

Improving Yields and Productivity of Microbe-Catalyzed Production of  
Targeted Bio-Molecules Using In-situ Adsorption.

by

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## ABSTRACT

With the aid of metabolic pathways engineering, microbes are finding increased use as biocatalysts to convert renewable biomass resources into fine chemicals, pharmaceuticals and other valuable compounds. These alternative, bio-based production routes offer distinct advantages over traditional synthesis methods, including lower energy requirements, rendering them as more “green” and “eco-friendly”. *Escherichia coli* has recently been engineered to produce the aromatic chemicals (S)-styrene oxide and phenol directly from renewable glucose. Several factors, however, limit the viability of this approach, including low titers caused by product inhibition and/or low metabolic flux through the engineered pathways. This thesis focuses on addressing these concerns using magnetic mesoporous carbon powders as adsorbents for continuous, in-situ product removal as a means to alleviate such limitations. Using process engineering as a means to troubleshoot metabolic pathways by continuously removing products, increased yields are achieved from both pathways. By performing case studies in product toxicity and reaction equilibrium it was concluded that each step of a metabolic pathway can be optimized by the strategic use of in-situ adsorption as a process engineering tool.

## DEDICATION

I have enjoyed the gift of having a wonderful family back home who have supported me through all my decisions. I could not have done this without you. This thesis is dedicated to you guys!

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# 1. INTRODUCTION AND BACKGROUND INFORMATION

## 1.1 Motivation

Mankind's quest for energy needs a resurgence, and with increasing global population there is a scarcity not only for fuels but also other products traditionally derived from the now ever-shrinking fossil reserves. Global populations grow at an alarming rate, which seems to have a cascading effect on global energy requirements. With increasing prices for fuels and chemicals derived from crude oil, problems like inflation and eventually recession can prove to be the bane of our existence. To counter this, scientists world over have been looking at alternative sources of energy, some of which are more promising than others. One of the most promising techniques is to use metabolic engineering (microbial cell factories) to produce chemicals and fuels. This is a low cost, sustainable and green method that has the potential to replace existing technology. The problems with this method of production include: low titers, low concentrations and toxicity. To counter this, development of effective separation processes would provide economic viability to biological production of biofuels. Production of certain first and second generation biofuels like butanol and ethanol biologically has been industrialized successfully, though inherent problems do exist. This thesis will address this concern and combat several issues faced in developing separation technologies for fuels and fine chemicals.

## 1.2 Global Population and Energy Demand

The current population of the world is seven billion people and is set to become eleven billion by year 2040. The burgeoning need for energy in the USA, BRIC nations (Brazil, Russia, India and China) and Mexico cannot be fulfilled by

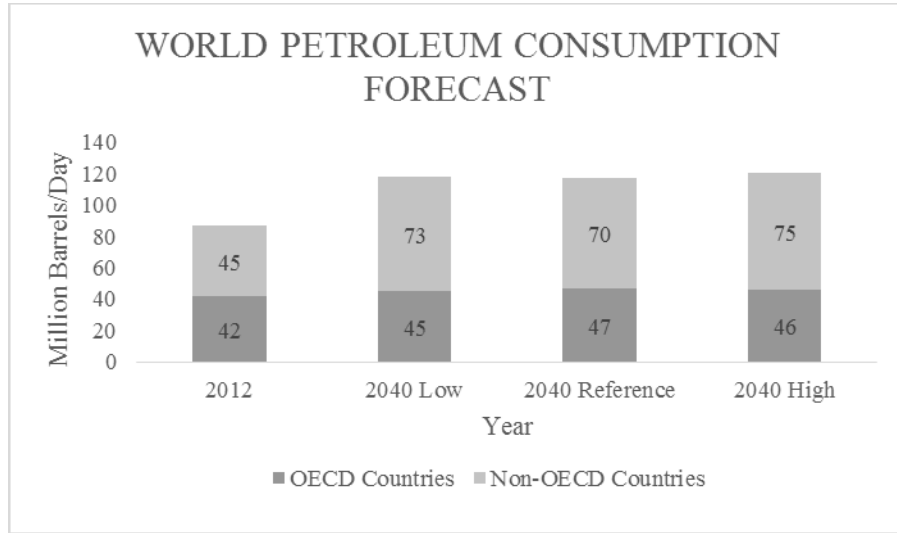


Figure 1: Depicts the world consumption of petroleum current and forecasts for 2040

the existing methods of energy production. Alternative sources of energy production are not a luxury anymore, rather a necessity. In the US alone, energy consumption across the four major sectors (transportation, residential, commercial and industrial) is expected to grow at 0.4%/year to 106.3 quadrillion BTU in 2040 from 82.6 quadrillion BTU in 2012. Our production capacity with current infrastructure cannot match this demand, and compounded with the problem of dwindling fossil reserves, we must shift our focus to other lucrative options. Some of the options that have been explored are solar powered technology, wind energy, natural gas. These technologies while green and environmentally friendly choices, cannot sustainably replace fossil fuels. This is due to

two major reasons: existing engine technology does not support them and capital costs are extremely high.

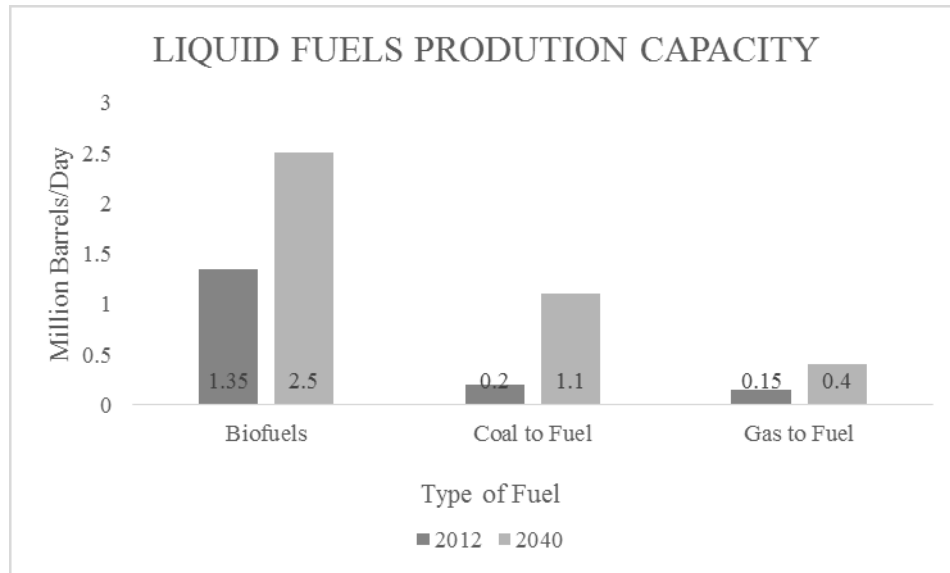


Fig 2: Depicts the liquid fuel production capacity and forecasts to 2040

### 1.3 Metabolic Engineering: Replacing Traditional Production Methods

These inherent problems are overcome by microbial production of fuels and chemicals by metabolic engineering. Any organism has a set of biological pathways in place to produce energy from food sources to sustain itself; in this process it can also produce other valuable chemicals and fuels that it secretes out as by-products. Metabolic engineering makes use of biological tools to modify these pathways to target the production of certain bio-molecules by ensuring only pathways that the organism needs for its growth and production of the bio-product (which maybe a non-natural metabolite) exist while deleting or silencing other pathways. In other words, these microbes become green factories for the production of a certain compound. Essentially, the microbe produces

certain amino acids and proteins (enzymes) that catalyze the substrate selectively to produce the bio-product of interest. The advantages enzymes offer over other catalysts are: high selectivity, bio-degradability, non-toxic to the environment and operation at near ambient temperatures. Microbial production is not only limited to production of biofuels, since these organisms potentially can be modified to produce a vast number of enzymes, they can be engineered to produce precursors to petroleum derived chemicals, fine chemicals, pharmaceutical chemicals and other value added compounds. This is the need of the hour as it will reduce our dependence on crude oil for production needs. Therefore, the prospects of microbial production of chemicals are vast and promising.

