Executive Summary

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1. **Title of the Project:**CO_2 to Value-added Products: Sustainable Process Development for Succinic acid and Hexanol Production through Hybrid Fermentation Route

2. **Date of Start of the Project:**October 1, 2022

3. **Aims and Objectives:**

Objectives

- The proposal aims to develop a novel biotransformation process towards succinic acid and hexanol production via fermentation of CO_2 employing isolated/potential strains/enriched consortia as biocatalyst for translation (TRL6).

Sub-objectives

- Optimization of process for succinic acid production by isolated *Citrobacter amalonaticus*IICTSVMSA1 by CSIR-IICT (from TRL 4 to TRL 6)
- Optimization of process with Carboxydotrophs for CO_2 fermentation process towards hexanol production (from TRL 3 to TRL5)
- Design and operation of hybrid fermentation systems with the optimized parameters using the selected biocatalyst to achieve maximum hexanol and succinic acid production, respectively.
- Metabolomics studies of the carboxydotrophs/*Citrobacter amalonaticus*to identify the product specific pathway (hexanol and succinic acid production), gene/enzyme regulation.
- Electrochemical and analytical characterization.
- Demonstration of process at 100 L capacity for succinic acid and 50 L capacity for hexanol production
- Life cycle assessment (LCA) and Techno-economic analysis (TEA) for scaled up processes.
- DPR for low carbon process with net zero emission along with business model.

4. Significant achievements (not more than 500 words to include List of patents, publications, prototype, deployment etc.)

Succinic acidis a versatile chemical with various industrial applications, and its demand as a biobased sustainable alternative in place of petroleum-based feedstock is of much interest in the context of sustainability and green process development. The work revolves around the fermentation process of bio-succinic acid (SA) production with the wild/native strain *Citrobacte*r sp. (IICTSVMSA1) using glucose/glycerol, $MgCO_3$ and CO_2 as substrates. CO_2facilitates shift of glucose metabolism towards SA via phosphoenolpyruvate and oxaloacetate reactions. With this approach, glycerolin presence of CO_2produced good SAwith reduced by-products synthesis. Later on, the experiment designed with co-substrate fermentation specifically using the combination of glucose and glycerol as carbon sources were operated. Through a designed methodology, the ideal conditions that can maximize SA production was obtained while minimizing the formation of the by-products. With co-feedstock feeding (glucose and glycerol), keeping the initial ratio as 15+15 g/L of each substrate along with 10g/L of $MgCO_3$and CO_2, 13.9 g/L of SA was achieved. Later, with the loading rate of 30+30 g/L, SAproduction increased to 24 g/L followed by 26 g/L, 30 g/L and 37.3 g/L with loading rate of 30+40 g/L, 30+50 g/L and 30+60 g/L respectively. By keeping the glycerol constant at 60g/L, the glucose concentration was varied starting from 40 g/L, 50 g/L and 60g/L respectively, wherein 50+60g/L showed highest production (50.1 g/L) at 36h. The process was scaled up to a 25Lfermenter resulting in 73 g/L of SA (20h) with a productivity of 3.6 g/L/h (0.6g/g) using a non-genetic approach. Gene expression studied with specific genes (*pyk, frd, aceF, mdh,* and *ppc*) exhibited differential expression patterns (upregulated or downregulated) providing insights on the metabolic shifts during SA production. Hexanol, was also produced via non-genetic approach using strain *Clostridium acetobutylicum*ATCC-824. Hexanol production was achieved with glucose and ethanol (electron donor) via ketohexanoyl CoA. Butanol is mainly the precursor of hexanol which has multistep process primarily involving butanol dehydrogenation and butyraldehyde reduction. With glucose concentration of 40 g/Land 2 ml ethanol, the hexanol productivity was found to be 673mg/L. This study not onlyoffers a more sustainable and environmentally friendly method for biobased chemical production but also demonstrated the potential of non-genetic approaches as well as selective substrate feeding strategy towards enhanced biochemicals productivity.

Provisional patent under processing:

S Venkata Mohan, Triya Mukherjee, Amulya Kotamraju.Higher Titer Bio-succinic Acid Production by Co-substrate Fermentation with *Citrobacter* sp. IICTSVMSA1 and Thereof.

Expected publications:

Triya Mukherjee and S Venkata Mohan.Pathway Prediction for the production of succinic acid from *Citrobacter amalonaticus*(IICTSVMSA1) with glucose and glycerol feedstocks: Whole genome sequencing and Gene expression studies.Green Chemistry.

5. Concluding remarks

This work progress depicted a significant breakthrough in the production of biosuccinic acid (SA) using a non-genetic approach with the native strain *Citrobacter* sp. (IICTSVMSA1). Through a carefully designed fermentation process involving the use of glucose and glycerol as carbon sources, along with additional $MgCO_3$ and CO_2 as co-substrates, we have achieved good productivity of SA. The incorporation of CO_2 as a component in the metabolic pathway has led to the good production of SA with glycerol while also reducing the formation of by-products. By optimizing the ratio of carbon sources and maintaining the redox conditions, 50.1 g/L of SA production was achieved at 36h retention time with non-genetic approach. Scaling up this process to a 25L fermenter further demonstrated feasibility with a yield of 73 g/L of SA in20 hof time boasting a high productivity of 3.6 g/L/h and substrate conversion efficiency of 0.6 g/g.Gene expression studies with specific genes (*pyk, frd, aceF, mdh*, and *ppc*) exhibited differential expression patterns (upregulated or downregulated) providing insights on the metabolic shifts during the SA production. Hexanol which is an important chemical was produced by the strain *Clostridium acetobutylicum*ATCC-824with non-genetic approach. Hexanol production of 673mg/L was achieved with 40 g/L of glucose and 2 ml of ethanol (electron donor) primarily involving the genes butanol dehydrogenation and butyraldehyde reduction. In summary, the non-genetic approach used for SA production, not only offers a sustainable and environmentally friendly bioprocess but also underscores the potential of non-genetic strategies in enhancing the productivity.