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Application of Succinic Acid Produced Using *Phanerochaete Chrysosporium* MTCC787 with Glycerol as Substrate for Microbial Enhanced Oil Recovery Process in Bioremediation

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***Abstract*: Succinic acid is distributed in all living tissues and plays a significant role in intermediary metabolism. Succinic acid finds its applications in various fields such as pharmaceuticals, neutraceuticals, and agriculture and in downstream processing. As bio-based production of succinic acid is advancing in recent years, and to date, a variety of microorganisms has been engineered for the synthesis of succinic acid. Today, succinic acid is generated for human use synthetically or converted from biomass via fermentation, microorganisms, such as bacteria and fungi, has allowed for the high-yielding, commercial production from fermentation. *Phanerochaete chrysosporium* is a crust fungus, which forms flat fused reproductive fruiting bodies, which is studied most intensively and is very well known for its ability to degrade organopollutants. This study employs chemically assisted biological production of succinic acid using *Phanerochaete chrysosporium*, white-rot fungi. *Phanerochaetes chrysosporium* strain was obtained from MTCC 787 and was cultured initially in PDB broth for 7days at (38.4±1) °C. It was sub cultured in petriplates and was used in the inoculation of the optimized mandel's medium with glycerol as carbon source. Following the production of biomass, it was subjected to hydrogen peroxide treatment, which resulted in the production of succinic acid, which was extracted centrifugally and quantified titometrically. Production of succinic acid by *Phanerochaetes chrysosporium* was affirmed by UV-Visible spectrophotometric analysis. Succinic acid produced was further quantified by FTIR spectrophotometric analysis and gas chromatographic techniques. Sand packed column was designed to ascertain the effectiveness of the produced succinic acid in oil recovery rate. Statistical optimization for process efficiency was performed with Taguchi method of design.**

***Keywords*: Succinic acid, *Phanerochaetes chrysosporium*, Chemically assisted biological production.**

1. Introduction

Succinic acid, also called Butanedioic Acid, a dicarboxylic acid (chemical formula: $(CH_2)_2(COOH)_2$) that is widely distributed in almost all plant and animal tissues and that plays a significant role in intermediary metabolism. Succinic acid is not only the end product, but also precursor for variety of industrially important chemicals [1]. Succinic acid is mostly produced by chemical routes from n-butane and traditionally manufactured through routes using paraffin maleic anhydride, acetylene or acrylic acid as the starting material. Succinic acid is produced by pulverisation and distillation of amber, hence, it is known as 'Spirit of amber' [2]. Paraffin oxidation is the initial method to prepare succinic acid under catalysis of manganese or calcium by steam distillation. But the process purity is relatively low. Yet another methodology of production of succinic acid is maleic anhydride hydrogenation using noble metal catalyst such as palladium and ruthedium at high pressure and high temperature conditions. The major drawback in this process is not eco- friendly. Apart from this electrolytic reduction [3] of maleic anhydride also leads to succinic acid with higher conversion rates. The major disadvantage in this electrolytic process is heavy consumption of electricity and power making it more uneconomical.

Catalytically acetelene and acrylic acid is converted to succinic acid using economical feedstocks. Bio synthetic pathway for succinic acid production according to traditional methods is tri carboxylic acid cycle(TCA). Here, glucose is finally converted to phospoenol pyruvate and is further converted to oxaloacetic acid and maleic acid by citric acid cycle, which is reduced to fumerate and further reduced to succinic acid [4].

Traditional, large-scale, centralized chemical production plants stops modernization of process design and hinders for start-ups and new incomers. Instead, start-ups turn to biotechnological fermentation-based strategies with lesser requirements for capital investment, more dynamic market adaptations and better adjustment to niche requirements [5]. With globally decreasing fossil fuel and increasing crude oil price, traditional methods have become industrially void. As bio-based production of succinic acid is advancing in recent years, and to date, a variety of microorganisms has been engineered for the synthesis of succinic acid [6].

The biorefinery concept is an approach that strives to efficiently utilize biomass as a feedstock for integrated biofuels, energy, and chemical production. This approach is analogous to

current petroleum refineries, wherein, fuel production reduces overall costs while the co-production of value-added chemicals substantially enhances the economics and profitability of the process [7].