Metabolic engineering, once seen as an exclusive alternative to production of fuels, has now evolved into a field that could take mankind to the next level. Whether be it fuels or solvents, commodity chemicals or pharmaceuticals, metabolic engineering combined with synthetic biology possesses the tools and techniques necessary to produce varied metabolites of specific characteristics having similar properties all the time. Even certain chemicals that cannot be produced artificially can be produced in biological systems successfully. This research will focus on using process engineering as a tool to optimize metabolic pathways by identifying bottlenecks and troubleshooting specific issues related to pathways.

## 1.4 Bio-products of Interest in this Research

### 1.4.1 Phenol

Phenol is an economically important commodity chemical with an annual production of more than 7 million tons. Phenol has numerous applications: manufacture of plastics like polycarbonates, epoxies, bakelite and nylon; detergents, herbicides and pharmaceutical drugs. It is also a precursor to other important chemicals: bisphenol A and phenolic resins. Chemically produced by the oxidation of cumene (which in itself is produced from benzene), this process is very energy intensive and produces a lot of toxic waste, apart from intermediate cumene hydroperoxide which is toxic. With increasing demand for phenol and increase in oil prices, it is important to consider an alternative way to produce phenol. Metabolic engineering addresses all the current concerns with phenol production. Phenol can be produced by *Pseudomonas putida* and *Escherichia Coli*. This research will focus on the production of phenol in *E. Coli*. In *E. Coli*, phenol is produced by the action of the *tpl* gene encoding the enzyme tyrosine phenol lyase. The metabolic pathway is shown below:

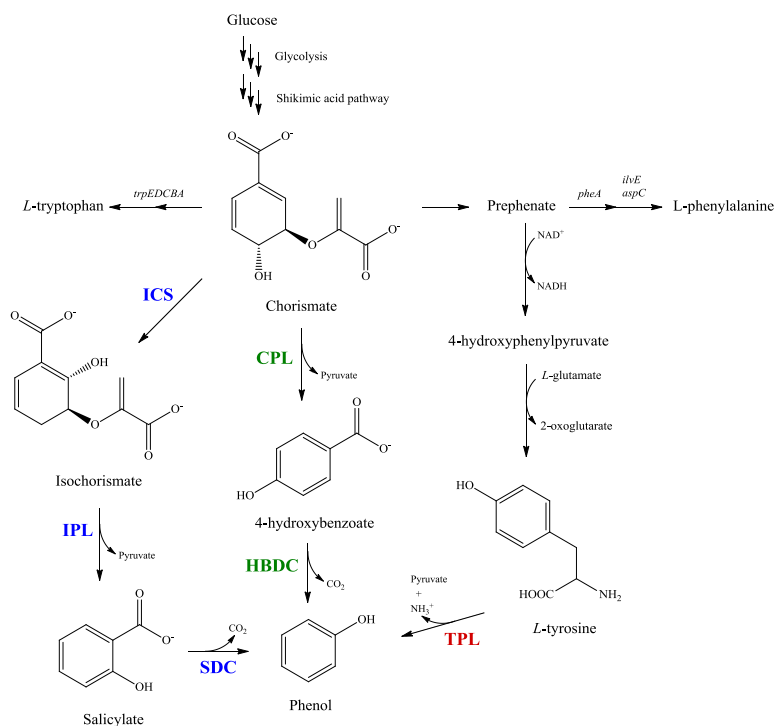


Fig. 3: Phenol Biosynthesis Pathways

### 1.4.2 Styrene Oxide

Styrene Oxide is an important value chemical that has several applications and is a precursor to several other important petrochemical derivatives. Chemical production of styrene oxide is done over a heavy metal catalyst and the disadvantage of this process is that only 48-57% of the product is of required stereo specificity, therefore the product obtained is a mixture of isomers. Styrene oxide is manufactured from styrene, which is manufactured from benzene, a petro-product. To reduce the pressure of petroleum and to achieve higher percentages of a single isomer, metabolic engineering offers an excellent alternative mechanism to produce styrene oxide. It is produced in *E. coli* from glucose by the conversion of chorismate to phenylalanine which then goes to trans-cinnamate,

styrene and then styrene oxide by the action of the genes *pal*, *fdc* and *styab* encoding specific enzymes in *E. coli* respectively. The metabolic pathway is shown below:

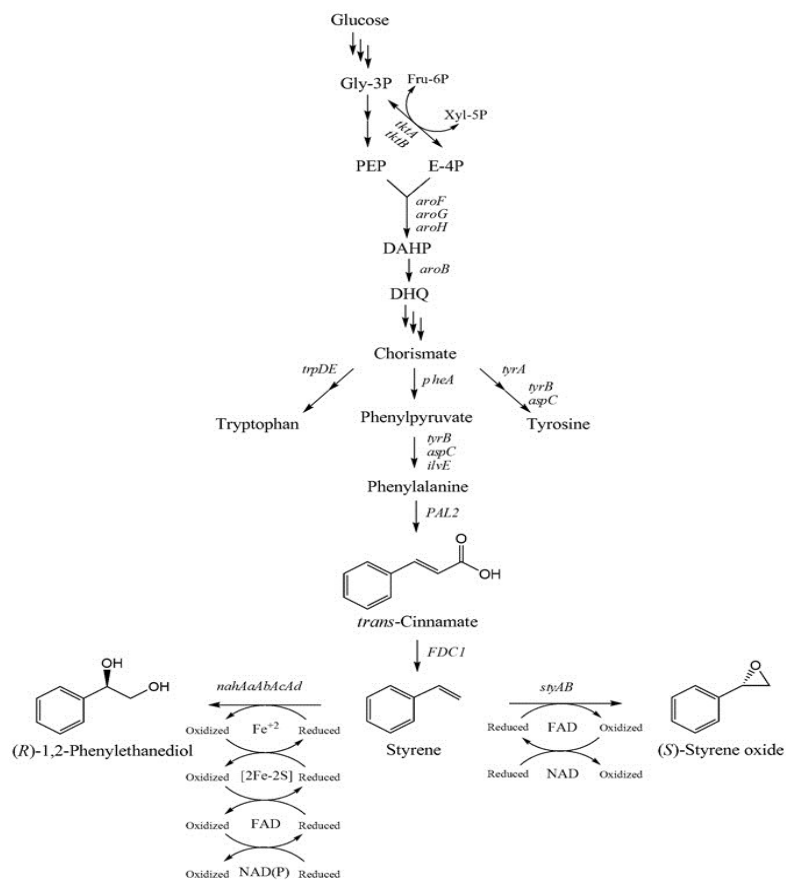


Fig. 4: Styrene Oxide Biosynthesis Pathway

### 1.4.3 Butanol

Butanol is considered a second generation bio-fuel and it was first manufactured in the 1916 by Chaim Weizmann from starch using the bacterium *Clostridium acetobutylicum*. Since its discovery as a byproduct of ABE fermentation, it has been subject to several research projects. Several recombinant organisms can now produce butanol, but all these processes are inhibited by the toxicity of butanol to the bacterial cells. Hence, product recovery techniques have been the focus of research in the second

half of the 20<sup>th</sup> century and the new millennium. Butanol is an important fuel as it offers several beneficial characteristics over its ABE counterparts: ethanol and acetone. ABE fermentation titers are typically in the ratio of 3:6:1 (Acetone: Butanol: Ethanol). This is one of the one of the major reasons why the focus has been steadily shifting from ethanol to butanol. Butanol also possesses excellent fuel characteristics: higher octane number, lower vapor pressure, higher flash point, less corrosive, lower water solubility. Higher octane number corresponds to higher compressibility of the fuel, which is generally preferred in a fuel engines as air-fuel mixture is compressed before the ignition stroke. Gasoline has a research octane rating of 90. Lower vapor pressure of butanol ensures that it stays in liquid phase and doesn't form an ignitable mixture with air in pipelines etc. Its lower miscibility in water makes it easier to use in existing infrastructure and no major overhauls are required to engine technology. Butanol is also less polar and resembles gasoline more closely than ethanol or methanol due to its longer carbon chain. Another reason butanol holds an inherent advantage over ethanol is blending costs, which is significantly lower than ethanol and even gasoline.

Butanol is produced in the solventogenic step where butyryl-CoA goes to butanol or butyrate. Butyryl-CoA is first dehydrogenated by the butyryldehyde (adhE) dehydrogenase and further dehydrogenated to butanol by bdhAB, both steps require the action of NADH which goes to NAD<sup>+</sup>.

Butanol will be used mainly as a comparison tool in this research to evaluate the performance of the powders and compare to existing literature on adsorption.



