METABOLIC ENGINEERING OF MICROORGANISM FOR THE PRODUCTION OF CARBOXYLIC ACID AND THEIR INHIBITORY EFFECTS.

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Abstract

To fulfill the requirement of futurre coming perspective for chemicals like carboxylic acid that are produced by microbial fermentation are an excellent biorenewable chemical. Due to their demand and usage as precursor for a number of industrial chemical, it can be produced through engineered microbes such as *Saccharomyces cerevisiae*, *E.coli* and *Bascilus subtilis*. Genetic engineering is now successfully and commonly used to change carbon to ethanol the end product of *Saccharomyces cerevisiae* to pyruvate. Genetic manipulation methods are useful for pathway design and for the production of several organic acids. Deletions of metabolic pathways that compete with the target compound are produces, can be a useful method for redirecting metabolic flux into the desired pathways. However carboxylic acids become increasingly inhibiting to the microorganisms. These inhibitors at their specific potency are commonly used as food preservative. The effects of carboxylic acids include cell membrane damage, change in microbial internal pH. Certain changes in cell membrane properties such as fluidity, composition, integrity and intracellular pH and hydrophobicity are associated with increase tolerance. These desirable products can use in the engineering of robust strains with improved industrial performance.

Keywords: fermentation, metabolic flux, membrane properties, metabolic engineering, precursors, inhibitors.

Introduction

Carboxylic acids are usefull compounds that play a role as a precursor for the industrial production of petroleum derived chemicals (Lennen *et al*., 2010; Shanks, 2010; Carlos Serrano-Ruiz *et al*., 2012) and biologically produced alcohols (Perez *et al*., 2013). and polymers (Wang *et al*., 2011). For the production of carboxylic acid, engineering progresses are used on *Escherichia coli* and *S.cervisiae*. (Ranganathan *et al*., 2012; Zhang *et al*., 2012a,b). Furthermore our renewed intrest in the compounds like carboxylic acids are now commonly used in preservation of food and in soaps (Russell, 1991; Ricke, 2003; Kabara and Marshall, 2010). The fact about the E.coli strain developed that they can produce 118 g/L lactic acid and 83 g/L succinic acid in the particular minimal media (Jarboe *et al*., 2010). That validates the organic acid tolerance may be increased by this organism. The mechanism of inhibition provide the guideline about the metabolic engineering technique.(Dunlop *et al*., 2011; Jarboe *et al*., 2011; Wang *et al*., 2013). That increase microbial strenghth and provide economically reasonable and industrially related process. Since we were basically interested in metabolic engineering of carboxylic acid production through the microorganism so we mainly focused on *E. coli* & *S. cerevisiae B.subtilis.* Carboxylic acids can be used as main chemicals to generate primary building blocks of industrial chemicals by both enzymatic and chemical catalysis. Currently, several carboxylic acids have been fermentatively produced (Table 1).

Selection-based strain improvement, often enabled by random mutagenesis, has been very successful for the making of carboxylic acids (Amiri *et al* 2006, Bai *et al* 2004). However, our ability to produce carboxylic acids and other fermentation products is often limited by complex cellular metabolism and regulations. Through new technologies like genomic sequencing and DNA recombination technology, we have the ability to overcome these limitations and improve microbial performance by fine-tuning enzymatic movement and controlling functions (Bailey 1991). Due to the recombinant DNA technology the classical strain improvement takes

place and that influence cellular activity, the metabolic pathway, enzymatic transport and the other regulatory functions of the cell.

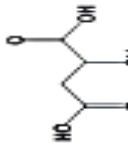
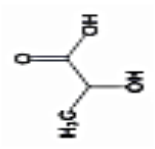
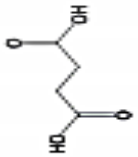
Saccharomyces cerevisiae widely used in industries like in food aplications and in making of primary metabolites that are the biomass products (Walker, 1998; Donalies *et al*., 2008). Metabolic engineering of S. cerevisiae produce pyruvate, dicarboxylic acids, monocarboxylic acids, malate lactate and succinate. While this paper central part is the production of these four compounds through metabolically engineered mirobes. (pyruvate, lactate, malate and succinate; Table 2).