Indeed, many challenges exist for making biochemicals from achieving sufficiently high yields in the conversion step, deploying cost-effective, sustainable separation processes that yield the product at the needed purity and high recovery yields, and competition with petroleum derived chemicals that often have many more decades of development work behind them.

Today, succinic acid is generated for human use synthetically or converted from biomass via fermentation, microorganisms, such as bacteria and fungi, has allowed for the high-yielding, commercial production from fermentation. Global production is estimated at 16,000 to 30,000 tons a year; with an annual growth rate of 10% [8].

Succinic acid is generally produced by number of anaerobic organisms such as species of *Anerobiospirillum, Propionibacterium, Pectinatus, Bacteroides, Ruminococcus, Actinobacillus, Prevotella, Succinimonas, Succinivibrio, Wolinella, Cytophaga* [9].

Many studies on bio-based production of SA utilize *Anaerobiospirillum succiniciproducens, some succinogenes* engineered strains of *Escherichia coli,* and *Mannheimia succiniciproducens* [10]. Among these strains, *A. succinogenes, rumen bacteria,* is often a top-performing microbe in terms of succinate titer, rate, and yield and positioned as a promising strain for industrial succinate production on lignocellulosic feedstocks [11]. These natural producers show excellent tolerance to osmotic pressure caused by high level of succinate. However, the cultivation of these natural producers always requires expensive nutrient media, thus increasing the production cost [12]. In addition to the natural producers, many microorganisms can be metabolically engineered to produce succinate as a fermentative end-product. These engineered producers are always model microorganisms since they are easy to be genetically modified [13]. A completely engineered pathway is required to render them capable of producing succinate thus making the process a bit difficult.

Phanerochaete chrysosporium is a crust fungus, which forms flat fused reproductive fruiting bodies instead of the mushroom structure. This fungi exhibit an interesting pattern of septate hyphae, giving a stronger line of defense in times of distress. The hyphae network has some branching, with diameters ranging from 3-9 µm. At the ends of the hyphae rests chlamydospores, thick-walled spores varying from 50-60 µm. The conidiophore gives rise to round asexual blastoconidia, which are 6-9 µm in diameter. It's the most intensively studied white rot fungus. Several studies showed that *Phanerochaete chrysosporium* is a model white rot fungus, because of its specialized ability to degrade the abundant hydrocarbons, while leaving the white cellulose nearly untouched. White rot fungi secrete an array of peroxidases and oxidases that act non-specifically via the generation of free radicals, which then undergo spontaneous cleavage reactions. The non-specific nature and exceptional oxidation potential of the enzymes has attracted considerable interest for application in bioprocesses such as organopollutants degradation and fiber bleaching. Due to *Phanerochaete chrysoporium*'s specialized degradation abilities, extensive research is seeking ways to understand the mechanism in order to enhance the bioremediation of a diverse range of pollutants [14]. Degradation of organics, dye and hydrocarbons is made possible by the production of extracellular enzymes, which take part in the remediation of various hydrocarbons and various toxic compounds [15]. This study employs biological production of succinic acid using *P. chrysosporium*, white-rot fungi.

2. Materials and Methods

A. Chemicals and raw materials

Chemicals such as PDB, Malt extract, Agar agar, ammonium sulfate, potassium dihydrogen phosphate, proteose peptone, ferrous and zinc sulfates, manganese and magnesium sulfate, calcium chloride, polysorebate etc., obtained from SRL chemicals were used. *Phanerochaetes chrysosporium* MTCC 787 strain obtained from MTCC. Conical flasks, petriplates, pipettes and other glassware's were used.

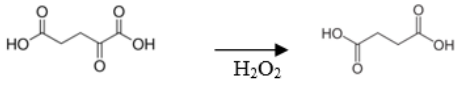
B. Strain, Media and Culture Conditions

P.chrysosporium MTCC 787 was inoculated from the vial in PDB in conical flask and was incubated at room temperature for 7 days and was sub cultured in petriplates using malt extract medium and agar as solid substrate [16]. Growth of *P.chrysosporium* MTCC787 was observed on petriplates. Growth of *P.chrysosporium* MTCC787 was measured colorimetrically at 405nm, and was plotted with time in hours on x- axis and optical density values on y- axis to determine the growth of organism. Percentage of growth of the organism was calculated using the formula:

$$\% \text{ Growth} = \left[\frac{\text{Sample OD}}{\text{Control OD}} \times 100 \right]$$

C. Extraction of succinic acid

Due to the thiamine added, α keto glutarate was produced extracellularly. To this liquid broth sample containing α keto glutarate, 1mL of hydrogen peroxide (H_2O_2) was added to produce succinic acid [17].



