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Application of calcium carbonate nanoparticles and microwave irradiation in submerged fermentation production and recovery of fumaric acid: a novel approach

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The aim of the present study was to explore the possible application of calcium carbonate nanoparticles (CCNPs) and microwave irradiation (MWI) in fumaric acid (FA) production and recovery, respectively. The fungal strain *Rhizopus oryzae* 1526 was employed as the biocatalyst for FA production. A glucose–basic salt medium was used as the fermentation medium. Scanning electron microscopy (SEM) analysis of CCNPs displayed the spherical shapes, while zetasizer measurements showed the CCNPs to be around 190 ± 20 nm in size. FTIR analysis of CCNPs confirmed the chemical composition. BET analysis confirmed higher specific surface areas of CCNPs (11.95 ± 0.03 m^2 g^{-1}) compared to calcium carbonate microparticles (CCMPs) (3.51 ± 0.02 m^2 g^{-1}). FA neutralization timing for CCNPs was much lower than CCMPs (190 and 350 seconds, respectively). CCNPs enhanced the volumetric productivity of FA from 0.47 g L^{-1} h^{-1} to 0.74 g L^{-1} h^{-1}. At 20, 40 and 60 g L^{-1} concentrations and at 25 °C, viscosities of the CCNPs were found to be lower than respective CCMPs. Moreover, the CCNPs did not exhibit any toxicity towards the fungus. FA production obtained with CC micro and CCNPs were 67.34 ± 2 g L^{-1} and 66.92 ± 2.7 g L^{-1}, respectively. Under MWI heating, 10 ± 1 min was found to be sufficient for recovery of FA and this was much lower than conventional heating timing of 28 ± 1 min.

1. Introduction

Fumaric acid (FA) [IUPAC ID: (*E*)-butenedioic acid] is an organic acid with diverse applications in the field of food, resin and dairy industries.[1–3] Because of the non-hygroscopic and non-toxic properties and higher buffering capacity than other food acids, FA is preferably used as an acidulant and nutritive additive in the food industry.[2] The chemical structure makes FA suitable for polymerization and esterification reactions and this has led to the extensive application of FA in resin industry. About 56% of the total annual production of FA is used in the resin industry for the production of paper resins, alkyd resins and unsaturated polyester resins.[4,5] In the dairy industry, FA has been recommended for use in ruminant diet for reducing enteric methane (CH$_4$) emission.[6,7] Moreover, FA has been recently experimented as highly efficient promoters of the Beckmann rearrangement (from benzophenone oxime Ia to benzamide IIa).[8] In the production domain, FA production through submerged fermentation (SmF) has emerged as the most explored biological alternative to chemical route. Active

research on different aspects of upstream and downstream processing of fermentative FA production has uncovered the key factors that contribute to the higher yield of this multifaceted organic acid.[9,10] Apart from the safety issues of chemically derived FA, newly explored bio-medical applications of FA and its ester derivatives has necessitated the biological production of FA.[11,12] In the last two decades, different research groups have explored the cost-effective FA production through SmF by introducing new concepts, such as carbon-economy and white biotechnology. Low cost carbon sources have been utilized for FA production. Different strains of the filamentous fungus *Rhizopus oryzae* have been employed as the most efficient bio-catalysts in SmF based FA production.[9,10] Strategies, such as immobilization of fungal strains on different solid supports, genetic engineering and metabolic shift in tricarboxylic cycle have been experimented for enhanced FA production under SmF conditions.[13–16] The overall progress made on the upstream processing of fermentative FA production is remarkable. However, fermentative approach of FA production is yet to address the bottlenecks in its downstream processing domain. This issue has been highlighted by different experts from time to time.[9,10] Efforts have been made for easy recovery of FA from fermentation broth. In a study by Zhang *et al.*, activated carbon was used as the adsorbent for FA recovery from the fermentation broth and later desorbed into acetone. The FA recovery

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yield was found to be around 93%.[17] In another study, a fixed bed column of amberlite ion exchange resin (IRA900) was applied with a stirred-tank bioreactor for intermittent *in situ* FA recovery from the fermentation broth. Compared to the results of the control experiment (without adsorption), this approach enhanced the recovery yield and productivity of FA by 25% and 59%, respectively.[18] Techniques such as nanofiltration and bipolar electrodialysis have also been applied for the recovery of FA from model solutions and real fermentation broth. The combination of nanofiltration (nanoporous ceramic membrane) with bipolar electrodialysis (membrane PC 200bip and the anion-exchange membrane PC 200D) efficiently recovered and concentrated FA from the fermentation broth.[19]