1. Metabolic engineering of Saccharomyces cerevisiae

The challenging task in the metabolic engineering of *S. cerevisiae* is the making of these compounds that may involve the Two steps: (1) removal of alcoholic fermentation, (Verduyn *et al*., 1984; Van Dijken & Scheffers, 1986; Postma *et al*., 1989) (2) engineered high-capacity glycolytic pathways are linked with the fast and high qualitative metabolic pathways.

Table 1: Production of Carboxylic acid, malate, lactate, succinate by *E.coli* and *S.cervisiae* from glucose.

Table 1. Production of the carboxylic acids malate, lactate and succinate by *E. coli* and *S. cerevisiae* from glucose.

Carboxylic acid	Organism	Condition	Titer (g/L)	Yield (g/g)	Productivity g/L/h
 Malate	*S. cerevisiae*	Aerobic flask	59	0.31	0.19
	E. coli	Two-stage process	34	1.05	0.47
 Lactate	*S. cerevisiae*	Anaerobic, batch	70	n/a	0.93
	E. coli	Anaerobic, batch	118	0.98	2.88
 Succinate	*S. cerevisiae*	Shake flask	3.62	0.1	n/a
	E. coli	Anaerobic, batch	83	0.92	0.88

n/a – not available

Table 2: Production and aplication of organic acids that widely produced in industrial scale with Saccharomyces cerevisiae**.**

	Pyruvate	Lactate	Malate	Succinate
Properties/description	C_3 carboxylic acid with a ketone group	C_3 carboxylic acid with a hydroxyl group	C_4 dicarboxylic acid with a hydroxyl group	C_4 symmetrical dicarboxylic acid
Current applications	Pharmaceuticals, polymers, cosmetics, food additives, agrochemicals	Polylactic acid, food preservative	Acidulant and flavor-enhancer in food and beverages	Acidulant, surfactant, ion chelator, antibiotics, pharmaceuticals
Possible applications	Flavoring agent	Polyesters, acrylates	Maleic anhydride, biodegradable polymers	Maleic anhydride, bionelle (biodegradable polyester), butanediol, biodegradable polymers
Key organisms	*Torulopsis glabrata*, *E. coli*	Lactic acid bacteria	*Aspergillus flavus*	Ruminant bacteria (*Actinobacillus succinogenes*, *Mannheimia succiniciproducens*), *E. coli*
Current production method	Chemically from tartaric acid or microbial conversion with *Torulopsis*	Bacterial conversion of sugars	Chemical conversion from petrochemically derived maleic anhydride	Chemical conversion from petrochemically derived maleic anhydride

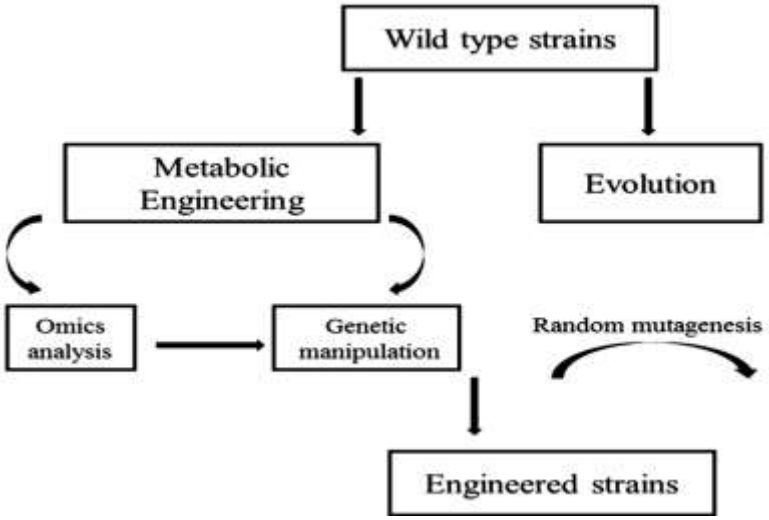


Figure 1. Strain development methods in carboxylic acid production.