αketoglutarate succinic acid

D. Selection of carbon sources

P.chrysosporium MTCC787 was cultivated in flask with mandel's medium. The mandel's medium prepared was

optimized by varying the concentration of the carbon substrate, which is glycerol, by varying the concentrations as 0.5mL, 1 mL, 2 mL, 3 mL, 4 mL and 5 mL. Among these variable concentration, 3mL glycerol was found to have profound growth of *P.chrysosporium* MTCC787 as shown in the table 1, and hence, it's considered for analysis..

Table 1
Percentage Growth

Sample	% Growth
Sample with 0.5 mL glycerol substrate	40.83 %
Sample with 1 mL glycerol substrate	40.44 %
Sample with 2 mL glycerol substrate	47.72 %
Sample with 3 mL glycerol substrate	55.19 %
Sample with 4 mL glycerol substrate	30.97 %
Sample with 5 mL glycerol substrate	20.40 %

E. Analytical Methods

1) UV Visible Spectroscopy

UV Visible Spectroscopy utilizes the principle of absorption or reflectance of light by the molecules in UV and Visible range. Electrons present in the molecules are excited and excited electrons absorb or reflect energy in the form of UV Visible light. Instrument used for UV Visible Spectroscopy is termed as UV Visible Spectrophotometer. It consists of light source or radiation source, beam chopper, grating diffractor, sample holder and detector. Most detectors are photomultipliers or photodiodes. Beam chopper splits the beam as it enters the spectrophotometer. Mostly cuvettes are used as sample holders. Cuvettes made up of quartz or glass is used. Liquid broth samples are subjected to UV-Visible spectrophotometric analysis. Absorbance was measured and readings were noted down and graphs were plotted.

2) Gas Chromatography

GC consists of column with inert solid absorbent packing as stationary phase and employs inert or unreactive gas such as helium, argon, nitrogen as carrier mobile phase. Sample to be analyzed interacts with walls of column and elutes out. It is temperature dependent, used to analyze presence of organic compounds in unknown sample. GC analysis is suitable for compounds with boiling point ≤450°C. As succinic acid has boiling point of 235°C, it's analyzed using gas chromatography.

3) Fourier's Transform Infrared Spectroscopy

FTIR Spectroscopy is an analytical technique used to identify organic compounds present in unknown sample. The FTIR analysis method uses infrared radiation to scan test samples and identify their functional groups. Infrared radiation from the source in the instrument is absorbed by the sample and it's converted to signal which is detected. Each molecule or chemical structure will produce a unique spectral fingerprint, which is identified by FTIR analysis.

4) Titrometric Assay

Liquid broth sample is taken for analysis. About 2 drops of phenolphthalein was added to it and it's titrated with 0.1 N sodium hydroxide (NaOH) till the production of pale permanent pink color, which is considered to be the end point of titration. [18]. Volume and normality were determined using the formula

$V_1N_1 = V_2N_2$

1ml of 0.1 N NaOH is equivalent to 5.905 mg of succinic acid.

Where $V_1$is the volume of 0.1 N NaOH required to neutralize succinic acid and N_1 is the normality of NaOH, which is 0.1N. V_2 is the volume of sample and N_2 is the normality of succinic acid.

The yield was calculated using the formula:

$Y = X/S$

Where

X = product concentration; S = substrate concentration.

5) Taguchi method and Statistical Analysis

A taguchi design was employed to screen and identify significant factors affecting succinic acid production from glycerol by *P.chrysosporium* MTCC 787. The investigated factors affecting succinic acid production were: carbon source, temperature and time for production of succinic acid. The Taguchi design is based on the model equation:

$Y = \lambda Y'$

Where $\lambda=1$

$Y=Y'$

Where Y is the response (yield of succinic acid), λ is linear coefficient and Y' is level of independent variable .Each of the variables in the Taguchi design was examined at two levels: 0.01 for low level and 2 for high level. The factors significant at the 95% level ($p \leq 0.05$) were considered to have a significant effect on succinic acid production by *P.chrysosporium* MTCC 787.

