JJBS

Volume 14, Number 2, June 2021
ISSN 1995-6673
Pages 261 - 266

Jordan Journal of Biological Sciences

Orange Peels Valorization For Citric Acid Production Through Single And Co-Culture Fermentation

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Received: May 14, 2020; Revised: September 10, 2020; Accepted: September 11, 2020

Abstract

The present work describes fermentation of raw materials to produce citric acid through single and co-culture process using indigenous strains of *Aspergillus niger* and *Aspergillus fumigatus*. For this purpose, orange peels, peanut shells and their homogenous mixtures were used as raw material using solid state fermentation (SSF). Incubation period, pH, temperature, moisture content, inoculum size and substrate concentrations were optimized simultaneously using response surface methodology (RSM) design followed by contour plot analysis. Citric acid was separated from other by products in a non-polar C-8 column and quantified using reversed phase high performance liquid chromatography (RP-HPLC) technique. It was found that more amount of citric acid (114.68±0.73 mg/mL) was produced during co-culture fermentation employing both *A. niger* and *A. fumigatus* simultaneously as compared to single culture fermentation inoculated with *A. niger* (94.92±0.46 mg/mL) and *A. fumigatus* (65.13±0.28 mg/mL), using 25 gms orange peels OPs as substrate, 60 % moisture content, 6 pH, 6 mL inoculum size, 6 days incubation period and 50 °C temperature as optimized fermentation conditions. The results also showed that RSM based co-culture SSF using cheap biomass can produce more amount of citric acid as compared to conventional fermentation.

Keywords: Raw materials, Citric acid, Co-culture fermentation, *A. fumigatus*, *A. niger*, HPLC, Orange peels.

1. Introduction

Citric acid is biodegradable, highly soluble and low toxic in nature (Yalcin *et al.* 2009; Artmaktadır 2010). It is considered among valuable commercial organic acids which are used in pharmaceutical, food and beverage industries (Abd El-Latif *et al.* 2020). There is a continuous need for research work to improve citric acid production systems and reduce the cost of substrates to fulfil application demands (Ema *et al.* 2020). More than two million tons of citric acid is being manufactured annually through fermentation technology (Ozdal *et al.* 2019) because production of citric acid by using chemicals is more expensive as compared to its production through biomass utilization using microbes either bacteria or fungi (Prado *et al.* 2005). This acid is used in various industrial processes, and 60 % of its production is being used in food industry with 5 % annual increase in production demand worldwide (Papagianni 2007).

By using fermentation techniques, agricultural biomass can be converted into useful products (Iqbal *et al.* 2013). Citric acid can be produced by using different raw products and agricultural wastes (Soccol *et al.* 2006) and milling products (Ema *et al.* 2020). Many organisms like bacteria, fungi and yeast are capable to produce citric acid in their culture medium through fermentation (Iqbal *et al.* 2015). As compared to bacteria, fungi especially species of *Aspergillus* have been preferred for citric acid production due to more yield (Angumeenal *et al.* 2013).

Both *Aspergillus niger* and *A. fumigatus* can grow on soil, dungs, compost piles and agriculture biomass (Cramer *et al.* 2006). The production of useful products like citric acid from biomass is influenced by fermentation parameters like incubation period, substrate, substrate concentration, inoculum size, moisture content, pH and temperature (Ali *et al.* 2002; Ajala *et al*. 2020). In this regard, Response Surface Methodology (RSM) approach during experimentation, helps to study the simultaneous interaction of various variables, optimization of different fermentation parameters as described above and their interaction with each other to provide statistically significant results (Wang *et al.* 2011).

HPLC being more reliable is an advanced technique to separate and detect the presence of citric acid in culture medium produced through biomass based fermentation as compared to conventional spectrophotometric detection. Therefore, this study has aimed to compare maximum production of citric acid through biomass valorization using single culture and co-culture fermentation techniques with different substrates and to quantify citric acid through HPLC technique.