1.1. Production of pyruvate exclusion of alcoholic fermentation.

During the production of organic acid, ethanol is produced as byproduct and this byproduct can decrease the product yield. To avoid the decrease in production in the ethanol coproduction, any enginnering strategy could be used for the high yield of organic acids production with *S. cerevisiae* but firstly ethanol formation should be removed. Between pyruvate and ethanol pathways there are two reactions that can be targeted to remove the ethanol formation. These two reactions are pyruvate decarboxylase and alcohol dehydrogenase. To remove the alcoholic formation in *S. cerevisiae* target will be the alcohol dehydrogenase enzyme. The deletion of ADH-1 gene did'nt help in the complete removal of ethanol, and in return the accumulation of large amount of glycerol and toxic acetadehydes may takes place (Drewke *et al*., 1990). Furthermore (Skory, 2003) describe that the ADH1 gene coding the main alcohol dehydrogenase enzyme in *S.cervisiae* strain and overexpressed the lactate dehydrogenase (LDH).

By removing pyruvate decarboxylase activity ethanol formation of alcohol dehydrogenase can be blocked in just a single step. *S.cerevisiae* contains genes so to remove the alcoholic fermentation three basic genes PDC 1,5 6 has been deleted that encode the functional pyruvate

decarboxylase isoenzymes (Hohmann, 1991). Mutant starain pdc1,5,6 deletion fails to grow on glucose in sybthetic medium and these are sensitive in high glucose concentration (Flikweert *et al*., 1996, 1997). Based on that specific point, the organism are not capable to use glucose as a sole carbon source shows that biosynthetic role of pyruvate decarboxylase enzyme in the formation of cytosolic acetyl-CoA (Flikweert *et al*., 1996). That is essential for lysine and lipid synthesis. The combination of C2 compounds and high glucose sensitivity is the main representative for the production of organic acids by the use of pdc starins (Flikweert *et al*., 1999; van Maris *et al*., 2003). The high titer pyruvate yield produced by these pdc strain of *S.cervisiae* and their great ability to produce organic acids..