6) Sand pack column experiment for microbial enhanced oil recovery process

Sand packed column was designed to determine the effectiveness of the produced succinic acid in oil recovery rate. A glass column of 10 cm length and diameter of 3 cm was packed with 33.9 g of sand material up to 6 cm of the column. Sand was first saturated with brine and then by the oil to reproduce the natural environment of petroleum reservoir. Column was flooded with brine first until no more oil was recovered in the effluent. Following this, water flooding was performed for oil recovery. Then its carried out with Triton-X (Chemical Flooding). Produced succinic acid was flooded and oil recovery rate was deduced.

3. Results and Discussion

A. Determination of Growth Curve

There was difference in growth rates and shapes of growth curves for varied concentrations. A marked difference is seen in growth phase for varied concentrations of the given substrate.

When glycerol is used as substrate at various concentration, (0.5mL,1 mL,2 mL,3 mL,4 mL,5 mL), *P.chrysosporium* MTCC 787 has shown considerable growth in mandel's medium. The average percentage growth [19] was found to be 40%. *P.chrysosporium* MTCC 787 has shown notable growth in mandel's medium with 3mL of glycerol as substrate

(56%).Hence, liquid broth sample of mandel's medium with 3mL of glycerol as substrate is considered for analysis. Liquid broth sample was subjected to centrifugation [20] at 5000 rpm and supernatant containing succinic acid is filtered using cellulose membrane. The resultant extract obtained is used for further analysis.

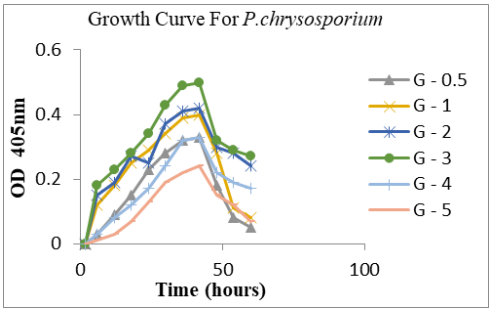


Fig. 1. Graphical representation showing growth of *P.chrysosporium* MTCC 787

B. UV Visible Spectrophotometric Analysis

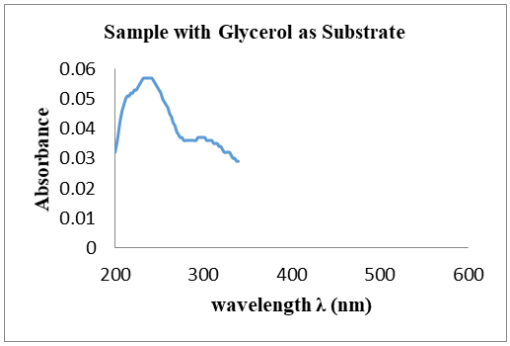


Fig. 2. Representation of UV Visible Spectroscopy showing absorbance

It's observed that a peak is seen at 240 nm of UV spectrum for sample with 3ml glycerol as carbon substrate. From UV-Visible Spectrophotometric Analysis, lower absorbance is observed widely for UV spectrum. A peak is observed at 240nm for the above sample which corresponds to the succinic acid range. This evidently shows that succinic acid is produced by *P.chrysosporium* MTCC 787 [21].

C. Fourier's transform infrared spectrophotometric analysis

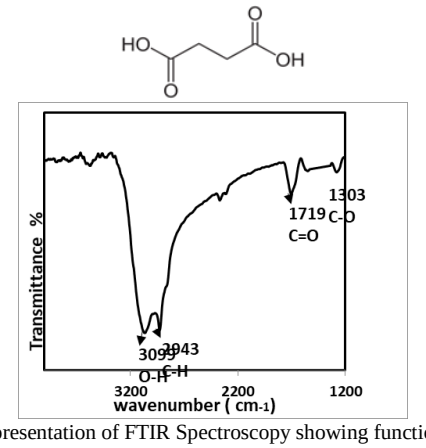


Fig. 3. Representation of FTIR Spectroscopy showing functional groups

Fourier Transform Infra-Red spectrophotometric analysis is used to analyze the presence of functional groups in unknown sample confirming the presence of specific organic compounds in unknown sample. FTIR employs beam of light containing various frequencies which is absorbed or reflected by the sample and amount of light absorbed or reflected by the sample is measured. In the above diagram, there's a peak observed at range of 1210-1320 cm^{-1} indicating the presence of C-O stretch. The above IR spectrum shows peaks at the range of 2500-3300 cm-1 and around 2900 cm^{-1} indicating the presence of O-H group and C-H stretch. There's a peak observed at the range of 1700 – 1725 cm-1 indicating the presence of C=O stretch in the above IR spectrum [22]. From the above IR spectrum diagram, it's evident that presence of C=O, O-H, C-H, C-O functional groups are observed confirming the presence of succinic acid**.**