To the specific interest of the present study, application of calcium carbonate (to be called as CC hereafter) as a neutralizing agent in SmF based FA production is unavoidable. There has been serious concern regarding the disadvantages of using CC over other neutralizing agents, such as Na_2CO_3, $NaHCO_3$, $(NH_4)_2CO_3$, and $Ca(OH)_2$. However, pertaining to the higher yield of FA, CC has been established as the default neutralizing agent in FA production.[9] Lower solubility and higher viscosity of CC slurries causes problems in the heat, mass and oxygen transfer throughout the broth during SmF. Non-stoichiometric addition of CC for neutralization of FA and maintenance of broth pH near to 6–7 is a regular practice during SmF based FA production. The fermentation product 'calcium fumarate' ($CaC_4H_2O_4$) has low water solubility (15.6 g L^{-1}) and this makes fermentation broth even more viscous.[9] $CaC_4H_2O_4$ has commercial application as calcium fortifier in beverages. Studies have been carried out on the relationship between the water solubility of $CaC_4H_2O_4$ and pH with different acidulants.[20] By stoichiometric ratio, 20 g of CC can neutralize 23.2 g of FA and this is irrespective of any SmF conditions. As FA concentration (g L^{-1}) in the SmF broth is not predictable, this stoichiometric ratio cannot be followed and this necessitates the addition of extra CC during SmF. Sticking to this principle, it might be possible to change the physical state of the CC but not the required concentration (g L^{-1}). Application of CC nanoparticles (CCNPs) instead of CC microparticles (CCMPs) can be an interesting and noble approach to experiment with. Nano dimension imparts new physical properties lacking in the bulk form (including micro size). Higher surface to volume ratio is one of the common advantages of nanoform over their micro-sized counterparts. For CCNPs, apart from higher surface area, lower viscosity and increased rate of FA neutralization might be the technical advantages over CCMPs. There has been no prior study in this sense but studies have been carried out on the advantages of using small sized CC in acid neutralizing kinetic studies.[21–23]

Apart from the CC associated bottlenecks of FA production, the fermentation products $CaC_4H_2O_4$ needs attention for easy recovery of FA. Conventionally, $CaC_4H_2O_4$ in broth is simultaneously heated (around at 80 °C) and acidified (mineral acids such as HCl or H_2SO_4) that release FA from $CaC_4H_2O_4$ and also increases the solubility of FA in the broth. However, this method is time consuming. The dissolution of FA into water depends on uniform heating and rate of heating. In this regard,

microwave irradiation (MWI) can be a good option for time-effective recovery of FA from the broth. MWI is an electromagnetic spectrum with a frequency in the range of 300 MHz to 300 GHz. For a variety of inorganic synthesis and biomedical applications, MWI is a well proven and widely accepted processing technology.[24,25] As compared to general heating treatment, a rapid and homogenous heating can easily be achieved by employing MWI even in materials exhibiting low heat conductivity as the transfer of energy does not rely on heat diffusion.[26,27] On the other hand, dissolution enhancement is an innovative concept which additionally accelerates the solubility and dissolution.[27,28] However, a good dielectric property is required for excellent efficiency of heat transfer.[29] Water (in the fermented broth) represents the solvent with a dielectric constant value of 80.37 (at 25 °C) and the major byproduct 'ethanol' formed during FA fermentation has a dielectric constant value of 24.3 (at 25 °C).[30] Both solvents can thus certainly enhance the MWI efficacy of heating the fermented broth in a shortened time. Moreover, acidic condition favors MWI heating. Considering the bottlenecks in the production and recovery of FA, CCNPs and MWI were thus investigated in the present study.

2. Experimental

2.1 Materials

The fungal strain, *Rhizopus oryzae* NRRL 1526 (to be called *R. oryzae* hereafter) was procured from Agricultural Research Services (ARS) culture collection, IL, USA. The inorganic salts ($CaCl_2$, Na_2CO_3, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and $CaCO_3$), urea and dextrose (dextrorotatory glucose) used in this study were of analytical grade (with min 99.5% purity) and purchased from Fisher Scientific (Ottawa, Ontario, Canada).

2.2 Culture and maintenance of *Rhizopus oryzae* 1526

For cultural practices (revival, sub-culturing and regular maintenance) of *R. oryzae*, the procedure mentioned by Das *et al.* (2014) was followed.[15] Briefly, PDA medium and incubation conditions of 37 ± 1 °C for 4 days were used for revival. Sporangiospores were collected by spread plate method after incubating 37 ± 1 °C for 72 h. Mycelium free spore suspension was kept at 4 °C and the glycerol stock (20%) was preserved at −80 °C. For inoculation, a stock of 1×10^8 spores per mL was maintained.