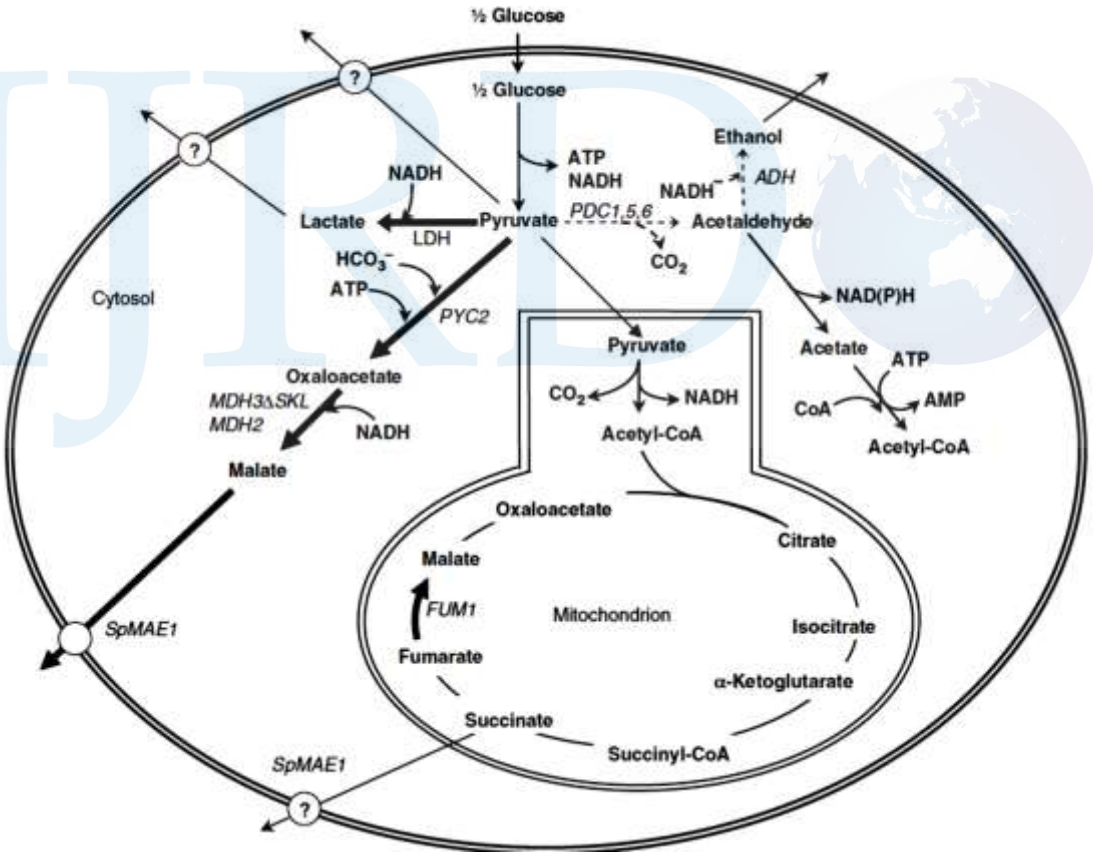


Figure 2: flow chart of various genetic changes used to improve the production of organic acids in S.cervisiae. Heterologusley expresed or overexpresed netive genes are denoted by bolld arows and limits or removal of genes expresion is denoted by deshed arows.

2. Metabolic engineering by genetic manipulation

2.1. Improvement of product formation by over expression of key Pathway enzymes

Increasing the work of enzymes in required metabolic pathway, as well as deletion of competing pathways, is often necessary to improve target production. There are huge exemples of these stretegies that are able to produce carboxylic acids. In this section, we review overexpression of both native and heterologous enzymes contributing to improved succinate production by E. coli and malate production by S. cerevisiae; Figure 4 shows a simplified overview of central carbon metabolism in E.colii in aerobic conditions the formation of succinate by E. coli is basically from the carboxylation of phosphoenolpyruvate (PEP) into oxaloacetate (OAA). This pathway is encoded by two enzymes: PEP carboxylase (PEPC, encoded by ppc) and PEP carboxykinase (PEPCK, encoded by pck).

Overexpression of ppc has been reported to significantly increase succinate production from the glocoze. However, no effect was found by overexpression of the native PEPCK in E. coli (Millard *et al*., 1996). Furthermore, overworking or expression of PEPCK from Actinobacillus succinogenes, the basic CO2-fixing enzyme in the A. succinogenes is succinate production pathway, in E. coli ppc-deficient mutant strains that increased the production of succinate by as much as 6.5-fold (Kim *et al* 2004). PEP may also be converted into pyruvate either by the phosphotransferase system (PTS) or by pyruvate kinase. In other organisms, pyruvate can be converted into OAA by pyruvate carboxylase (PYC) (Peters *et al*., 1997), that is not present in E.colii. Therefore, another way to produce more OAA is by the heterologous expressing of pyruvate carboxylase. The genes from Rhizobium etli pyc was expressed in E. coli, leading to an increase in succinate production from 1.18gL-1 to 1.77g L-1 (Gokarn and Altman, 1998). In succinate production by E. coli, NADH availability was reported to be a limiting factor. The

pathway to covert OAA to succinic acid needs 2 moles of NADH for one succinate production. However, through glycolytic pathway one moles of glucose provide 2 moles of NADH.

So the maximum theoretical yield of succinic acid is one mole per glucose consumed (Senchez *et al.,* 2005). The improved Yeild of succinate can be accomplished due to the availability of high NADH. Berríos-Rivera et al heterologously expressed NADH- forming compound like formate dehydrogenase by *Candida bondii* in *E.coli*. to generate 4 moles NADH per glucose consumed. Futhermore, this strategy was improved to produce more than 4 moles of NADH per glucose by combination with a more reduced carbon source (Berrios *et al*., 2004). Additionally, a novel pathway with decreased ratio of NADH/ succinic acid is now known to enhance succinc acid yield and and their production in *E.coli.* These (adhe ack-pta idha) genes involved in main CO2 dependent pathway and one gene (iclR) involved in regulation of the glyoxylate pathway under aerobic conditions were deleted to eliminate competing NADH pathways and redirect the ratio of carbon through the glyoxylate and fermentation pathway. Wild-type S. cerevisiae can naturally produce low levels of Lmalate as this compound is part of the central cyclic pathway like TCA cycle.