2. Materials and Methods

2.1. Cultivation of fungal species

Fungal species *Aspergillus niger* and *Aspergillus fumigatus* were obtained from Industrial Biochemistry Laboratory, Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat, Pakistan.

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These were cultivated on potato dextrose agar (PDA) medium (Sharma, 2010). The cultures were cultivated for 5 days in orbital shaker at 35 °C in order to obtain fresh colonies. Later, 100 µL inoculum of both *fungi* was spread into petri plates containing solidified PDA medium and placed at 35 °C for 5 days.

2.2. Screening of biomass

Biomass including Orange peels (OPs), peanut shells (PSs) and their mixture were employed as substrate for citric acid production. Three flasks containing 5g of OPs, PSs and their mixture containing 2.5 gms of each substrate along with 5mL of water were autoclaved at 121 °C temperature, 15 psi pressure for 20 min. A total of 3 mL inoculum was added to each flask then incubated at 35 °C for 5 days. The inoculum comprised of 1.5 mL overnight inoculum of each fungal strain (*A. niger* and *A. fumigatus)*. After screening, the substrate producing maximum citric acid was further employed in RSM trial.

2.3. Response Surface Methodology (RSM)

In order to study and optimize the effect of incubation period, pH, temperature, substrate concentration, inoculum size and moisture content for citric acid production, solid state fermentation was performed using statistical design of Response Surface Methodology through Minitab software (version 17) (Wang *et al.* 2011). Accordingly, under RSM, fermentation experiment was carried out using different combinations of pH, temperature and incubation period and moisture content, substrate concentration and inoculum size.

2.4. Optimization of fermentation parameters through RSM

For the determination of optimum values, different concentrations and ranges of fermentation parameters were tested for maximum citric acid production. For optimum incubation period, biomass substrates were inoculated with 1.5 mL of single and co-culture consortia after addition of 5 mL distilled water to give moisture content and incubated for 1, 2, 4, 6 and 7 days. For optimum pH, inoculated substrates were treated at different values of pH (3-9) using phosphate buffer. For optimum temperature and moisture content, experiment was conducted at different range of temperature (20-55 °C) and moisture content (10-90 %). For inoculum size, inoculated biomass was treated with 1-11 mL inoculum size and for optimization of substrate, different concentrations (3-58 gms) of substrate were used to obtain maximum production of citric acid.

2.5. Fermentation

Single and co-culture fermentation was performed in separate flasks. For single culture experimentation, 5 gms of powdered OPs were moistened with 3 mL of phosphate buffer, and 1.5 mL inoculum of *A. fumigatus* and *A. niger* was added in separate flasks. For co-culture experimentation, same amount of substrate (OPs) was added to 3mL of phosphate buffer, and 1.5 mL inoculum of both *A. niger* and *A. fumigatus* was added in same flasks. The selected values of fermentation parameters including pH, temperature, incubation period, inoculum size, moisture content and substrate concentration were further employed in single and co-culture fermentation. After incubation, 50 mL of distilled water was added, and cultures were placed in orbital shaker at 37 °C for 30 min at 180 rpm. The culture was filtered with Whatsman filter paper (Number 42, pore size 2.5 µm), centrifuged at 4000 rpm for 15 min, then the supernatants were collected and used for detection of citric acid through HPLC analysis.

2.6. HPLC analysis

HPLC grade standard of citric acid was purchased from Sigma-Alrich (USA). A stock solution (1000 µg/mL) of citric acid standard was prepared and further diluted (100, 200, 300, 400 and 500 µg/mL) to draw the standard curve. The samples were analyzed on reversed phase high pressure liquid chromatograph (RP-HPLC) system (Hitachi, Japan) at room temperature using isocratic mode with distilled deionized water as mobile phase. The flow rate was set to 1.5 mL/min and absorbance of citric acid was noted at 212 nm using UV/visible detector. The stationary phase comprised of C-8 column (15cm x 4.6 mm x and 5µM). The qualitative and quantitative detection of citric acid was performed using retention time and peak area information of chromatograms, respectively.