2.3 Media and inoculum preparation

Glucose-basic salts medium consisting of glucose (50 g L^{-1}), urea (2 g L^{-1}), KH_2PO_4 (0.6 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.11 g L^{-1}) and $FeSO_4 \cdot 7H_2O$ (0.0088 g L^{-1}) was used for spore germination. The medium final pH (4.6) was not adjusted, unless specifically indicated. To avoid Maillard reaction, the two media components: (a) glucose; and (b) urea + salts, were heat sterilized (20 min, 15 psi, 121 ± 1 °C) separately and mixed aseptically at room temperature. For SmF medium preparation, the same procedure was followed but with a higher

carbon (80 g L^{-1} glucose) to nitrogen (0.2 g L^{-1} urea) ratio (C : N = 400 : 1) was maintained.

Around 50 mL of sterilized growth medium was inoculated (2%, v/v) with sporangiospores of *R. oryzae* in a 250 mL Erlenmeyer flask and then incubated at 30 °C, 200 rpm for 24 h.

2.4 SmF conditions

Around 7.9 mL (final inoculum concentration 5%, v/v) of cell pellet inoculum was transferred into 500 mL Erlenmeyer flasks containing 142.1 mL of SmF medium. The inoculated flasks were incubated at 25 °C, in a rotary shaker at 200 rpm for a maximum of 84 h. Heat sterilized CCMPs (5–10 μm) and CCNPs (190 ± 20 nm) were used as neutralizing agents at 60 g L^{-1}.

2.5 Preparation of CCNPs

The CCNPs were prepared following the method of Ueno *et al.* with some modifications.[31] Briefly, 6.50 mL of 5 M $CaCl_2$ and 25 mL of 1 M Na_2CO_3 were mixed and stirred vigorously for 20 min, followed by addition of 50 mL of d. H_2O. The large CCNPs were first discarded by precipitation without centrifugation. The suspension was centrifuged (34 000 × *g*, 10 min) and the supernatant and precipitate (CCNPs) was separated. The prepared CCNPs were then dispersed in d. H_2O and freeze-dried into powder form for further application in SmF.

2.6 Analytical methods

Physicochemical characterization of the prepared CCNPs was carried out by different instrumental analysis. The size and shape of the CCNPs was investigated by a scanning electron microscope (Zeiss EVO® 50 Smart SEM system). For average size (diameter) measurement, zetasizer nanoZS (Malvern instruments Ltd., UK) was employed. Fourier transform infrared (FTIR) analysis of the CCNPs was carried with a Nicolet™ iS™50 spectrophotometer (Thermo Scientific) with He–Ne laser, DTGS detector and KBR beamsplitter with a built-in Miracle-ATR (Diamond). The ATR-FTIR spectra of the CCNPs samples were collected and analyzed using OMNIC Specta™ software. The Brunauer–Emmett–Teller (BET) surface area analysis of CCMPs and CCNPs samples was carried out with a Quantachrome, AUTOSORB-1 series model instrument. The changes in the pHs of FA solution after addition of CC (CCNPs or CCMPs) were recorded as a function of time (seconds, s) and compared for CCNPs and CCMPs. Viscosities of the CC slurries, $CaC_4H_2O_4$ solutions and broth samples were measured by a rotational viscometer (Fungilab, Premium Series, NY, USA). L1 spindle was used with a sample cup volume of 30 mL. Viscosities were expressed in the unit of cP. Acidified broth samples were irradiated for different time durations (2–20 minutes) at the maximum power output (900 W) of the microwave oven. For comparison, conventional heating method was followed for FA recovery.[32] HPLC analysis was carried out for quantification of FA in the fermented broth samples. For HPLC sample preparation, the method of Zhou *et al.* was followed.[33] The technical details of the HPLC were as follows: System: DIONEX DX500, with an Acclaim OA, 5 μm, (4.6 × 150 mm) column with a refractive index detector (PDA-100 DIONEX, UV, 210 nm). 2.5 mM methanesulfonic acid (CH_3SO_3H) at a flow rate of 1 mL min^{-1} was used as the mobile phase and column temperature was 30 °C.

2.7 Statistical analysis

Data are represented as mean ± SD of three independent experiments. Correlations were considered significant at $p < 0.05$.

3. Results and discussion

3.1 Characterization of CCNPs

SEM analysis displayed roughly spherical CCNPs with solid dense structure. A SEM image of the CCNPs is shown in Fig. 1. As no stabilizing agent was used during the preparation of CCNPs, aggregation of CCNPs was observed after drying of the sample on SEM grid. The size distribution curve of CCNPs originating from the zetasizer nano analysis indicated uniform size distribution of particles with a mean size of 190 ± 20 nm as shown in Fig. 2. FTIR analysis of CCNPs sample showed the three distinctive infrared transmission bands corresponding to their chemical composition. The results are displayed in Fig. 3. The infrared bands at 1452 cm^{-1}, 871 cm^{-1} and 714 cm^{-1} wavenumbers corresponded to the asymmetric stretching vibration (v_3), asymmetric stretching vibration (v_2) and symmetric stretching vibration (v_4) of CO_3, respectively; and also confirmed the calcite crystalline state of CCNPs.[34–36] The BET analysis confirmed the larger specific surface area of CCNPs as compared CCMPs. The specific surface areas measured for CCMPs and CCNPs were 3.51 ± 0.02 m^2 g^{-1} and 11.95 ± 0.03 m^2 g^{-1} respectively. Previously, it has been shown that changes in the specific surface area of CCNPs depend on preparation method and concentration of the inorganic salts ($CaCl_2$ and Na_2CO_3 in the present study).[37]