Although four pathways are known in S.cervisiea for malate formation most promising route for malate production from glucose is from the end product of glycolysis pyruvate leads to the reduction of OAA to malate that results maximal production of 2 mol of male and mol per glucose. Cytosolic iso-enzymes Mdh2p overexpressed and increased 12 g per liter malate production (Pines *et al.,* 1997). But Mdh2p is subject to repression by glucose, both at the enzyme and transcript level (Minard and McAlisterhenn. 1992). The stretagy increase the malic acid concentration more than 3-fold in shake flask experiments. However, overexpressing pyruvate carboxylase (PYC2) did not significantly improve malate production. Malate transport is also an important strategy to improve malate production. S. cerevisiae does'nt have transporters for malic acid and the diffusion across the plasma membrane is slow

(Volschenk *et al.*, 2003). Thus, there has been interest in the use of heterologous transporters. SpMAE1 the malate transpoters were first reported to mediate the import of malate in S. cerevisiae later studies showed that expression of SpMAE1 also helps and capable in incressing the malate production (Camarasa *et* al., 2001).

2.2. Improvement of product formation by inactivation of competing pathway

Deletion of metabolic pathways that compete with the target compound that are produced can be a useful method for redirecting metabolic flux into the desired pathway. Anaerobic production of succinate by E. coli is normally associated with co yield of acetic acid formic acid lactic acid and the ethanoll. Preventing the formation of these byproducts would improve succinate production by both increasing product purity and hopefully increasing product yield and concentration, though this is challenging given the constraints of maintaining redox balance and the need for a net generation of ATP. Deletion of lactate dehydrogenase (ldh) eliminates the pathway that converts pyruvate to lactate (Matjan *et al.*, 1989 48).