3. Results

3.1. HPLC detection of citric acid

The amount of citric acid was quantified through HPLC analysis. The standard curve of citric acid was obtained using HPLC grade standards having different concentrations. A typical chromatogram of stock standard of citric acid has been shown in Figure 1 which shows elution of citric acid from HPLC column at 2.0 min retention time.

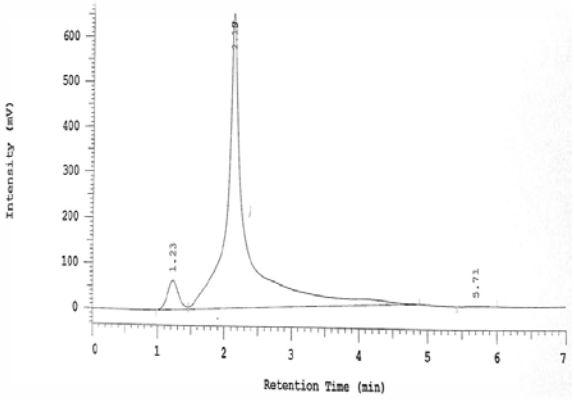

Figure 1. HPLC chromatogram of stock standard of citric acid (1 mg/ml). The analyte was eluted at retention time of 2 min and detected at 212 nm wavelength using UV/Visible detector through isocratic mode of analysis.

3.2. Biomass screening

The growth of *A. niger* and *A. fumigatus* was visible after 5 days of inoculation in separate flasks containing three different biomass (OPs, PSs and uniform mixture of OPs and PSs). The maximum production of citric acid (114.68±0.73 mg/mL) was observed in OPs substrate as analyzed through HPLC (Fig 2) as compared to PSs and their mixture by co-culturing (*A. niger* and *A. fumigatus)* technique.

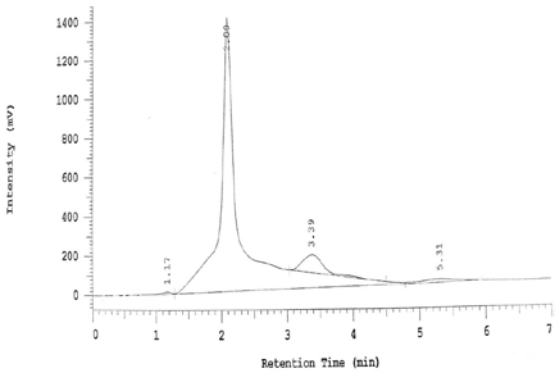

Figure 2. HPLC chromatogram of maximum amount of citric acid (114.68±0.73 mg/mL) produced in orange peels (substrate) based fermentation culture using co-culturing of *A. niger* and *A. fumigatus*. The analyte was eluted at 2 min retention time and detected at 212 nm wavelength using UV/Visible detector.

3.3. Optimization of fermentation parameters through RSM

Different fermentation parameters were optimized using RSM, and effect of pH, temperature and incubation period for citric acid production through co-culture technique was observed (Table 1). The maximum production of citric acid (94.92±0.46 mg/mL) as determined by HPLC analysis (Fig 3) was noted after 6 days of incubation during single culture fermentation using *A. niger* while single culture fermentation using *A. fumigatus* produced 65.13±0.28 mg/mL citric acid. However, co-culturing using both *A. niger* and *A. fumigatus* yielded more amount of citric acid (114.7±0.73 mg/mL) after 6 days of incubation period. For determination of optimum pH, the cultures were treated with different pH values using phosphate buffer and maximum production of citric acid was observed at pH 6. Regarding temperature, the maximum production was observed at 50 °C during co-culture among different ranges of temperature. The moisture content of 60% maximally produced citric acid during co-culture experimentation. An inoculum size of 6 mL was found optimum. In addition, upon using different concentrations of biomass substrates OPs, PSs and their mixture, maximum production of citric acid was observed at 25 gms concentration of OPs during co-culture fermentation employing both fungal strains (Table 2).

