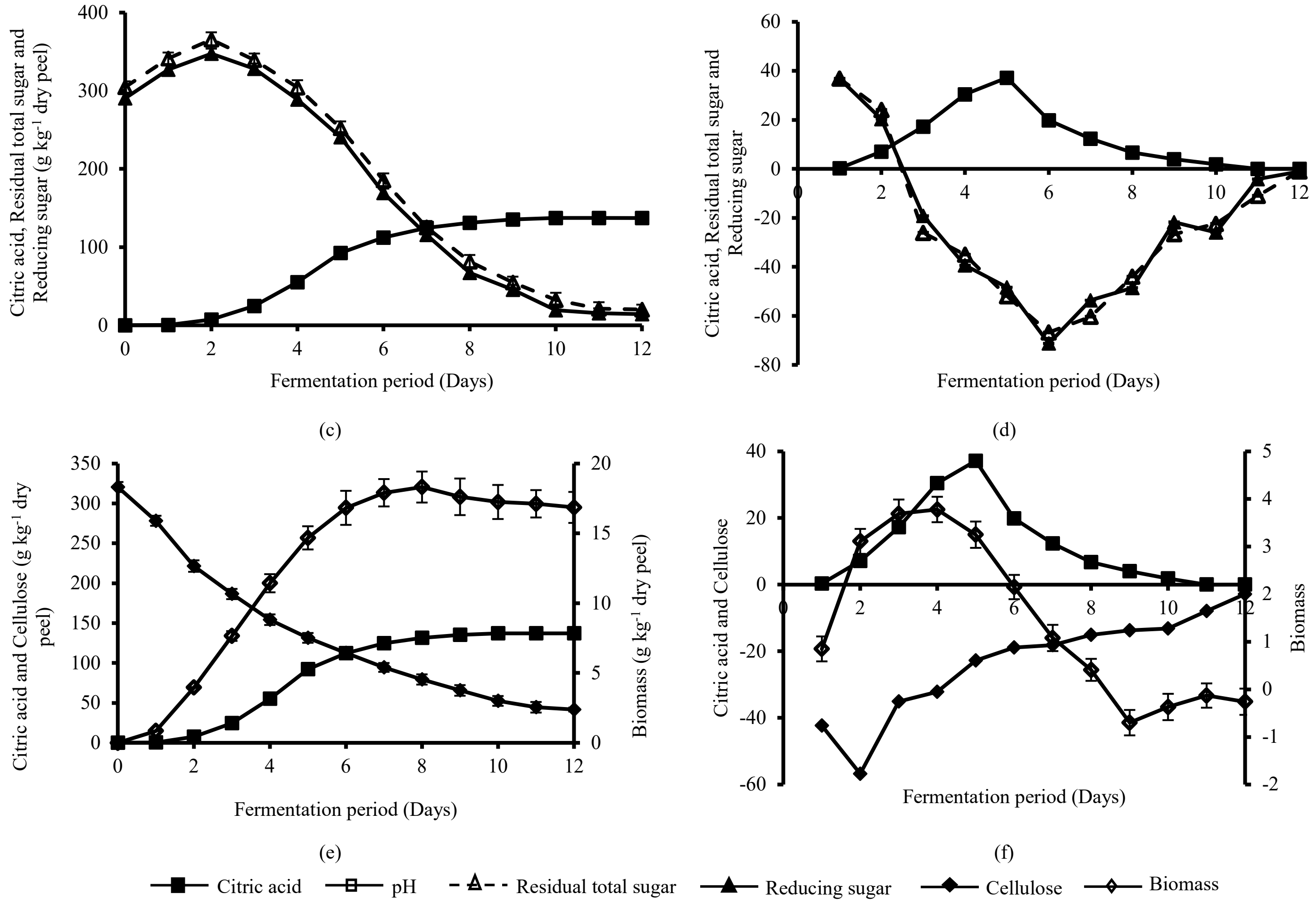


Figure 2 Variation in (a) citric acid and pH, (b) kinetic rate of citric acid and pH, (c) citric acid, residual total sugar and reducing sugar, (d) kinetic rate of citric acid, residual total sugar and reducing sugar, (e) citric acid, cellulose and biomass, (f) kinetic rate of citric acid, cellulose and biomass with fermentation period (Days)