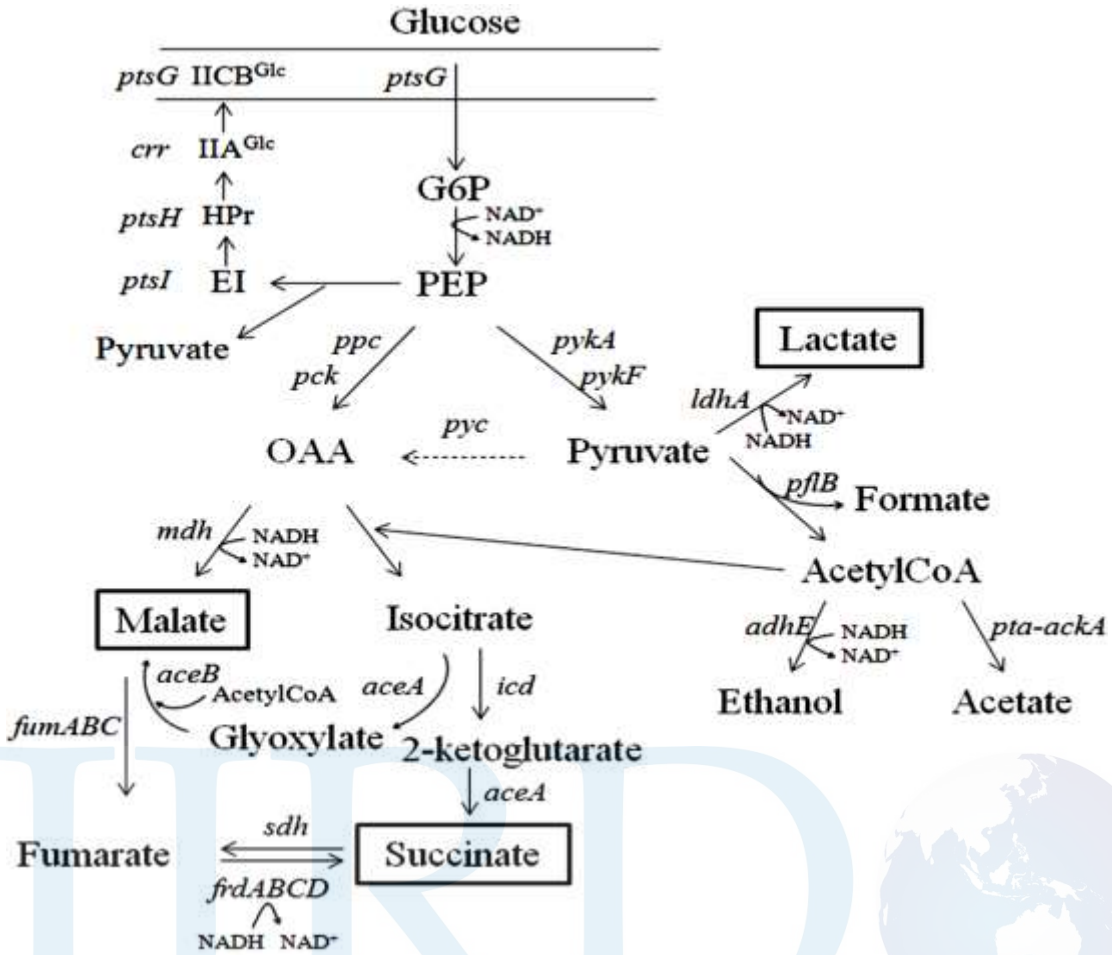


Figure 4: *E.coli* metabolic pathway for the production of lactic acid malic acid and succinc acid. For simplicity, cofactor usage is not shown. Heterologous genes expression is indicated by a dashed line. Genes: crr, glucose-specific phosphotansferase enzyme IIA component; fumABC, fumarase isoenzymes; frdABCD, fumarate reductase; ppc, phosphoenolpyruvate carbolxylase (PEPC); (PYC); pykA and pykF, pyruvate kinases; ptotein phosohotransferase; sdh, succinate dehydrogenase. Formation of the other three byproducts (ethanol, formate and acetate) is dependent on pflB which change pyruvate into actyle Co-A and formic acid. Although simultaneous inactivation of pflB and ldhA resulted in the intended decrease in production of lactate, acetate and ethanol, unfortunately this double mutant strain was unable for glucose fermentation. However mutation in this ΔpflB ΔldhA strain helps them to ferment glucose and produce all the products like ethanol succinate and acetate in 1:2:1 ratios. which was an improvement relative to the wild-type ratio of 1:2:2 (Donelly *et al.*, 1998).

Furthermore, the causative mutation restoring glucose fermentation to gene mapping of ptsG genes encoding a membranebound, permease enzyme in PTS system that specifically inactivate the ptsG genes in the double mutant strain that helps to improve the ability to ferment glucose and enhance the production ofsuccinate.

Redox balance is also a great factor in pathway enginerring. The double mutant (ΔpflB ΔldhA) resulted in a NADH/NAD+ 2:1 imbalance, which can limit growth. Singh et al identified a series of genes related to NADH oxidation: grxB, hyfF, yhcA, argA, pfkB, marA, moaE, ygfT, and nuoC. Triple deletion mutants for three pyruvateforming enzymes (ptsG, pykF and pykA) produced 2.05 g L-1 succinic acid, a more than sevenfold increase over the wild type (0.29 g L-1) (Lee *et al*., 2005). Under aerobic conditions, the most effective way to produce succinic acid is through the glyoxylate cycle, in which iso-citrate is converted into succinic acid and glyoxylate through (aceA). Disruption of succinate dehydrogenase (sdh), isocitrate dehydrogenase (icd), glyoxylate operon aceBAK repressor and acetic acid pathway redirect the flux of carbon through glyoxylate bypass, resulting in production of 5.08 g L-1 (43mM) succinate in an aerobic batch fermentation (Linn *et al*., 2005).

The same strategy was applied in yeast: genes encoding succinate (SDH1, SDH2) dehydrogenase and isocitrate dehydrogenase (IDH1, IDP1) were deleted from S. cerevisiae, increasing succinate production. (Raab *et al*., 2010). Ethanol is often produced as an undesirable byproduct during carboxylic acid production by yeast. There are two enzymes associated with ethanol production: PDC and ADH . the first attempt to eliminate ethanoll formation was conducted in a lactate-producing strain. The ADH1 gene encoding ADH, which converts acetaldehyde into ethanol, was deleted. However, the decreased ethanol titer in the adh1- deletion strain did not result in increased accumulation of lactate (Skory *et al*., 2003). While deletion of all three PDC genes (PDC1, 5 and 6) encoding PDC isozymes completely eliminated ethanol formation and increased the accumulation of pyruvate, the mutant strains

showed defects in growth that produce on glucose as the only C source. This weakness was addressed by directed evolution of a PDC knock-out strain(Van *et al*., 2004).