3.2 Measurement of pH of FA solutions

Solubility of FA in water changes with temperature and this in turn, controls the final pH of the solution. In the present study, different amounts (g L^{-1}) of FA were dissolved in water at 25 °C
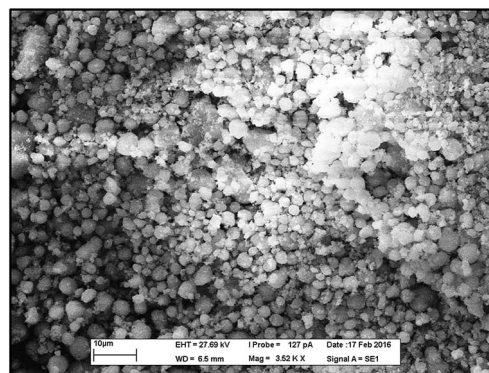

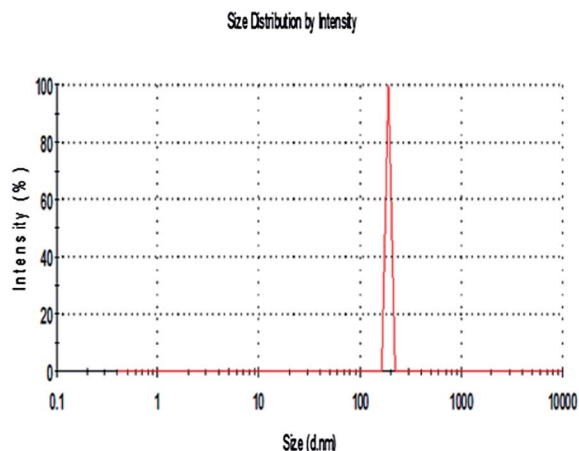**Fig. 1** SEM micrograph of $CaCO_3$ nanoparticles prepared in the present study.

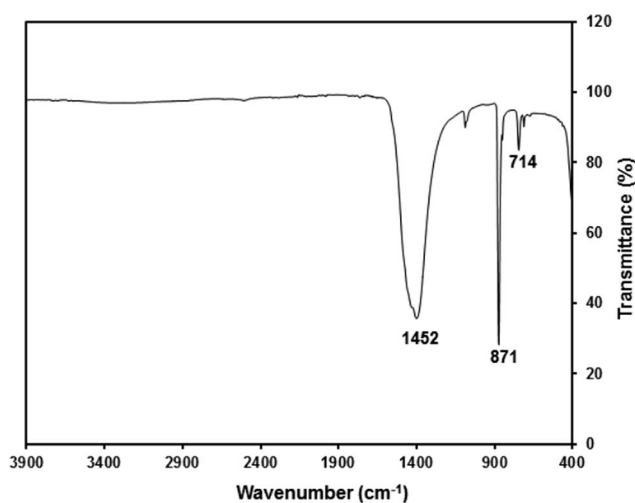**Fig. 2** Size distribution of CaCO₃ nanoparticles prepared in the present study.

Fig. 3 ATR-FTIR spectrum for CaCO₃ nanoparticles prepared in the present study.

and the corresponding pH was measured. The results are presented in Table 1. The solubility of FA in water was around 6.1 g L^{-1} at 25 °C and it corresponded to the pH of 2.1 ± 0.2. Lower concentration (3.05 g L^{-1}) increased the pH to 2.4 ± 0.3. However, above 6.1 g L^{-1} FA, the pH remained almost unchanged. These results were in accordance with the literature on FA properties.[38] After reaching the maximum solubility of

Table 1 pH of different fumaric acid aqueous solutions measured at 25 °C

Fumaric acid concentration (g L^{-1})	pH
3.05	2.4 ± 0.3
6.1	2.1 ± 0.2
10	2.2 ± 0.1
20	2.1 ± 0.1
30	2.1 ± 0.2

6.1 g L^{-1} at 25 °C, the pH was not affected by further addition of FA. At half (3.05 g L^{-1}) of the maximum soluble concentration, less production of H^+ ions from the dissociation of FA caused higher pH (2.4 ± 0.3). This basic information was extrapolated for the pH *vs.* FA production under SmF conditions.