## **1.5 Separation Methods Used in Bio-Processes**

Most bio-processes are run in batch mode or very rarely semi-continuous mode. This is because cells need to be replaced periodically to obtain optimum titers. Designing a bio-process should take into account the organism used, the bio-product produced, substrate used, conditions for growth and knowledge of culturing the organism. Since various factors like pH, temperature, ambient conditions, anaerobic nature of organism affect the growth and consequently titers of bio-product, care should be taken to check all these boxes. An important factor that ties into the final titer is the activity of the enzyme which can be hindered by high concentrations of inhibitory compounds, which can then result in low titers. This can harm the overall viability of the process. For this reason, one of the most important parts of the bio-process is to design a separation mechanism not only for the recovery of our bio-product but also removal of the inhibitory compound. Some of the various separation technologies that have been used are:

### **1.5.1 Pervaporation vs Membrane Evaporation**

Pervaporation is the process of separating a bi-component or multi-component mixture by partial vaporization through a membrane. Membranes are designed to be permeable to the product of interest with the upstream end at atmospheric pressure and downstream side at vacuum, this allows for the evaporation of a particular component on the downstream side. Separation occurs based on permeability and not volatility, driving force being the difference in activities or fugacities of the bi component mixture. Fugacity of the components can be calculated based on Raoult's Law for liquids and Dalton's law of partial pressures for gases.

Traditional membranes used in separation have been PDMS (Poly di-methyl Siloxane) and zeolite. Selectivity of a membrane can be increased by adding in compounds like ionic liquids (IL), IL-supported PDMS has been shown to exhibit improved selectivity. Ionic liquids used were 1-ethenyl-3-ethyl-imidazolium hexafluorophosphate and tetrapropylammonium tetracyanoborat (Casson et.al). Toxicity assays have been performed to determine the partition coefficient of these membranes. It followed the expected trend: butanol>acetone>ethanol. A problem with this IL supported membrane is impregnation specifically stabilizing the IL on the porous matrix to prevent leaks. At low differential pressures, the mechanical stability is a concern. Another research group (Plaza et al.) used what are called ionic liquid gel (IG) membranes for their pervaporation setup over the over traditionally used PDMS and Zeolite membranes. They compared the performance of the membrane with and without IG support. They reported that selectivity of butanol extraction increased with the use of IG supported membrane. It went up from a measly 40% removal to 90% at 5000s. The mass transfer resistance to butanol was lower with IG and the diffusion coefficient of butanol was found to be  $3.9 \times 10^{-10} \text{ m}^2/\text{s}$ . Although, this is promising the diffusion of acetone and ethanol are still higher than butanol. Some of the other problems faced was solubility of butanol in the ionic liquid, fouling of the membrane and separation issues with regards to the new ABE mixture obtained on the membrane side.

Membrane evaporation or membrane distillation is based on the concept of separation based on phase change, the difference from pervaporation being that this phase change is based on volatility and not activity. A hydrophobic membrane will allow the water vapor to pass through while the fuel permeates slower thereby cooling off further

and collected easily on the other side. Usually a membrane made of PTFE is used in these applications, but this method is not preferred because it involves heating the culture to high temperatures.

### **1.5.2 Liquid-Liquid Extraction vs Perstraction**

Liquid-Liquid Extraction is an age old extraction method. Despite its inherent weaknesses, it is a widely used process as an easy downstream step to concentrate the bio-product before more energy intensive step is used to purify the bio-product. Liquid-liquid extraction is based on the difference in solubility of each solute (constituent of bioprocess) in the extractant. Since butanol has a higher octanol-water coefficient than acetone and ethanol, it readily enters the extractant usually a polar organic solvent. The major disadvantage of this method is the inhibitory effects of extractants and the formation of emulsions.

The advantage that perstraction offers over liquid-liquid extraction is the use of a membrane to remove the extractant, the disadvantages being membrane fouling and clogging.

### **1.5.3 Partial Vaporization vs Gas Stripping**

Partial Vaporization has been used as a method to separate ethanol from fermentation broths. This type of separation is based on the difference of volatility between different components in a multi-component mixture with the more volatile components separated on top by a throttling valve. Some of the problems faced while using this method include high energy intensity requirement. Approximately 10% of the

ethanol produced in the bio-process would have to be expended to this process of separation. While this method may be used in batch production, it cannot be used in continuous or semi-continuous processes as the heat from the separation would ruin the cells as well as the culture media (carbohydrates, proteins and other organic components).

Gas Stripping with an inert gas such as Nitrogen has been tried to drive the toxic product out of the reactor, but the low volatility of butanol, styrene oxide and phenol make this method ineffective. Also, the purge gas may affect the nutrient balance in the media and change some parameters like pH that could affect final titers.

#### **1.5.4 Separation based on solid phase adsorption**

Separation using solid phase adsorbents has been of interest in the recent past with a lot of work being done with various types of adsorbents, both commercial and those produced in lab scale. This seems to be an attractive method to concentrate the bio-product in the reaction vessel or outside by pumping and then desorbing at a higher temperature. This method was initially thought to be ineffective due to issues like fouling of the adsorbent, stability of the adsorbent structure, regenerative capacity of the adsorbent but over the years several research groups as well as for-profit organizations have been developing technologies to overcome these issues.

##### ***1.5.4.1. Comparison of different configurations***

Packed bed adsorption is a commonly used method to separate the bio-product of interest outside the fermenter in a column that has a packed bed of the adsorbent. Usually, a pump sends the culture through the bed and then this allows the biofuel to be adsorbed

from the broth, this biofuel-free culture is then sent back to the fermenter or phased out. While this offers the option of running a semi-continuous process in theory, the practicality still leaves much to be desired. This is because the cells get caught in the void spaces creating serious fouling problems in the adsorbent. This also inhibits the flow through the column and huge pressure differences inside the column which are not ideal conditions in a bio-process. To overcome this, expanded bed adsorption has been investigated as a possible alternative to the packed bed configuration.

An expanded bed is typically a fluidized bed of adsorbent in a column outside the fermenter where the culture is pumped in an up-flow direction fluidizing the bed. Though, not as effective from a separation standpoint, this configuration offers a larger void space between adsorbent particles resulting in better flow through the column and better contact between biofuel containing culture media and adsorbent particles.

The simplest of configurations though is the addition of the adsorbent directly to the fermenter containing the bacterial culture. This requires no additional column thereby allowing for enormous savings in capital. The problems attributed to configuration is the difficulty of recovering the adsorbent from the column, fouling of the adsorbent and disruption of the process. If these problems can be overcome, the potential of this configuration is very exciting. This method could help to reduce capital costs, make the process less energy intensive hence more economically viable, help reduce the effects of inhibitory compounds as they are being produced, thereby potentially improve titers well above other configurations, achieve a truly continuous bio-process.

In the search for an ideal candidate for a material that can overcome most of the problems with this configuration, the Vogt lab at University of Akron has developed a

magnetically responsive mesoporous carbon powder with high surface area. Nielsen Lab at ASU (through collaborative NSF grant funding with Vogt lab), has been investigating the use of these powders as potential adsorbents in the removal of biofuels and other compounds of interest from bacterial culture. This research will focus on the use of these powders applied to pathways developed in the Nielsen Lab to improve titers and yield of the products of interest: butanol, styrene oxide and phenol.

#### ***1.5.4.2 Adsorption using Mesoporous Carbon Powders vs Other Materials***

The magnetic mesoporous carbon powders (MMCPs) contain a cobalt oxide metal framework usually prepared from  $\text{Co}(\text{acac})_3$  precursor on a phenolic resin that promotes the formation of a graphene like layer to ensure that metal oxide does not leach out in acidic solutions.