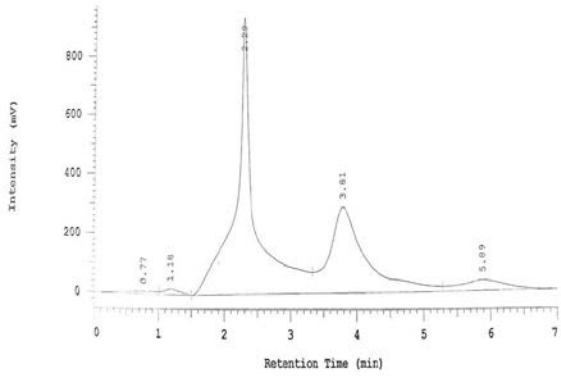

Figure 3. HPLC chromatogram of citric acid (94.92±0.46 mg/mL) produced in orange peels (substrate) based fermentation culture using single culture of *A. niger*. The analyte was eluted at 2 min retention time and detected at 212 nm wavelength using UV/Visible detector.

Table 1. RSM based optimization of pH, temperature and incubation period for citric acid production through co-culture fermentation using *A. niger* and *A. fumigatus*

S #	pH	Temperature (˚C)	Incubation period (Days)	Amount of citric acid (mg/mL)
1	3	55	2	50.86
2	6	35	1	29.53
3	8	50	2	42.51
4	8	20	2	11.19
5	3	20	2	10.09
6	5.5	35	4	56.18
7	6	35	4	52.09
8	5	35	4	26.89
9	9	35	4	29.07
10	3	35	4	0.572
11	3	20	6	30.50
12	3	50	4	45.28
13	8	20	6	17.43
14	6	50	6	70.76
15	5.5	35	7	16
16	6	60	4	49.28

Table 2. RSM based optimization of moisture level, substrate concentration and inoculum size for citric acid production through co-culture fermentation using *A. niger* and *A. fumigatus*

S #	Moisture level (%)	Substrate concentration (g)	Inoculum size(mL)	Amount of citric acid (mg/mL)
1	90	45	9	72.55
2	60	25	9	76.91
3	60	25	6	11.85
4	30	5	9	30.64
5	90	5	3	28.42
6	60	25	6	114.68
7	60	58	6	41.72
8	30	45	3	47.82
9	60	3	6	0.48
10	30	5	3	42.83
11	60	25	1	23.02
12	30	45	9	43.01
13	10	25	6	43.26
14	90	45	3	25.13
15	60	25	11	28.11
16	90	5	9	18

3.4. Interaction among fermentation parameters

The interaction of fermentation parameters like incubation period, pH, temperature, inoculum size, moisture content and substrate concentration were observed through RSM design and contour plots were prepared using Minitab software (version 17) to observe significant interaction among various parameters. A significant interaction shows dependency of one parameter with other while non-significant interaction shows non dependency of parameters with each other regarding production of citric acid during single and co-culture experimentation.

3.5. Interaction among parameters during single culture using A. niger

A significant interaction was observed between substrate concentration and moisture content during single culture inoculation with *A. niger* (Fig 4). It was observed

that maximum citric acid (94.92±0.46 mg/mL) was produced with 58 gms substrate concentration and 60 % moisture content. However, the interaction between inoculum size and moisture content was non-significant (Fig 5), which shows non-dependency of both parameters for production of citric acid.

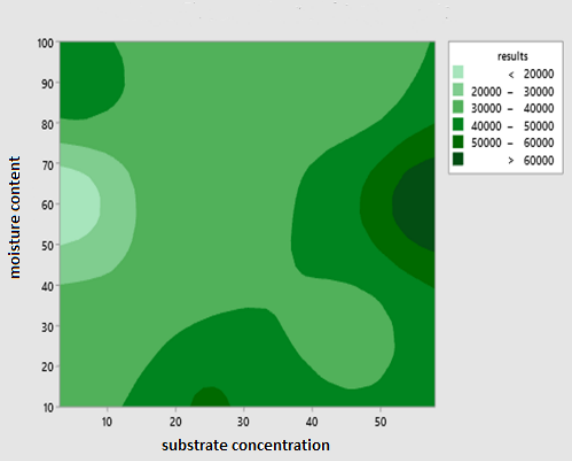

Figure 4. Contour plot showing significant interaction (p-value<0.05) between substrate concentration and moisture content for citric acid production from orange peels (OPs) using single culture (*A. niger*). The light green colour is representing minimum and dark green colour represents more production of citric acid.