3.3 Formation of CaC₄H₂O₄ and reaction time

The reaction between CC and FA can be represented as follows in eqn (1):

$$CaCO_3 + C_4H_4O_4 \rightarrow CaC_4H_2O_4 + H_2O + CO_2 \qquad (1)$$

This reaction confirms that 20 g (200 mM) of $CaCO_3$ (CCNPs or CCMPs) neutralizes approximately 23.2 g (200 mM) of FA. In the present study, CC was used as CCMPs and CCNPs against FA at above mentioned stoichiometric ratio. The time (s) required for change in the pH (2.5) of FA aqueous solution was recorded and the results are shown in Fig. 4. The neutralization reaction between FA and CCMPs took around 350 ± 19 s and the pH reached to 6.2. Thereafter, changes in pH were negligible and indicated completion of reaction. When experimented with CCNPs, the changes in pH were rapid and a stable pH of 6.23 was achieved after 190 ± 12 s. Thus, in comparison to CCMPs, the neutralization reaction time for CCNPs was reduced by 160 s. Particle size of neutralizing agent can have impacts on the reaction time. Previously, it has been shown that when applied as antacids; acid-consuming capacity and acid neutralizing velocity are influenced by the available specific surface area of the particles of CC. As the specific surface areas of CCNPs (11.95 ± 0.03 m² g^{-1}) were larger to CCMPs (3.51 ± 0.02 m² g^{-1}), the FA neutralization reaction was faster.[21–23]

3.4 Viscosity measurement

Measurements of viscosities (cP) of different concentrations of the CCMPs and CCNPs slurries were carried at 25 °C and the
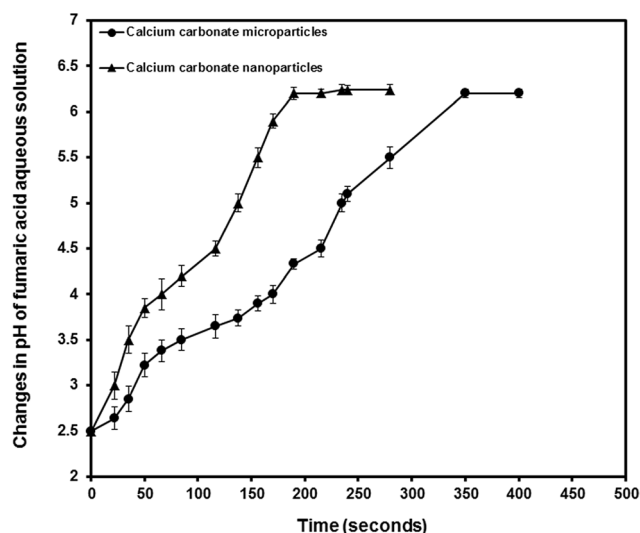

Fig. 4 Effect of CaCO₃ particle size on the neutralization time (seconds) of fumaric acid aqueous solution.

results are displayed in Table 2. The concentrations (20, 40 and 60 g L^{-1}) of CC was selected on the basis of the stoichiometric ratio to FA. For CCMPs, the viscosity was 1.39 ± 0.12 cP at 20 g L^{-1} and increased with further increase in CC concentration. At 60 g L^{-1}, viscosity reached 1.9 ± 0.11 cP. Compared to CCMPs, the viscosities of CCNPs slurries at same corresponding concentrations were lower. At 20 g L^{-1} of CCNPs concentration, the viscosity was 1.15 ± 0.1 cP and at the next two applied concentrations (40 and 60 g L^{-1}), the viscosities recorded were 1.48 ± 0.15 cP and 1.72 ± 0.1 cP, respectively. The lowering of viscosity of CCNPs slurries can be discussed on the ground of general acceptance of solid particle properties *vs.* viscosity. It has been shown that small particles generate larger inter-particle free space that helps in lowering viscosity. For larger particles, due to the greater inertia of interaction, more energy is required to momentarily accelerate or retard. This energy accounts for the extra viscosity.[39,40] Moreover, recently it has been proven that CCNPs can enhance surface hydrophobicity and decreases the yield stress and viscosity.[41] Based on these theories, it can be ascertained that lowering of viscosity of CCNPs slurries, as was found in the present study, was pertinent.