*Table 1: Materials used in Bio-product Separations with their Properties*

Material	Brand	Pore Size (Å)	Specific Area (m <sup>2</sup> /g)	Freundlich Parameters		Langmuir Parameters		Reference
				K <sub>F</sub> (mmol/kg)	N	K <sub>L</sub> (L/kg)	N	
Mesoporous Carbon Powder	CS-68-800	82	1287	245±59	1.92±0.14	-	-	Levario, et.al. 2010
Mesoporous Carbon Powder	FDU16-800	58	671	708±66	4.61±0.34	-	-	Levario et.al 2010
Mesoporous Carbon Powder	CS-Co-10-800	43	1125	169.82±25.12	1.39±0.107	-	-	Dai et.al 2012 and this research
Zeolite	Zeolyst CBV901	24.24	700	-	-	0.168±3.6E-03	3.14±0.94	Oudshoorn et.al, 2009
Polymeric Resin	Dowex L-493	46	1100	446±15	2.22±0.26	-	-	Nielsen et.al, 2009

The powder is prepared by a tri-constituent assembly: a cobalt acetylacetonate(acac) precursor, tetra ethyl orthosilicate (TEOS) and commercially available triblock copolymer (Pluronic F127). Evaporation induced self-assembly of these constituents followed by pyrolysis at 600 degrees results in the formation of a highly ordered mesoporous carbon powder. The cobalt oxide yields magnetic properties to the MMCP, cobalt was found to be more stable and had higher magnetization compared to iron oxide and nickel oxide. Powders produced by EISA possessed increased surface area compared to etching and also had superior resistance to leaching. The powder that was used in this research was the CS-Co-10-800, which had the highest cobalt content (10%) compared to other powders. The cobalt content was the differentiating factor on loading, having a higher impact than even surface area. The properties of various materials used in adsorption are listed along with their properties in Table 1.

## **1.6 Research Objectives**

Metabolic pathways face two major issues: toxicity of inhibitory compounds to cells and metabolic flux related issues. This research will focus on these two issues related to the pathway in *E. Coli* NST74 that goes from chorismate to two products: styrene oxide (via phenylalanine) and phenol (via tyrosine).

With titers of tyrosine already at 1.3 g/l (McKenna et.al), styrene oxide production is limited by the toxicity of styrene oxide at 1.6 g/l. Removal of styrene oxide from culture should drive the production of phenylalanine resulting in a overproduction styrene oxide above current titers, thereby increasing the yield of the product to above 0.083 g/g

glucose. Phenol production via tyrosine in NST 74 is limited by the metabolic flux in the conversion of tyrosine to phenol by the *tpl* gene. Current titers stand at 249 mg/l with a yield of 0.0125. This equilibrium limited reaction can be forced to completion by the systematic removal of phenol as it is being produced.

Addressing these two problems will be the major focus of this research. Determining the isotherm fit for these compounds to understand their equilibrium behavior is essential to the thesis. Once, the equilibrium behavior is established, powders will be employed in the culture test the hypothesis.

The thesis is split into 4 chapters: Chapter II will address all the materials, methods and protocols used in the experiments including information about analytical methods, culturing methods and experimental design for isotherms. Chapter III and IV will be case studies on styrene oxide and phenol respectively that will go into results from characterizing isotherms and washes to culturing and calculation of yields.



## **2. MATERIALS AND METHODS**

### **2.1 Structure and Background**

In this section, the main materials and methods or protocols followed will be described in detail. First, the protocols for the adsorption isotherms as well as kinetics experiments will be described along with the chemicals used. Next the culturing methodology for the *E. Coli* will be explained. Explanations and background information shall be provided wherever applicable. This section is built in to give the reader an understanding of the standards used and to establish a standard of consistency required in culturing micro-organisms. All chemicals used for making standard solutions, stocks, model solutions and media were purchased from Sigma Aldrich, St. Louis, Missouri, USA.

### **2.2 Adsorption Isotherms**

#### **2.2.1 Protocol for the isotherm**

Adsorption isotherms for various compounds on the magnetic mesoporous carbon powders are the first step in characterizing the behavior of these powders. Adsorption isotherms were made by making up some model solutions of known concentrations and then adsorbent is added before they are setup in a shake table for a specified time. The protocol for butanol is explained in detail here while the same protocol may be applied across the board to all compounds, it is important to design the experiment based on what is necessary to be characterized.

The factors that go into designing the isotherms are: range of operation in bioreactor setup, isotherms in the range of concentrations of what the bio-production of the same compound are more relevant to us than other concentrations. The conditions for the isotherm should match the conditions used in actual culturing. Keeping all this mind, for the adsorption of butanol, 100 mL of 3 stock solutions of 40.5 g/l, 20.25 g/l and 8.1 g/l were prepared. Then, model solutions of 27 g/l, 16.2 g/l, 13.5 g/l, 10.8 g/l, 3.24 g/l, 2.43 g/l, 1.62 g/l, 0.81 g/l, 0.405 g/l and 0.2025 g/l were prepared by diluting from stock solutions. 0.5 mL of all these solutions were sampled and stored in HPLC vials for sampling as controls for the calibration curve. Around 75 mg of magnetic mesoporous carbon powders were weighed out in a weigh scale and added to 15 bottles, then 20 mL of the model solutions are added separately to different bottles. These bottles containing a specific concentration of the model solution and around 75 mg of powder are then closed tight with septa caps to prevent losses to atmosphere. Once all this done, they are placed on a shake table for around 24 h at 200 RPM. 0.75 mL of sample was then collected in a 1.5 mL minifuge tube and was centrifuged at 14000x g for 7 minutes. 0.500 mL of this then added to a HPLC vial for HPLC analysis. The centrifugation step is added in to ensure that no particulates enter the HPLC column.

### **2.2.2 Protocol for Regeneration**

The steps in the regeneration of the powders were:

- Pour off model solution or culture broth from which bio-product was adsorbed.
- Perform appropriate washes for the removal of adsorbate.

- Once washes are performed, as much of supernatant is removed as possible, then powders are placed in the oven at 120°C for 24-48 hours depending on amount of powder used. If more than 0.5 g powder is dried, 48 hours is chosen.

## 2.3 Culturing

### 2.3.1 Strains/Plasmids Used

Table 2: Strains and Plasmids used in this research

Strain/Plasmid	Antibiotic	Promoter	Target Compound
NST 74	-	-	Styrene Oxide, Phenol
NST 74 $\Delta$ tyrA	-	-	Styrene Oxide
NST 74 $\Delta$ pheA	-	-	Phenol
BW25113	-	-	Phenol, Styrene Oxide
Fdc1	Amp	P <sub>trc99A</sub>	Styrene
Pal2	Amp	P <sub>trc99A</sub>	Transcinnamate
Styab	Kan	P <sub>trccolK</sub>	Styrene Oxide
TutA	Kan	P <sub>trc99A</sub>	Phenol

Plasmid isolation is an important step in culturing as storing the plasmid in the native *E. Coli* degrades the plasmid over time, hence it is usually stored in a storage strain. For styrene oxide production, 3 plasmids need to be added to the native organism using 2 promoters. The promoter p<sub>trc99A</sub> can be used with plasmids pal2 and fdc1, they are stored in *E. coli* BW25113. The promoter p<sub>trccolK</sub> was used with styAB. To isolate these 2 plasmids, the plasmid mini-prep kit was used.

### **2.3.2 *Escherchia Coli* NST 74**

Two strains were used for styrene oxide production, NST 74 and NST 74  $\Delta$ tyrA, the latter being the strain with the competing tyrosine pathway knocked out. These strains were used from their frozen stock stored at  $-80^{\circ}\text{C}$ . These cells first need to be transformed with the necessary plasmids (prepared by PCR and stored in storage strains) before culturing. First step in transformations is the preparation of chemically competent cells that can be used to uptake the plasmid. These are prepared by first growing up a 5 mL seed of the required cells overnight, the seed is then used to inoculate a flask containing 50 mL LB media and grown to an OD<sub>600</sub> of 0.375. Once this is done, cells are added to pre-chilled Falcon tubes, spun down at 14000xg for 7 min at  $4^{\circ}\text{C}$ , supernatant poured off and then 10 mL of ice cold 15% glycerol containing calcium chloride solution is added. This step is repeated once more. The cells are spun down finally at 11000xg for 4 min at  $4^{\circ}\text{C}$ , supernatant poured off and 2 mL of cold 15% glycerol containing calcium chloride solution is added to it and several 100  $\mu\text{L}$  aliquots are made in 1.5 mL centrifuge tubes. These aliquots contain the chemically competent cells. One tube of chemically competent cells is then allowed to thaw on ice for 10 min, then 1.5  $\mu\text{L}$  of each plasmid is added for a 2 plasmid mixture (1  $\mu\text{L}$  for single plasmid and 2.5  $\mu\text{L}$  of each plasmid for 3 plasmid mixture), this is allowed to sit on ice for 30 min. Cells containing plasmid are then heat-shocked for 30s in a water bath at  $42^{\circ}\text{C}$ . Once this is done, 0.5 mL of SOC media is added to the tube and this is incubated at  $37^{\circ}\text{C}$  for at least 30 min (usually 60 min), before it is plated (on a plate with corresponding antibiotics) and incubated overnight. A lawn of cells should be visible after 12 hours.