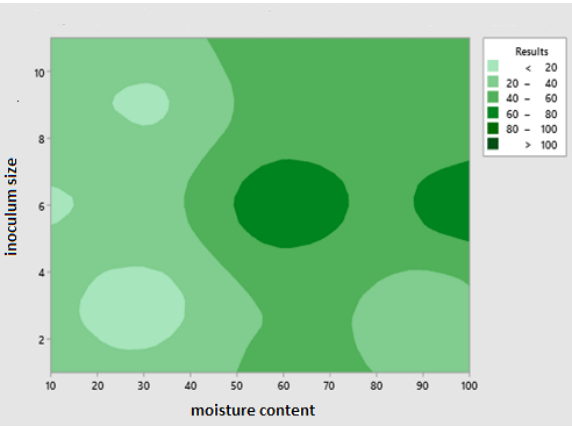

Figure 5. Contour plot showing non-significant interaction (p-value>0.05) between moisture content and inoculum size for citric acid production from OPs using single culture (*A. niger*). The light green colour is representing minimum and dark green colour represents more production of citric acid.

3.6. Interaction among parameters during single culture using A. fumigatus

The maximum production of citric acid (65.13±0.28 mg/mL) was observed at pH 6 and 60 °C temperature during single culture inoculation using *A. fumigatus*, showing more significant interaction between pH and temperature (Fig 6) as compared to interaction between moisture content and substrate concentration during which maximum production was observed at 60 % moisture content and 58 grams of substrate concentration however their interaction was less significant (Fig 7).

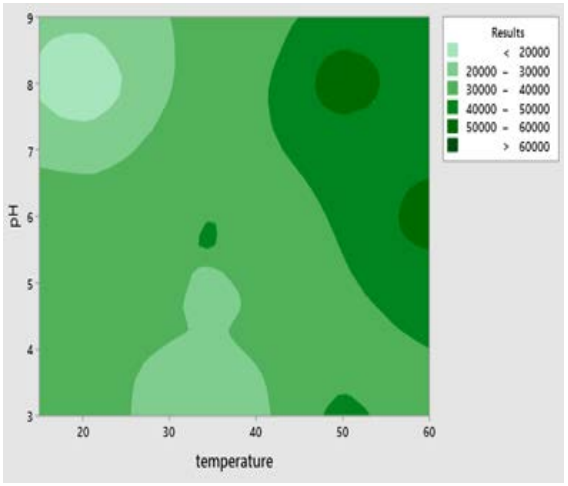

Figure 6. Contour plot showing significant interaction (p-value = 0.001) between temperature and pH for citric acid production from OPs using single culture (*A. fumigatus*). The light green colour is representing minimum and dark green colour represents more production of citric acid.

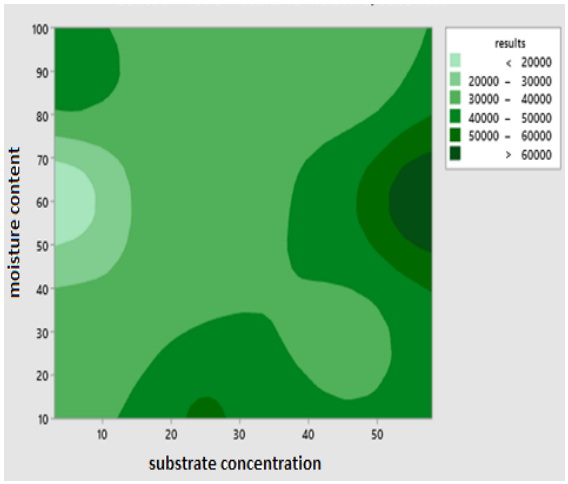

Figure 7. Contour plot showing non-significant interaction (p-value=0.12) between substrate concentration and moisture content for citric acid production from OPs using single culture (*A. fumigatus*). The light green colour is representing minimum and dark green colour represents more production of citric acid.