Apart from CCMPs and CCNPs slurries, viscosities were also measured for the reaction products (*i.e.* calcium fumarate, CaC$_4$H$_2$O$_4$) formed after the reactions between different applied concentrations of CCMPs and CCNPs and FA in a stoichiometric ratio. As shown in Table 2, the reaction product CaC$_4$H$_2$O$_4$ formed from the reaction between 20 g L^{-1} of CCMPs + 23.2 g L^{-1} of FA showed a viscosity of 2.2 ± 0.28 cP at 25 °C. At double stoichiometric concentrations of CCMPs and FA, the viscosity increased to 2.67 ± 0.21 cP. For the reaction product of 20 g L^{-1} of CCNPs + 23.2 g L^{-1} of FA, the viscosity recorded was 2.18 ± 0.19 cP and increased to 2.58 ± 0.26 cP at 40 g L^{-1} of CCNPs + 46.4 g L^{-1} of FA stoichiometric ratio. Overall, there was no effect of the size of CC on the viscosity of the reaction product. Previous study confirmed that the maximum solubility of

CaC$_4$H$_2$O$_4$ in water at 25 °C was 1.56 grams per 100 grams of water.[42] Thus, the viscosities measured for CaC$_4$H$_2$O$_4$ in water at 25 °C actually represented the rheological behaviour of CaC$_4$H$_2$O$_4$ with a solubility of 15.6 g L^{-1} in water solvent. The formation of CaC$_4$H$_2$O$_4$ in water at 25 °C was found to be irrespective of the size (MPs and NPs) of CC. As pH lowered, viscosity increase/decrease and formation of CaC$_4$H$_2$O$_4$ in water at 25 °C during FA production should follow the same stoichiometric ratio, the information on the viscosities of CaC$_4$H$_2$O$_4$ solutions was vital before real SmF application of CCNPs.

3.5 SmF with CCMPs and CCNPs and effects on FA production

It is a consensual view that broth viscosity can influence the productivity of FA during SmF.[43] Previous studies have confirmed that CC slurries (concentration dependent) increase broth viscosity which leads to oxygen transfer problem throughout the broth.[9,10] In an aerobic process such as FA production, high viscosity of CC slurry can lower FA production. Meanwhile, pellet morphology of FA producing strains is considered as the most favourable form that allows easy mass, heat and oxygen transfer during SmF. Thus, SmF with pellet rules out morphological interference in FA production. To practically correlate all these vital factors to FA production profile under real SmF conditions, viscosities of different samples were measured before and during SmF. The obtained results are shown in Table 3. The glucose basic-salt medium with around 80 g L^{-1} of glucose showed viscosity of 1.25 ± 0.12 cP at 25 °C. This value was in accordance with previous findings.[44] After addition of 5% pre-cultured cell pellets (inoculum), the viscosity of the medium changed marginally. However, mixing of CCMPs (60 g L^{-1}) considerably increased the viscosity to 3.27 ± 0.18 cP. In case of CCNPs, mixing of same amount to the medium increased the viscosity upto 2.98 ± 0.1 cP. In the first 24 h of SmF, the viscosity changes were monitored after every 12 h. For CCMPs, the initial (0 h) viscosity lowered from 3.27 ± 0.18 to 3.1 ± 0.08 cP after 12 h of SmF. It further decreased to 2.28 ± 0.2 cP after 24 h of SmF. From 24–48 h, the viscosity increased again and reached 2.41 ± 0.18 cP. There was a marginal increase (0.14 cP) from 48–72 h. CCNPs also showed similar changes of viscosity during SmF. However, in the first 12 h of SmF with CCNPs, the change in the viscosity was around 0.6 cP and this was much higher than 0.17 cP, as was obtained with CCMPs. The decrease in the viscosity during zero to 12 h of SmF for both CCMPs and CCNPs was caused by the consumption of CC by FA. This trend continued upto 24 h of SmF. After reacting with FA, CCMPs and CCNPs formed CaC$_4$H$_2$O$_4$ and this product has higher solubility (15.6 g L^{-1}) as compared to CCMPs or CCNPs (6.1 g L^{-1}) at 25 °C. Further accumulation of CaC$_4$H$_2$O$_4$ caused viscosity increase from 24–72 of SmF for both CCMPs and CCNPs.

The production profile of FA obtained with glucose basic-salt medium and CCMPs and CCNPs, is shown in Fig. 5. In the first 12 h of SmF, the concentrations of FA were 6.44 ± 1.23 and 5.6 ± 1.8 g L^{-1} for CCMPs and CCNPs, respectively. From 12–24 h of SmF, as compared to FA concentration (11.34 ± 2.4 g L^{-1}) obtained with CCMPs, production of FA was higher (17.89 ± 3.3

Table 2 Viscosities of different samples of calcium carbonate measured at 25 °C[a]

Sample analyzed	Viscosity (cP)
Water	0.85 ± 0.08
CCMPs (20 g L^{-1})	1.39 ± 0.12
CCMPs (40 g L^{-1})	1.7 ± 0.18
CCMPs (60 g L^{-1})	1.9 ± 0.11
CCNPs (20 g L^{-1})	1.15 ± 0.1
CCNPs (40 g L^{-1})	1.48 ± 0.15
CCNPs (60 g L^{-1})	1.72 ± 0.1
Calcium fumarate (20 g L^{-1} of CCMPs + 23.2 g L^{-1} of FA)	2.2 ± 0.28
Calcium fumarate (40 g L^{-1} of CCMPs + 46.4 g L^{-1} of FA)	2.67 ± 0.21
Calcium fumarate (20 g L^{-1} of CCNPs + 23.2 g L^{-1} of FA)	2.18 ± 0.19
Calcium fumarate (40 g L^{-1} of CCNPs + 46.4 g L^{-1} of FA)	2.58 ± 0.26

[a] CCMPs, calcium carbonate microparticles; CCNPs, calcium carbonate nanoparticles; FA, fumaric acid.