Once the cells are transformed, a seed is prepared by using one colony to inoculate a 5mL seed containing required antibiotics and grown overnight. After 12 h of growth, this seed is used to inoculate a flask containing a media containing MM1 salts, 20% glucose, supplemented with necessary amino acids if necessary and required antibiotics. 10  $\mu$ L of 1M IPTG is added after flask reaches an OD600 of 0.5. This flask is cultured for 72-96 hours adjusting pH regularly to 7.4. Samples are drawn typically at 24 h intervals. The flasks are placed on a shake table at 200 RPM, incubated at 32°C during this time period.

## **2.3 Analytical Methods**

### **2.3.1 HPLC detection of Phenol and Styrene Oxide**

A hypersil gold C-18 column was used in a 1100 series Hewlett Packard high performance liquid chromatograph. Separation was performed on a reverse-phase Hypersil Gold SBC18 column (4.6 mm  $\times$  150 mm; Thermo Fisher, USA) operated at 28°C. The column was eluted at a total constant flow rate of 1.0 mL/min using “solvent A” (consisting of 5mM sulphuric acid solution) and “solvent C” (consisting of acetonitrile, HPLC grade). The eluent was used as a mixture of 85% solvent A and 15% solvent C for 12 minutes. The eluent was monitored using a diode array detector set at 215 nm. The peaks for the required compounds were observed at 2.265 minutes, 3.6 minutes, 6.8 minutes and 10.8 minutes for tyrosine, phenylalanine, styrene oxide and phenol respectively.

### **2.3.2 HPLC detection of Glucose**

Glucose analysis was performed on the same HPLC system; however, now using an RID detector and an anion exchange column (Aminex HPX-87H; BioRAD, Hercules, CA, USA) operated at 35 °C. The column was eluted with 0.005 M H<sub>2</sub>SO<sub>4</sub> at a constant flow rate of 0.8 mL/min. Glucose peak was observed at 14 minutes.

### **3. LEVERAGING IN-SITU ADSORPTION TO IMPROVE STYRENE OXIDE BIOSYNTHESIS**

#### **3.1 Introduction**

Styrene Oxide is produced from the amino acid phenylalanine, which occurs naturally in *E. coli*. Phenylalanine is an essential amino acid that is a precursor to several commercially valuable chemicals, food supplements, etc. Several years of research has been devoted to improving yields in *E. coli*. Styrene Oxide is produced from phenylalanine by the action of enzymes PAL2, FDC1 and StyAB that help catalyze steps that go to transcinnamate, styrene and styrene oxide respectively. These steps all have high negative values of Gibbs energy, suggesting spontaneity and high metabolic flux through the pathway.

Therefore, the major problem for this pathway is expected to be the toxicity of styrene oxide to the culture once maximum titers are reached. MMCPs when used as in-situ adsorbents of styrene oxide should be able to drive the production of phenylalanine through this pathway resulting in enhanced titers and yields for styrene oxide.

#### **3.2 Characterization of the MMCPs**

The first step in this process was to characterize the MMCPs to find the equilibrium parameters for styrene oxide. Surface phenomena are best understood via isotherms, indicating the loading capacity of the adsorbate (styrene oxide) on the adsorbent (powder).

To compare the performance of these powders to other products used previously and to give the reader a background, isotherms for butanol were fit and then a comparison is shown.

Since these powders are basically a carbon matrix, they function on the basis of hydrophobic attraction. They have a high surface area, very porous, high porous volume. The goal of this work is to fit the data obtained on a simple and explicit isotherm. Of all the models that are used, the Langmuir and Freundlich models are the most commonly used. Based on the properties of the compounds used and the powder, the Freundlich model provides the best fit. Regression was performed using the data analysis package on Microsoft Excel and a 95 % confidence interval was established for error. Parameters were calculated by the data analysis and error was established upto one standard deviation.

The equation for the Freundlich model is given by:

$$q = k_F C_{eq}^{1/n}$$

Where  $q$  = specific loading capacity at a particular concentration (mmol/kg),

$C_{eq}$  = equilibrium concentration of adsorbate (mmol/L)

$K_f$  and  $n$  are the Freundlich model parameters with units mmol/kg and dimensionless units respectively.

Specific loading is determined by the following equation from experimental data.

$$q = \frac{C_{aq} - C_{eq}}{m} * V$$

Where  $C_{aq}$  = aqueous concentration of adsorbate before adsorption in mM

$m$  = mass of powder used in kg

$V$  = volume of solution used in L



Error analysis was done by repeating one point in triplicate and applying the standard deviation as error on the experimental data.

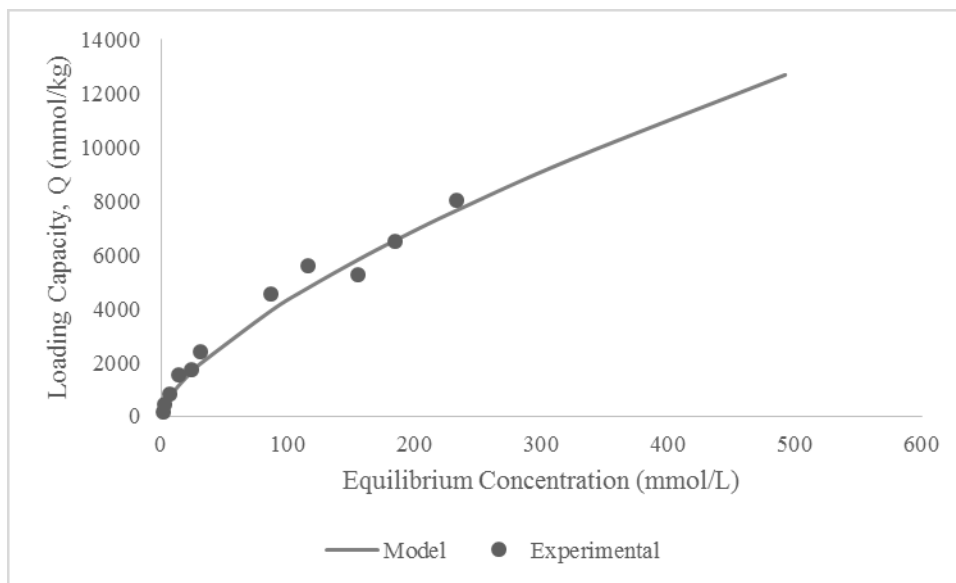


Fig. 5: The figure depicts the adsorption of butanol onto mesoporous carbon powder CS-Co-10-600 with the specific loading plotted vs equilibrium concentration. Solid line is the best fit isotherm and points are the experimental data.

The isotherm fits the Freundlich model, this is expected considering the large surface area of the powder and the hydrophobic nature of the longer chain of the  $-R$  group in butanol. The next step was to compare this performance to other powders and commercially available products. Among the current commercially available products, the resin Dowex Optipore L-493 has been used widely as it is one of the better adsorbents available commercially. It will also be interesting to see how the addition of cobalt affects the performance of the powders in terms of adsorption.