3.7. Interaction among parameters during co-culture (A. niger and A. fumigatus)

It was further found that during co-culturing using both *A. niger* and *A. fumigatus*, a significant interaction appeared between temperature and pH parameters as shown in Fig 8, yielding maximum amount of citric acid at 50°C and pH 8. However, when temperature was employed as parameter with incubation period, a non-significant interaction was observed through contour plot analysis (Fig 9) producing maximum citric acid at 50°C for 6 days of incubation period.

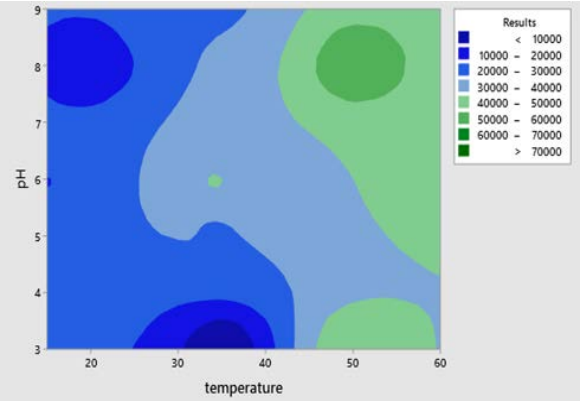

Figure 8. Contour plot showing significant interaction (p-value<0.05) between temperature and pH for citric acid production from OPs using co-culture (*A. niger* and *A. fumigatus*). The dark green colour shows more production of citric acid while light blue colour shows less production.

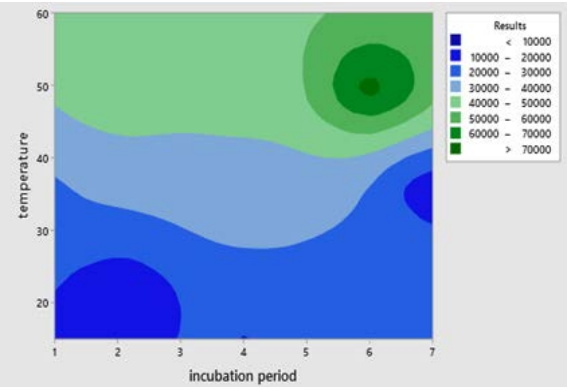

Figure 9. Contour plot showing non-significant interaction (p-value>0.05) between temperature and incubation period for citric acid production from OPs using co-culture (*A. niger* and *A. fumigatus*). The dark blue colour is representing less production while dark green colour shows more production.

4. Discussion

The results of this study showed that as compared to single culture fermentation, more amount of citric acid was produced during co-culture fermentation using indigenous strains of *A. niger* and *A. fumigatus*. It was also observed that citric acid was successfully separated from other byproducts present in the fermentation culture medium, using reversed phase (non-polar) stationary phase with polar mobile phase (distilled deionized water) in isocratic mode at 212 nm wavelength. Hence, RP-HPLC technique was found reliable to quantify organic acids produced through fermentation using different biomass.

OPs were found best biomass substrate to produce citric acid, and the possible reason for this is presence of high content of carbohydrates in its peel, which facilitates microbial growth and enhances fermentation process. Particularly, pectin is present in maximum concentration (42.5%) among components of OP as compared to other components including cellulose, hemicellulose and soluble sugars like glucose, fructose and sucrose (Rivas *et al.* 2008). Pectin mainly consists of sugar acid (galacturonic acid) units. The sugars are used as carbon source during fermentation of citric acid and concentration of these sugars is enhanced due to breakdown of pectin content of OPs through fungal pectinases. The OP substrate was more degraded due to increased production of pectinases during co-culture as compared to single culture fermentation. Therefore, more the availability of substrate and its respective enzyme, there will be enhanced breakdown and ultimately release and consumption of monosaccharides which enhances citric acid production during fermentation.