Table 3 Viscosities of different samples at various time intervals (h) of submerged fermentation[a]

Sample analyzed	Viscosity (cP)				
	0 h	12 h	24 h	48 h	72 h
Glucose basic salt medium	1.25 ± 0.12	NA	NA	NA	NA
Glucose basic salt medium + 5% (v/v) cell pellets	1.28 ± 0.05	NA	NA	NA	NA
Glucose basic salt medium + 5% (v/v) cell pellets + CCMPs (60 g L^{-1})	3.27 ± 0.18	3.1 ± 0.08	2.28 ± 0.2	2.41 ± 0.18	2.55 ± 0.13
Glucose basic salt medium + 5% (v/v) cell pellets + CCNPs (60 g L^{-1})	2.98 ± 0.1	2.38 ± 0.19	1.65 ± 0.23	1.95 ± 0.14	2.11 ± 0.15

[a] NA, not applicable; CCMPs, calcium carbonate microparticles; CCNPs, calcium carbonate nanoparticles.

g L^{-1}) with CCNPs. This corresponded to the increase of volumetric productivity from 0.47 g L^{-1} h^{-1} to 0.74 g L^{-1} h^{-1}. From 24–36 h of SmF, the FA product yields for CCMPs and CCNPs were almost similar (around 12.33 and 13.11 g L^{-1}, respectively). However, the most productive phase of FA was in between 36 and 48 h that resulted in around 31.55 and 30.65 g L^{-1} of FA for CCMPs and CCNPs, respectively. Overall, FA production was found to be higher from 12–60 h of SmF with CCNPs. The application of CCNPs as neutralizing agent in the SmF production of FA has not been carried out earlier and thus the discussion needs to be made for higher FA yield with CCNPs can only be referred to the other supporting experimental results obtained during the present investigation. As shown earlier, CCNPs slurries were found to be less viscous to CCMPs slurries at same applied concentration (60 g L^{-1}) and temperature (25 °C). As FA production is influenced by the broth viscosity during the entire time frame of SmF, there was high possibility that FA production was favoured by the use of CCNPs. Moreover, as the highest FA concentrations (67.34 ± 2 and 66.92 ± 2.7 g L^{-1}, respectively) obtained with CCMPs and

CCNPs were comparable, it strongly suggested that the metabolic performances of the used fungus *R. oryzae* 1526, was not influenced by the replacement of CCMPs with CCNPs. It also ruled out any associated nano-toxicity of CCNPs on the fungus or in other words, application of CCNPs was safe for this fungus mediated FA production through SmF technology. Nanoparticle concentrations such as 60 g L^{-1} used in the form of CCNPs, was an exceptionally higher concentration to be tolerated by any microorganisms including *R. oryzae* 1526. From the FA production profiles obtained with CCMPs and CCNPs, it was concluded that volumetric productivity of FA was enhanced by CCNPs, but not the final FA concentration.

3.6 MWI *vs.* conventional heating in FA downstream processing

The two well-known FA recovery methods proposed by Gang *et al.* (1990) and Dang *et al.* (2009) make use of heat energy and CC neutralizing properties of mineral acids (such as HCl or H$_2$SO$_4$).[32,45] Acidification neutralizes unused CC, while heating increases the solubility of both FA and CaC$_4$H$_2$O$_4$. Technically, both methodologies facilitate the separation of soluble FA from the solid mass (fungal biomass and CaSO$_4$). However, due to the technical advantages such as no need of special heating equipment and lower consumption of heat energy, the FA recovery method of Dang *et al.* (2009) is mostly followed.[32] In the present study, MWI was intended for application in FA recovery. For a comparative study, fermented broth samples were processed through conventional method and studied parameters were recorded.[32] The results are presented in Table 4. SmF broth samples from both CCMPs and CCNPs neutralizing agents mediated SmF of FA production were considered for this investigation. The broth samples were also heated under MWI and required time was monitored. For both conventional and MWI heating, acidification and pH adjustment were similar. For MWI heating, an experiment was carried out on the optimization of minimum time required for maximum FA recovery from the broth. A time range of 2–20 min was applied and correlated with the recovered FA concentration (g L^{-1}) (data not shown). From this optimization study, it was concluded that under MWI heating conditions, a minimum of 10 ± 1 minute was required for maximum FA recovery. Although, the
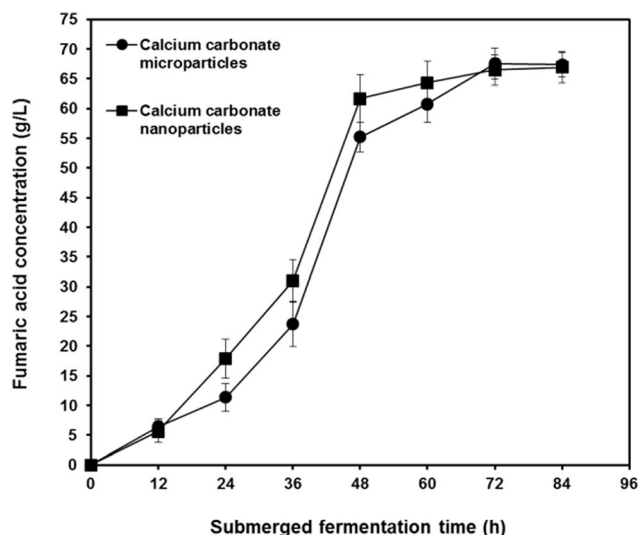