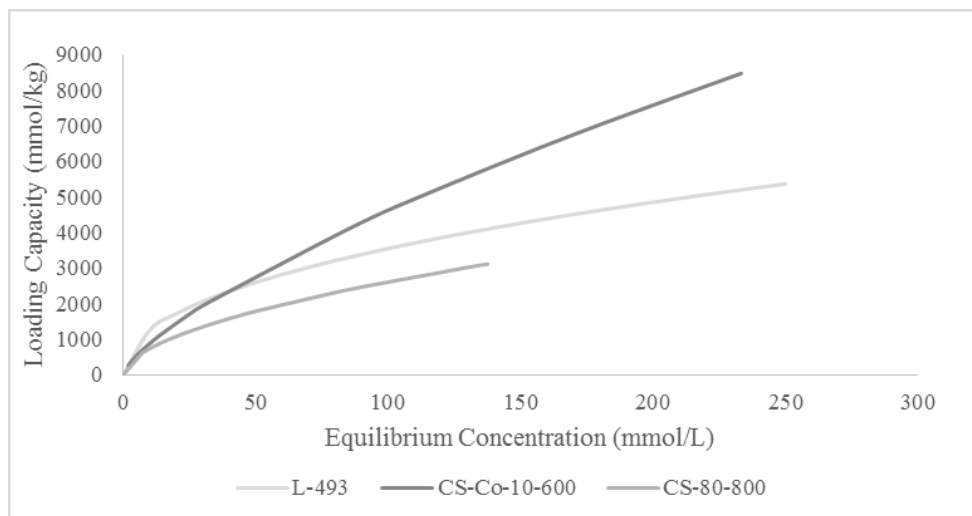
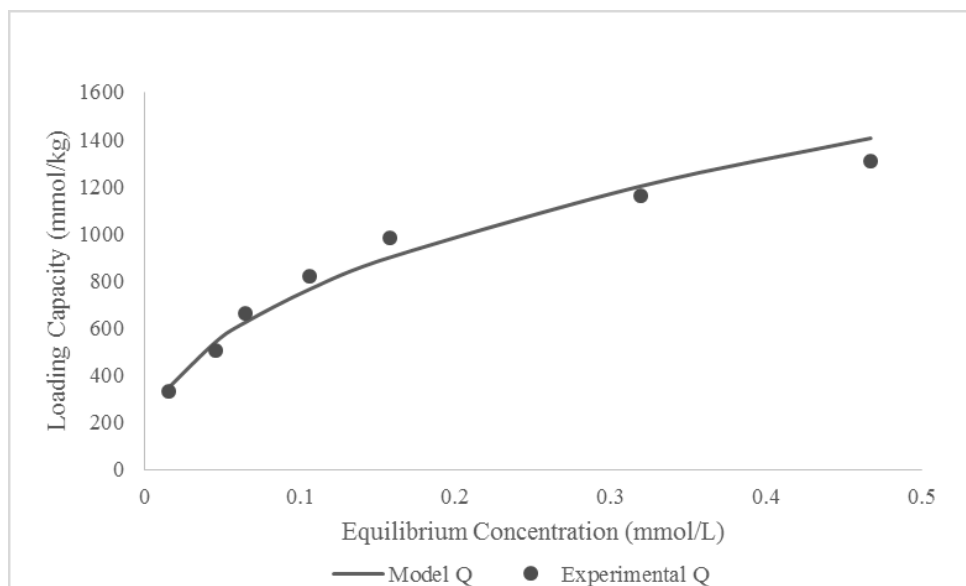


Fig. 6: This figure compares the adsorption isotherms of butanol onto three adsorbents: L-493 Resin, CS-Co-10-600 powder and precursor CS-80-800 powder.

From figure 6, we notice that addition of cobalt has only improved the performance of the powder compared to its precursor and the L-493 resin which show almost similar adsorption capacities. Now that it was established that the powders had better adsorption capacity than the older materials, an isotherm for styrene oxide was also characterized by the same protocol used for butanol.



7: This figure depicts the adsorption of styrene oxide onto the CS-Co-10-600 powder

Table 3: Freundlich parameters for the different adsorbents and adsorbates

Adsorbate	Adsorbent	Freundlich Parameters	
		$K_f$ (mmol/kg)	N
Butanol	L-493	446±115	2.22±0.26
	CS-81-800	446±95	2.39±0.19
	CS-Co-10-600	169.82±25.12	1.397±0.107
Styrene Oxide	CS-Co-10-600	4581.42±539.55	2.43±0.154

From the table, the striking feature is that the n value for the styrene oxide in the new MMCPs is similar to the older n parameters of butanol with the other adsorbents, whereas the K value is almost ten times larger. This would mean a ten time higher loading capacity on the MMCPs for styrene oxide compared to butanol on L-493 or older powder. This may be explained by the higher electronegativity of styrene oxide and its ability to form resonance structures by transferring charge onto the benzene ring, this may form stronger bonds or higher number of molecules/site than butanol which has close to zero electronegativity. This is ideal as we work with very low concentrations of

styrene oxide in microbial cultures. At higher concentrations, butanol outperforms styrene oxide or phenol as it forms more layers, due to less bulky nature of the -R group in butanol compared to styrene oxide and phenol

Although the isotherm looks promising, the real challenge lies in the recovery of the styrene oxide once it is adsorbed on the powder. This is done by multiple elutions of water and methanol to displace styrene oxide. Now for the elution of styrene oxide, methanol was chosen as displacer as it is commonly available solvent that is small enough to just displace the styrene oxide compared to say ethanol which may form secondary adsorption layers on the powder. Also, methanol is easy to wash off on the HPLC column during analysis unlike ethanol which does not separate very well. Over time, the protocol was perfected by trial and error. The current protocol involves the use of 1 water rinse off followed by 3 washes with methanol.

Mass recovered in the elution is calculated by using a simple mass balance across both sides:

$$\text{Mass Eluted} = \sum_{i=0}^n C_i V_i$$

$$\text{Total Mass Recovered} = \text{Mass Eluted} + \text{Mass left over in Solution}$$

$$M = \sum_{i=0}^n C_i V_i + C_{eq} V$$

Where,  $C_i$  = concentration of adsorbate in the displacer for each elution

$V_i$  = Volume of displacer used for each elution

$V$  = Volume of the solution used for adsorption.

Table 4: Percentage and Overall Recovery of Adsorbate Styrene Oxide from Methanol Elutions

Initial Mass In (mg)	Initial Mass Left after Adsorption (mg)	Water Rinse Mass Obtained (mg)	Methanol Elution Mass of Styrene Oxide (mg)			Total Eluted Mass (mg)	Percentage Eluted	Percentage Recovered
			Elution 1	Elution 2	Elution 3			
8.416	1.060	0.254	3.380	2.412	0.757	6.8034	80.83	93.42
6.312	0.780	0.208	2.674	1.978	0.389	5.249	83.15	95.51
5.260	0.394	0.169	2.270	1.614	0.346	4.4	83.65	91.22
4.208	0.475	0.149	1.564	1.355	0.296	3.363	79.93	91.11
3.156	0.548	0.145	1.186	0.883	0.205	2.418	76.62	93.98

It is very clear from the table that the protocol that exists for the washes that they are very successful in recovering almost all the mass of styrene oxide that is adsorbed on the powder. On an average around 83 % of the mass of styrene oxide going in is eluted and around 92-95% of the total styrene oxide mass is recovered from the process. Therefore, this is a very efficient way to recover the styrene oxide we may derive from a bio-process. The data from the washes also makes a good case for the initial hypothesis about the electronegativities of styrene oxide and phenol being the reason for their increased performance at lower concentrations. A strong displacer like methanol had to be used three times to get all of the compounds off. Most of the butanol was recovered from powder after water rinse and a single elution of methanol, a second elution was required only for the removal of traces of butanol.

### 3.3 An Analysis on Titrers, Yields and Effect of using the powder

#### 3.3.1 Titrers and Yield from Flasks

Table 5: Titrers and yields of styrene oxide

		NST 74		NST 74 $\Delta$ tyrA	
		Titer (mg/L)	Yield (g/g)	Titer (mg/L)	Yield (g/g)
Culture	Aqueous Conc.	632.23 $\pm$ 17.28	0.040	837.53 $\pm$ 11.27	0.054
Culture	Aqueous Conc.	646.64		815.23	
+	Adsorbed Conc.	401.26		462.57	
Powder	Total Conc.	1047.9	0.067	1277.8	0.082

A yield of 0.040 g/g of styrene oxide was obtained with respect to glucose in NST74 which went up to 0.067 g/g when the powder (1 g/L) was used to continuously remove the styrene oxide produced. This drove the reaction towards completion resulting in more flux through the phenylalanine route which means there was increased production of phenylalanine as well. In NST74  $\Delta$ tyrA, the competing tyrosine pathway is knocked out. This ensured that more flux was available through the phenylalanine pathway initially, which resulted in higher concentration of 837.53 mg/L. The yield for this pathway stands at 0.054. By the addition of the powder and driving the pathway further, the yield increased to 0.082 resulting in an overall titer of 1.277 g/L which is close to the toxicity limit although aqueous concentration was only 812 mg/L.