As the maximum production of citric acid using solid state fermentation was observed with OPs as substrate, it was therefore selected to optimize other parameters of experiment. Like our strategy of SSF, Ajala *et al.* 2020 have also preferred SSF to produce citric acid and obtained more yield as compared to sub-merged fermentation. Various agro-waste bioresidues have been used by many researchers as substrates in solid state fermentation for production of useful products through microbes (Uma & Rita, 2008; Chinnasamay *et al*. 2011; Rajashri & Anandrao, 2012). Particularly for citric acid production, biomass based substrates have been used by Kareem *et al.* 2010 using single culture solid state fermentation; however, we observed more production yield using co-culture fermentation. Regarding incubation period, better results were observed with decreased incubation period (6 days) in our study as compared to results reported by other researchers. Ambati *et al.* 2001 and Cevrimli *et al.* 2009 found 7 days as optimum incubation period for citric acid production using *A. niger*. The results of study also indicated that an acidic culture medium with pH ≤ 6 leads to more production of organic acids using co-culture technique. Shabaan *et al.* 2020 also favored an acidic medium to enhance the production yield using potato peels and mixed grasses as raw materials. Other researchers have also favored an acidic medium for citric acid production using *Aspergillus* species (Cevrimli *et al.* 2009, El-Gamal *et al.* 2018, Ambati *et al.* 2001, Bhattacharjee *et al.* 2015).

Our findings suggest temperature of 50°C as favorable to obtain more production yield of citric acid using biomass; however, El-Gamal *et al.* 2018 found 45 °C as optimum temperature during single culture fermentation by *A. fumigatus* for citric acid production. Similarly, 60 % content was found as optimum moisture content for production of citric acid in our study while Kareem *et al.* 2010 reported 65 % content as optimum during single culture fermentation using *A. niger*. The reason for decrease in required moisture content is use of two fungi simultaneously in same fermentation medium using co-culture technique.

For substrate concentration, it is important to describe that optimum concentration of substrate depends upon composite nature of substrate and type of fermentation used during culturing. The amount of substrate (OPs) used in our method is less than that of Solomon *et al.* 2018 which used banana peel as substrate. The co-culture technique is favored due to less consumption of raw substrates (Ali *et al.* 2016). Accordingly, results of the present study indicate that co-culture fermentation should be preferred as compared to single culture fermentation because it requires less amount of substrate and yields more production of organic acids by using microbes particularly fungi.

The understanding of impact of applying multiple parameters and their combined effect on production of fermentation products in presence of different microbes is essential in order to obtain best results within least time period, and contour plot analysis is an excellent tool to

observe such effect of multiple parameters in a fermentation experiment as described above. Therefore, contour plot analysis was applied accordingly, and the results showed that a significant interaction exists between pH and temperature for production of citric acid either during single culture fermentation or co-culture fermentation and the possible reason might be interdependency of both parameters during RSM based experimentation.

5. Conclusion

The findings of present study indicate that as compared to single culture fermentation using only *A. niger*, choice of co-culturing using both *A. niger* and *A. fumigatus* is a successful strategy to degrade biomass and to obtain maximum amount of citric acid (114.68±0.73 mg/mL). Among various biomass like OPs, PSs and their mixture, OPs as raw substrate can produce more amount of citric acid. In addition, described RP-HPLC technique could be more useful as compared to spectrophotometric detection of citric acid as it facilitates separation of organic acid from other products produced during fermentation, and separated analyte can be collected using fraction collector. Hence, proposed co-culture solid state fermentation method using RSM design can be employed to enhance the production of citric acid using cheap and easily available biomass resources.

Acknowledgments

This research work was funded by Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat, Pakistan.

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