Fig. 5 Production profile of fumaric acid under submerged fermentation conditions of 25 °C, 200 rpm and 84 h with CaCO$_3$ micro and nanoparticles as neutralizing agent.

Table 4 Comparison of the conventional and microwave irradiation methods applied for fumaric acid downstream processing[a]

Parameter studied	Conventional method		Microwave irradiation	
	Broth with CCMPs	Broth with CCNPs	Broth with CCMPs	Broth with CCNPs
Heating temperature (°C)	80 ± 1 °C	80 ± 1 °C	95 ± 1 °C	95 ± 1 °C
H_2SO_4 concentration	5 N	5 N	5 N	5 N
Final pH	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Heating time required (min)	28 ± 2 (until clear)	27 ± 2 (until clear)	10 ± 1	10 ± 1
FA ($g\ L^{-1}$) recovered	67.55 ± 2	66.45 ± 2.7	65 ± 2.56	68.5 ± 1.7

[a] CCMPs, calcium carbonate microparticles; CCNPs, calcium carbonate nanoparticles.

minimum MWI heating time required for the visual manifestation of broth clarity was around 6–7 min, this resulted in lower FA recovery for both CCMPs and CCNPs (data not shown). Thus, on an average, 10 min MWI heating time was found to be optimum for maximum recovery of FA (65 ± 2.56 g L^{-1} and 68.5 ± 1.7 g L^{-1} for CCMP and CCNPs, respectively). The comparative outcome of this study confirmed the lower time consuming FA recovery through MWI. The heating time was almost lowered by 2.8 folds without affecting the recovery of FA. This was conclusive for both CCMPs and CCNPs. The results also suggested that application of CCNPs in FA production did not influence the downstream processing of FA under conventional or MWI heating conditions. With higher or lower broth volumes, heating time will be changed accordingly. It was concluded that irrespective of CCMPs and CCNPs, the well-known technical advantages of MWI over conventional heating resulted in FA recovery in lesser time. A rapid and homogenous heating of the broth and dissolution enhancement of FA under MWI, shortened the FA recovery time by almost 2.8 folds.[26–28] MWI energy heated the broth samples directly, while under conventional heating condition, heat energy first had to conduct through the walls of the Erlenmeyer flasks containing the broth samples. It delayed the heating of the broth samples. In general, MWI heating takes place through ionic conduction and dipole rotation.[27] In the present study, presence of polar groups such as –COOH and $>C=O$ (in FA) and –OH (in water) in the broth samples, accelerated (rotational alignment) the conversion of MWI into heat energy. Moreover, the high dielectric constant value (80.37, at 25 °C) of water enhanced the MWI efficacy of heating the fermented broth in a shortened time.

4. Conclusions

Application of calcium carbonate nanoparticles was found to be advantageous over micro form of calcium carbonate during submerged production of fumaric acid. Fumaric acid neutralization timing for calcium carbonate nanoparticles was much lower than microparticles (190 and 350 s, respectively). Higher specific surface area of calcium carbonate nanoparticles (11.95 ± 0.03 m^2 g^{-1}) compared to micro form (3.51 ± 0.02 m^2 g^{-1}), caused faster consumption of fumaric acid and resulted in the lowering of neutralization timing. Calcium

carbonate nanoparticles enhanced the volumetric productivity (from 0.47 g L^{-1} h^{-1} to 0.74 g L^{-1} h^{-1}) of fumaric acid in the first 12–24 h of fermentation. Nanoformulation of calcium carbonate did not exhibit toxicity towards the fungus *Rhizopus oryzae* 1526. Microwave irradiation shortened the time required for the maximum recovery of fumaric acid from 28 to 10 min.

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