D. Gas chromatographic analysis

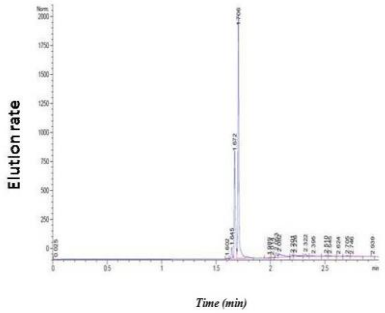


Fig. 4. Representation of Gas Chromatography showing succinic acid peak

From the above chromatographic diagram, it's evident that peaks are observed around 2 mins of elution time. So, we can conclude that presence of succinic acid is affirmed in the broth [23]. This shows that *P.chrysosporium* MTCC 787 produces succinic acid.

E. Titrometric Assay for Succinic Acid

Based on titrometric assay done for succinic acid, volume of 0.1 N NaOH required to neutralize succinic acid and normality of succinic acid was calculated, and volume of 0.1 N NaOH was found to be 16 ml and normality of succinic acid was found to be 0.016N. The concentration of succinic acid produced was estimated as 94.48 mg/ml. The yield of succinic acid was determined to be 2.01 ml. It's observed that *P.chrysosporium* efficiently produces succinic acid (67%) when 3mL of glycerol is fed as substrate.

F. Statistical optimization for process efficiency taguchi design

The statistical design of experiment (DOE) can be used to design a scheme of experiment under dissimilar conditions. A wondrous statistical technique known as Taguchi method, was proposed and established by Genichi Taguchi. This method is generally used for optimizing the design variables with two main advantages: (i) significantly reducing the overall testing time (ii) minimizing of experimental cost. The selection of

control factors is a prime stage for application of Taguchi method. This method has two crossed array layout namely an inner array and an outer array. The inner array is made up of the orthogonal array selected from all possible combination of the controllable factors. This orthogonal array can be properly designed from the application of Taguchi method by which the experimental condition can be optimized. To evaluate the experimental results, analysis of data is needed [24]. In this study, carbon source, temperature, time were used to compose the Taguchi design. Effects of the variables on the response and the estimated values for the effect of each of the independent factors are shown. The highest succinic acid concentration produced by *P.chrysosporium* MTCC787 was observed in experiment, in which all the variables were at their highest levels.

Table 1
Taguchi design for three variables with the corresponding experimental values of succinic acid concentration

Std	Run	Block	Factor 1 A:carbon sour ml	Factor 2 B:temperature degree celsius	Factor 3 C:time hours	Response 1 yeild of succin ml
7	1	Block 1	1.00	37.90	168.00	1
3	2	Block 1	1.00	65.80	96.00	0.75
11	3	Block 1	3.00	10.00	168.00	0.01
12	4	Block 1	3.00	65.80	168.00	0.025
6	5	Block 1	5.00	37.90	24.00	0.5
17	6	Block 1	3.00	37.90	96.00	2
9	7	Block 1	3.00	10.00	24.00	0.01
14	8	Block 1	3.00	37.90	96.00	2
8	9	Block 1	5.00	37.90	168.00	1.15
13	10	Block 1	3.00	37.90	96.00	2
4	11	Block 1	5.00	65.80	96.00	0.025
15	12	Block 1	3.00	37.90	96.00	2
10	13	Block 1	3.00	65.80	24.00	0.01
2	14	Block 1	5.00	10.00	96.00	0.01
16	15	Block 1	3.00	37.90	96.00	2
5	16	Block 1	1.00	37.90	24.00	0.025
1	17	Block 1	1.00	10.00	96.00	0.01

All studied factors exerted a positive effect on succinic production. Carbon source, temperature, time had the strongest impact on the level of *succinic acid* concentration.

The calculated R^2value of 0.9499 suggests that this model is well fitted to the experimental data.