Table 7 Pearson's correlation coefficient for variation in different attributes of fermentation media

	Citric acid	pH	Residual total sugar	Reducing sugar	Cellulose	Biomass
Citric acid	1					
pH	-0.9978	1				
Residual total sugar	-0.9373	0.9448	1			
Reducing sugar	-0.9387	0.9459	0.9998	1		
Cellulose	-0.9584	0.9643	0.8763	0.8763	1	
Biomass	0.9713	-0.9653	-0.8361	-0.8380	-0.9636	1

Table 8 Pearson's correlation coefficient for kinetic rate of variation in different attributes of fermentation media during citric acid production

	Citric acid	pH	Residual total sugar	Reducing sugar	Cellulose	Biomass
Citric acid	1					
pH	-0.9306	1				
Residual total sugar	-0.5805	0.5543	1			
Reducing sugar	-0.5870	0.5744	0.9885	1		
Cellulose	-0.2070	0.2019	-0.4652	-0.4330	1	
Biomass	0.7923	-0.7475	-0.1506	-0.1676	-0.6728	1

Production of citric acid in the fermentation media increased exponentially from 3rd day (24.76±0.54 g kg^{-1} of dry matter) of fermentation, and continued till 8th day (131.34±4.29 g kg^{-1} of dry matter). The rate of production decreased for the next consecutive 3 days, and there was no significant ($p \leq 0.05$) difference in variation (Figure 2a). The final yield obtained was 137.2±3.13 g kg^{-1} of dry matter. Change in pH of the fermentation media followed the opposite kinetic pattern of citric acid production throughout the process of fermentation (Figure 2a). The pH of the fermenting substrate decreased rapidly from 3^{rd} day (3.74±0.11) to 8^{th} day (2.58±0.21) of fermentation, and at the later stage, the reduction in rate of decrease of pH was pragmatic (Figure 2b). Pearson's correlation coefficient of variation (-0.9978) and kinetic rate of variation (-0.9306) between citric acid production and pH indicated a direct negative relationship among these two variables (Tables7 and 8). Although *A. niger* has higher tolerance to low pH than other microorganisms, excess acidification is well established to affect the growth of microorganism and citric acid production (Torrado et al., 2011).

The interrelationship between citric acid production and consumption of residual total sugar and reducing sugar during fermentation period is shown in Figure 2c-d. Residual total sugar and reducing sugar content in the fermentation media increased from 304.5±7.55 and 290.35±7.18 g kg^{-1} dry matter to 365.45±9.33 and 347.43±6.41 gkg^{-1} dry matter, respectively till the end of 2^{nd}day of fermentation, even though there was some detectable citric acid production at that stage. This can be happened only when additional sugar is produced from other sources in the media. It may be due to the ability of *A. niger* to produce various enzymes to break cellulose and pectin. *A. niger* produces pectinases and cellulases to catalyze the partial or total hydrolysis of the substrate, leading to their solubilization and release of galacturonic acid and simple sugars, respectively (Torrado et al., 2011). Reduction in sugar content in the media was observed from 3^{rd}day of fermentation. As production rate of citric acid increased with time, the consumption rate of sugar also increased exponentially till 10^{th}day of fermentation (Figure 2c-d). More than 93% of residual total sugar and 95% of reducing sugar was consumed at the end of fermentation. Very close interrelationship between citric acid production and consumption of residual total sugar and reducing sugar was established with correlation coefficient of -0.9373 and -0.9387 respectively (Table 7). Based on the amount of fermentable sugar consumed, yield of citric acid was more than 94% under optimum fermentation condition. This signifies that almost all consumed fermentable sugar was utilised in the production of citric acid. Pattern of kinetic rate of citric acid production and sugar consumption is not closely correlated. Correlation coefficient of kinetic rate of citric acid and residual total sugar as well as reducing sugar was obtained as -0.5805 and -0.5870 (Table 8). This phenomenon can be explained by the fact that citric acid is produced from simple sugars but this is not the only path of sugar consumption. Several other enzymes of *A. niger* also contributed in sugar balance by hydrolysing pectin and cellulose.