Further flux through this pathway is possible through the consumption of all phenylalanine and glucose. This would mean titers of styrene oxide reaching toxicity limits. It is hypothesized that if the styrene oxide were to be removed as it was produced at concentrations above toxicity, it would not lead to cell death and may pull more on the phenylalanine to produce even more styrene oxide. So a toxicity assay was performed in NST74 by exogenous addition of styrene oxide to show that the powders were capable of removing the styrene oxide quick enough before cell death.

### 3.3.2 Toxicity Assay for Styrene Oxide

A toxicity assay was performed in NST74 by exogenous addition of styrene oxide solution. The experiment was designed as a 3 way test to study the toxicity of powder, powder added along with styrene oxide and just styrene oxide and a control flask of NST74 was run in the background to use as comparison.

Powders in large quantities are known to inhibit cell growth by binding to certain essential proteins or form complexes with the cell, inhibiting membrane transport functions, therefore a dedicated study needs to be performed to study the effect of powder on the cells. Initial results from studies show that powders are not inhibitory up to 150 mg/50 ml of culture, which is much higher than the amount needed for this study, which typically 30-70 mg/50ml culture flask. The study was restricted to this range due to the limited availability of powders.

Similarly, the toxicity limit of styrene oxide is around 1.4-1.6 g/L to *E. Coli* and this must be investigated, to check if the powders can remove the inhibitory product fast enough before it results in mass cell death.

So respective flasks containing 50 mL LB were inoculated with 1% (v/v) seed of BW25113 grown overnight. They were grown to an OD of 0.75 after which necessary powders and styrene oxide were added in. The 4 flasks used were:

- BW25113 culture (flask 1)
- BW25113 culture + powder (flask 2)
- BW25113 culture + powder + styrene oxide (flask 3)
- BW25113 culture + styrene oxide (flask 4)

The clock is now reset to time 0 h.

Before all exogenous additions, all flasks were mixed and split up again to ensure the same starting OD for all flasks. Two sets of experiments were designed one with styrene oxide concentration at 1.3 g/L and one with styrene oxide concentration at 1.6 g/L. These levels were designed in relevance to our titers which are at these levels. The flasks that contained powder all had 50 mg of powder.

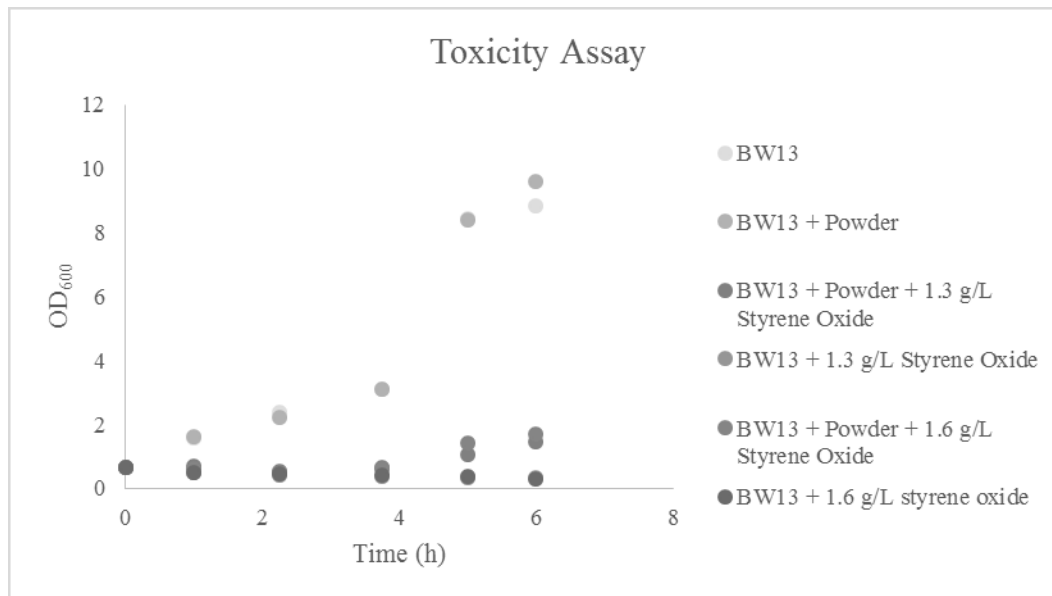


Fig. 8: Toxicity Assay showing the growth of cells as a function of exogenous addition of powder and styrene oxide in various combinations.

From the results of the toxicity assay, it is clear that styrene oxide is toxic to cells at high concentrations and has severe inhibitory effects. This toxicity may hinder the flux systematically through the pathway, thereby resulting lower titers. The control flask with just culture grew up to an OD of 8.85, while the flask with cells and powder did even better going up to 9.61. The difference is clearly not during exponential growth where similar growth was noticed. At stationery phase, with powders, the OD did not flatline as quickly as the Flask 1. The powders had a positive effect on cell growth by pulling more



flux through the pathway by adsorbing some of the pathway chemicals resulting in higher consumption of substrate to build cell walls. This is a pleasant surprise as more cells mean more product eventually increasing yields and productivity.

There is a trend of decreasing OD for the flask containing just the styrene oxide, this is true for both concentrations. Cell death seems imminent right from time 0 and by the halfway point, clearly there is a drop in OD indicating the start of cell death due to toxicity. This decrease in OD is negated in flasks containing the powder and is characterized by an increase in OD at the halfway point of the experiment. This shows that removal of styrene oxide helps in relieving the toxicity. HPLC sampling was performed to study the adsorption of styrene oxide on the powders. Adsorption of styrene oxide takes longer than 6 hours but this is expected to be different in MM1 media as it lacks the large nutrients present in LB that might bind onto the powders. With more and more styrene oxide adsorbed, it is seen that by 3 hours there is a spike in OD for these 2 flasks.

While this removal of styrene oxide reduces the inhibitory effects on the cells, one may also hypothesize that this will pull more flux through the entire pathway by driving the equilibrium in the forward direction. This will result in even higher titers of aromatics in this pathway.

## 4. TROUBLESHOOTING THE PHENOL BIOSYNTHESIS PATHWAY USING MAGNETIC MESOPOROUS CARBON POWDERS

### 4.1 Introduction

The other bio-product of interest in this research is phenol and as we know it is produced from tyrosine by the action of the enzyme *tpl*: tyrosine phenol lyase. Tyrosine is a  $\beta$ -amino acid, a non-essential amino acid in humans but nevertheless one of the most important biological compounds. The reaction from tyrosine to phenol is severely equilibrium limited in that the Gibbs energy change for the reaction is positive. It is clearly a reaction that is non-spontaneous and needs a driver for the reaction to go to completion. With the powders, shown to improve yields in the phenylalanine pathway, experiments were designed to be performed with this pathway to increase the titers of phenol.

### 4.2 Isotherm

The phenol isotherm was developed the same way as the others. The same equations for  $q$  were used and the experimental data was fit to a Freundlich fit.

The isotherm is below and the parameters are in the table.

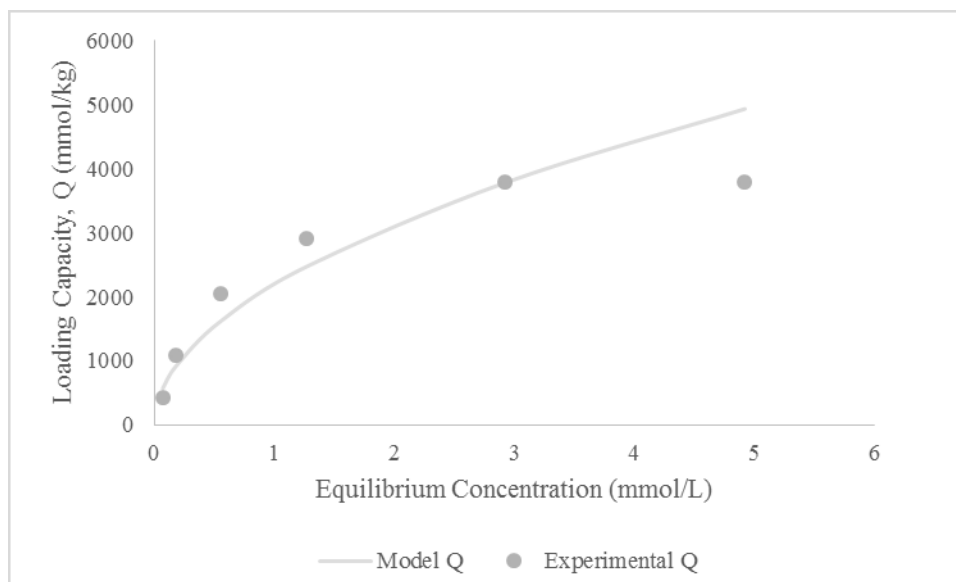


Fig. 9: This figure depicts the specific loading for phenol plotted against the equilibrium concentration

The isotherm fits the Freundlich model and parameters are shown in the table 6 below:

Table 6: Freundlich Parameters for Phenol Adsorption onto CS-Co-10-600

Adsorbate	Adsorbent	Freundlich Parameters	
		$K_F$ (mmol/kg)	N
Phenol	CS-Co-10-600	2187±195.2	1.96±0.112

From the table it is clear that these powders help in the superior adsorption of aromatic compounds at low concentration due to some kind of physical interaction force formed in resonance structures. The  $k$  value is significantly higher than value for  $k$  for butanol on the older materials. The value of  $n$  is higher than 2 which signifies a plateauing after certain concentration. This is in concurrence with data from the styrene oxide where we had similar isotherm behavior.