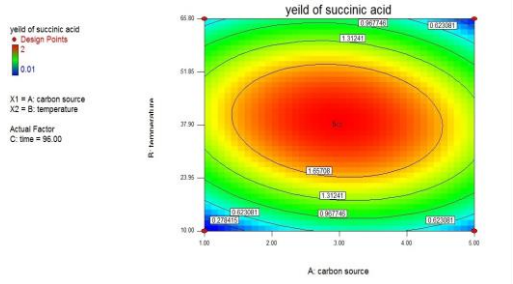


Fig. 5. Taguchi design model for production of succinic acid

The response surface plot is shown in Fig. 5. This plot demonstrate that succinic acid concentration was affected by investigated factors namely carbon source and temperature. In the case of time, no influence was observed in the studied range of its concentrations. The selected range covered the optimum condition for carbon source and temperature. Optimum succinic acid production by *P.chrysosporium MTCC787* occurs at a concentration of glycerol of 3mL at a temperature of 37.9°C for 96 hours. The mean concentration of the obtained succinic acid produced by *P.Chrysosporium* MTCC787 was 2mL which is higher than the predicted value and confirms the validity of the model [25].

G. Sand pack column experiment for microbial enhanced oil recovery process

Succinic acid produced by *Phanerochaete chrysosporium* was subjected to experiment for its probable application in microbial enhanced oil recovery by using sand packed column on laboratory scale. Water injection which is a secondary recovery method, can only achieve a certain amount of oil recovery; beyond which, no more oil could be recovered due to high capillary force, which restricts the mobility of oil. As succinic acid produced has bio-surfactant properties [26] that can reduce the capillary force by reduction of interfacial tension between oil and water, this technique was employed to study its influence on oil recovery.

The column was packed with sand and it was saturated with brine water followed by 19 ml of oil. Water flooding was carried out with 10 ml of distilled water in the column, and about 6ml of oil was recovered during this process. After no oil was observed in the effluent using the water, tertiary process was carried out. About 5 ml of Triton-X, a chemical surfactant, was flooded into the column and checked for oil recovery [27]. About 1.8 ml of crude oil was recovered. During this process, a certain amount of oil was recovered. Finally, 10 ml of cell free supernatant containing the produced succinic acid was flooded into the column and checked for oil recovery [28]. About 2ml of crude oil was recovered. From the above fig. 5, it's evident that succinic acid shows efficient oil recovery more close to that of chemical surfactant.

4.Conclusion

Traditional, large-scale, centralized chemical production plants stops modernization of process design and hinders for start-ups and new incomers. Instead, start-ups turn to biotechnological fermentation-based strategies with lesser requirements for capital investment, more dynamic market adaptations and better adjustment to niche requirements. With globally decreasing fossil fuel and increasing crude oil price, traditional methods have become industrially void. As bio-based production of succinic acid is advancing in recent years, and to date, a variety of microorganisms has been engineered for the synthesis of succinic acid. Today, succinic acid is generated for human use synthetically or converted from biomass via fermentation, microorganisms, such as bacteria and fungi, has allowed for the high-yielding, commercial production from fermentation. Global production is estimated at 16,000 to 30,000 tons a year; with an annual growth rate of 10%. Succinic acid is generally produced by number of anaerobic organisms such as species of *Anerobiospirillum*,

Propionibacterium, *Pectinatus*, *Bacteroides*, *Ruminococcus*, *Actinobacillus*, *Prevotella*, *Succinimonas*, *Succinivibrio*, *Wolinella*, *Cytophaga*.

This study employed biological production of succinic acid using *P. chrysosporium*, white-rot fungi. *P.chrysosporium* MTCC 787 was cultured in mandel's medium prepared and growth of *P.chrysosporium* MTCC 787 and production of succinic acid was observed. Growth curve for *P.chrysosporium* MTCC 787 was determined and growth % was calculated. The yield of succinic acid was determined to be 2.01 mL, titrometric and spectrophotometric analysis was done. FTIR and GC analysis were performed confirming the production of succinic acid. Construction of a sand pack column is easy, rapid and inexpensive and the problems associated with core flood studies like preservation of live cores. This makes the sand pack column a suitable bench-scale technique for microbial enhanced oil recovery. Sand packed column was designed to ascertain the effectiveness of the produced succinic acid in oil recovery rate. Statistical optimization for process efficiency was done with Taguchi design.

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