Kinetics of biomass and cellulose content with time, along with their interrelationship with citric acid production is represented in Figure 2e-f. Correlation coefficient of citric acid production with biomass growth (0.9713) and cellulose (-0.9584) revealed a very close positive and negative relationship between these variables, respectively (Table 7). It was observed that cellulose content of fermentation media reduced drastically from the initiation of fermentation process and it continued till the end of the process. But, the rate of reduction became slower at the later stage (Figure 2f). The pattern of growth kinetics of biomass closely followed the ideal microbial growth curve with shorter lag phase. The pH value maintained at the beginning (till 3^{rd} day of fermentation) of fermentation favoured the specific biomass formation by facilitating the synthesis of essential enzymes, nucleic acids and other required substances for cellular growth and multiplication. The rate of change of citric acid is moderately correlated (0.7923) with kinetic rate of biomass growth and poorly correlated (-0.2070) with cellulose breakdown (Table 8). It can be explained with the fact that citric acid is produced from fermentable sugars as a secondary metabolite of *A. niger*. Hence, the production rate of citric acid is not directly correlated with the fungal growth rate and cellulose breakdown rate. Rather, it depends upon fungal cell concentration, the intensity of cellular stimulation to produce citric acid as well as available sugar in the media.

Kinetic rate of citric acid production increased from 1st day and reached the maximum value of 37.18 gkg^{-1} per 24h on 5th day of fermentation. Growth rate of biomass increased initially and then decreased after 4th day of fermentation. This rate of decrease of biomass continued, and it became negative growth rate from 9th day onwards to till the end. Interrelationship of kinetic data between citric acid and biomass growth suggests that the production of citric acid is a two-stage process. In the first stage, slow production of citric acid at rapid biomass growth was observed, whereas the second stage revealed very rapid citric acid production at declined biomass growth rate. This fact strengthened the findings of previous researchers (Auta et al., 2014; Panda, 2017) that citric acid production was a type-II fermentation process. The increased amount of citric acid accumulation with time perhaps raised the toxic effect for *A. niger*, and started to hamper its metabolic activity.

Fermentation kinetics revealed that the performance of the fermentation process could be improved by monitoring and controlling of parameters like pH, residual total sugar, reducing sugar, cellulose and biomass content of the media, and using any of these parameters as multi-objective parameters for the identification of the optimal level of process parameters.

3.7 Characterisation of citric acid

Chromatograph developed in paper chromatography revealed the R_f value of 0.35 for experimental citric acid as compared to the R_f value of 0.34 measured for standard citric acid. Accuracy of 98.6% strengthened the evidence of the sample as citric acid. Thin layer chromatograph developed on TLC plate did not show any spotting. Spectral output of the sample showed the highest absorbance peak at 575 nm. This result was very close to the absorbance peak of standard citric acid at 576 nm. Thin layer chromatography and spectral analysis strengthened the claim that the substance produced was citric acid (Patil and Patil,2014).

4 Conclusion

The mixed substrate fermentation of tangerine and pineapple peel waste in the proportion of 1:1 improved the yield of citric acid as compared to mono-substrate fermentation without any fortification of media. The optimum initial fermentation medium pH of 4, and addition of 5% (w/w) methanol and 15% (w/w) sucrose to the medium was found to enhance the synergistic effect of the substrates, and resulted in the consumption of more than 94% of fermentable sugar to produce 137.2 g citric acid per kg of dry matter. Fermentation kinetics revealed that the production of citric acid was a two-stage process. In the first stage, the rate of production of citric acid was slow whereas the growth rate of biomass was rapid. In the second stage, the production rate of citric acid was rapid whereas growth rate of biomass was slow. Production of citric acid from the peel wastes of tangerine and pineapple adds values to the peel wastes, and reduces the risk of environmental pollution, carbon emissions and greenhouse gas effects to a great extent.

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