### 4.3 Analysis from Culturing and Resting Cell Assay

Phenol is produced from tyrosine by the action of tyrosine phenol lyase (*tpl*) enzyme. Titters stood at 224 mg/L at a yield of 0.012 g/g glucose for phenol in the NST74 strain and at 249 mg/L of phenol at a yield of 0.013 g/g glucose in the NST74  $\Delta$ pheA. Both yields and titters increased when powders were added, titters were up to 408 g/L in NST74 and upto 502 g/L in NST74  $\Delta$ pheA. These are close to double the titters. It is interesting to note that all flasks had significant tyrosine build-up which indicates that this reaction is inhibited by equilibrium. Conversion to phenol resulted in more tyrosine flux through the pathway, which is expected but continued flux with more and more phenol consumption is encouraging, but also indicates an inherent equilibrium issue with the last step on the pathway. A resting cell assay was performed to better understand the dynamics of this step.

Table 7: Titters for production of phenol from tyrosine with the effect of adsorption

		NST 74		NST 74 $\Delta$ pheA	
		Titer (mg/L)	Yield (g/g)	Titer (mg/L)	Yield (g/g)
Culture	Aqueous Conc.	223.7	0.012	249.1	0.013
Culture	Aqueous Conc.	201.7		271.06	
+	Adsorbed Conc.	206.7		231.3	
Powder	Total Conc.	408.4	0.021	502.36	0.027

Titters are almost double with the powder, with the presence of equal stockpiles of tyrosine. Tyrosine is insoluble in water at 0.4 g/L which poses another problem for this pathway. The kinetics of the powder might not be quick enough to adsorb all the phenol produced when tyrosine goes into solution, hence limiting the flux through once the equilibrium limits are reached.

Having hypothesized that the pathway is equilibrium limited in the previous step, a resting cell assay was performed to establish this condition. In the resting cell assay, it was noticed that at equilibrium, the conversion of tyrosine to phenol was not complete at 72 hours. When 1.6 g/L tyrosine was added, more than 0.5 g/L tyrosine was left over in flask, and when 1.3 g/L of tyrosine was added, 0.143 g/L of tyrosine was left over, these 2 cases indicate an inherent equilibrium limitation in the system.

When powders are added, it results in pulling more flux through the pathway and results in higher phenol titers. There is a doubling of yield for both the strains with the highest yield at 0.026 (g/g) for the NST74  $\Delta$ pheA when powders were added in. This is characterized by an increased production of tyrosine as well, as we see no reduction in tyrosine titers when there is increased phenol production. The issue was therefore a fundamental flux issue that is prevalent. A resting cell assay was then done to troubleshoot this issue.

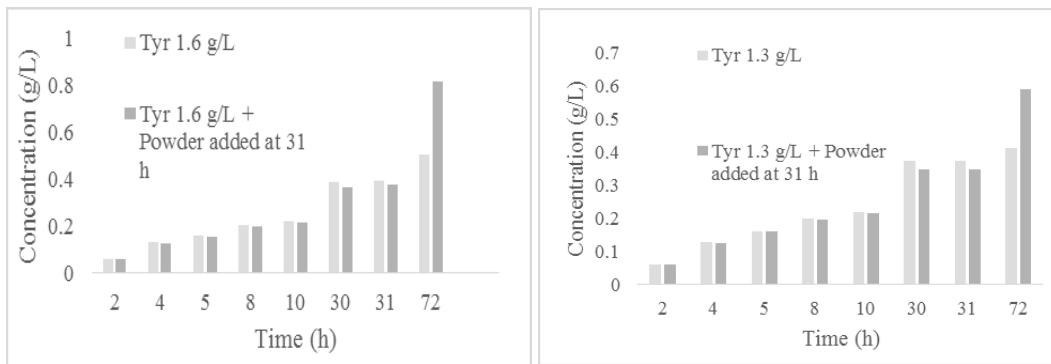


Fig. 10: Depicts the resting cell assay for phenol production from tyrosine, the darker bars indicate the powder added at 31 h.

BW25113 with tutA plasmid added in was grown for 36 hours in LB before suspending the “resting cells” in PBS. To PBS, tyrosine was added and so was IPTG, to make sure that cells make enough protein that help in conversion of tyrosine to phenol. No glucose/phenylalanine was fed/supplemented and only the action of this step tyrosine to phenol was isolated from the pathway. This study confirmed the hypothesis that it is possible to isolate and troubleshoot a problem in a pathway.

To overcome this limitation, powders were added at 31 hours when the equilibrium sets in to see any change in titers or behavior. Addition of powders did have an effect on the consumption of tyrosine, in the 1.6 g/L flask tyrosine consumption was increased and more phenol produced, 0.140 g/L was left over in flask, but this is because the powders could be at saturation or near loading capacity. In the 1.3 g/L flask, almost all the tyrosine was consumed, only 0.02 g/L was left over. Phenol titers increased 50% from 0.6026 g/L to 0.9 g/L in the 1.6 g/L tyrosine flask and in the 1.3 g/L tyrosine flask, phenol titers were at 0.64 g/L up ~50% from 0.41175 g/L. This makes a case for the use of the powder as an efficient analysis tool to troubleshoot issues such as equilibrium flux.

It is clear that the flask without powder added in was not able to convert all the tyrosine to phenol due to limitation in metabolic flux. The flask with the powder added at 31 hours, produced 4 times as much phenol as the flask without the powder. This is clearly a sign that a major bottleneck was removed by the powder. The only variables in this process were tyrosine consumption and phenol production, which are complementary. Therefore, clearly with the addition of powder the tyrosine was converted to phenol as more and more phenol was removed by the powder.

#### 4.4 Conclusions

Process Engineering is traditionally looked at as a field that is applicable to chemical manufacture of a product, not as a tool or method used in metabolic engineering. This thesis addressed the concerns with metabolic pathways: namely toxicity and equilibrium metabolic flux related issues by using solid phase adsorption, a process engineering tool, to look for solutions. Through a systematic breakdown of the pathways, each step of the pathway can be optimized to perform more efficiently and therefore provide higher total yields. Process engineering also offers the opportunity to identify certain problems that may go beyond the scope of metabolic engineering.

In the styrene oxide pathway, it was found that toxicity of styrene oxide can be overcome by the powders. With the addition of powders three issues were addressed:

- i. toxicity issue of styrene oxide, resulting in increased production of styrene oxide as cells can still be active after reaching toxicity thresholds.
- ii. increasing flux through the pathway by pulling more phenylalanine through this route
- iii. consumption of residual all glucose and phenylalanine in the flask.

Therefore, the potential is a three way increase in styrene oxide titers which is exciting for future work involving this pathway.

In the phenol pathway, addition of powders results in more phenol and more tyrosine production, which has the potential to achieve highest titers through that pathway, therefore making it an attractive candidate in case a strain that were to be developed for tyrosine overproduction.

Some of the future work could involve the optimization of production of certain high value products by predicting recovery rates or controlling titers by optimizing substrate use. Development of an in-house automated electromagnet in on the cards and is currently an active project, this will be implemented along with use in the bioreactor for use along with the powders.



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