3. Inhibitory Effects of Carboxylic Acid

3.1. Inhibition and the pH

By comparing the carboxylic acid with other compounds like sorbic acid and 2,4-dinitrophenol revealed that the carboxylic acid cause immediate cell death than other inhibitors and the inhibition effect of carboxylic acid is different from others. Our research is on the increased toxicity to *S.cervisiea* that the molar base chain size is increased from 6-8 carbons. (Liu *et al*., 2013b) but this chain dependent toxicity not seen in E.coli (Royce *et al*., 2013). At lower pH and specifically at the molecular pKa value of the media pH may increase the toxicity of the carboxylic acids (Stratford and Anslow, 1996; Liu *et al*., 2013b; Royce *et al*., 2013). Carboxylic acid toxic power is associated with hydrophobic nature and their toxic nature (Zaldivar and Ingram, 1999). These aspects of toxicity help them to transport into the cell.

3.2. Movement from the cell

Carboxylic acid move in out from the cell through diffusion process and through transporter proteins (Nikaido, 2003). Limiting the steps of permeation of carboxylic acid from the membrane is due to their chain length function (Evtodienko *et al*., 1996). 2-6 carbon chain length limit transport through diffusion and the pH is below their pKa value. But the longer chain carboxylic acid limit their movement in al pH values. Abundance of anions within the cell play an important role in inhibition by carboxylic acids (Carpenter and Broadbent, 2009). Specific membrane transporters helps in the movement of carboxylic acid like pdr12ABC transporters are found in the study of other inhibitors like sorbic acid etc (Piper *et al*., 1998).

3.3. Damage to the membrane

Cell membrane is permeable towards carboxylic acid so this is the indication that these compounds are soluble and this is their important characteristic. Microorganisms are inhibited due to the presence of carboxylic acid that permeates and cause damage to the cell membrane (Ricke, 2003; Desbois and Smith, 2010). The omics study of microbes like *E.coli* revealed that the membrane damage is the major stress that organisms have faced. Membrane damage is accessed by the greater penetrability of the inner membrane (Lennen *et al*., 2011). Through N.A dye and cell viability is also decreased that can be tested by the colony forming units that are linked with the non-producing strain in the same conditions. Mg+2 used as a descriptive small molecule that engaged within the cell but from damged cell it leaks out and predicts the damged membrane of the cell (Liu *et al*., 2013b). When *E.coli* is treated with octanic acid at minimum media pH 7 and at 37◦C resulting in increasing the fluidity and decreasing the membrane efflux (Royce *et al*., 2013).

3.4. Changes in Membrane characteristics that increase the tolerance

The change in the cell membrane due to carboxylic acid increases the tolerance of the membrane against inhibitors. So metabolic pathways through metabolic engineering gives a better product with increased tolerance (Ramos *et al*., 2002). It's been found that the mutant strain with decreased hydrophobicity have the increasing amount of tolarnce towards organic solvents. While understanding the mechanism of that changes the role of enginnering efforts helps a lot. When hydrophobicity decreases the contents of cell membrane changes into lipopolysaccharides and LPS amino acids (Aono and Kobayashi, 1997). Lennen and Pfleger (2013) hypothesized that the decreasing concentration of hydrophobicity and saturated fatty acids is due to the toxicity of carboxylic acid and it is not the mechanism of microorganism for

increasing the tolerance. While engineering technology helps to overcome from the carboxylic acid toxicity and by increasing the amount of saturated fatty acids (Lennen and Pfleger, 2013).

4. Conclusion

Past researches describe that the microorganism like S.cervisiea have the ability to produce organic acid at very low pH. *S.cervisiea* is actually considered as safe because they are governmentally approved, and because of their simple structure they are largely used in industrial scale fermentation and their genetic manuplation is easy so they are commoly used for the production of organic acids like carboxylic acid. Metabolic engineering of microorganisms is effective system that provide a high products and product tolerance. Metabolic engineering helps to play a major role in improvement of the yeast physiology. Like transcription enginnering techniques helps to produce increasing amount of ethanol production and tolerance in *S.cervisiea* and *E.coli.* Metabolic engineering in the form of overexpression of key pathway genes, as well as deletion of competing pathways, has proved quite effective for improving carboxylic acid production. Improved tolerance to carboxylic acids is a key aspect of this area that needs further attention to enable production of these chemicals at higher titer. Carboxylic acids that are produced by the microorganism also have some inhibitory effects against these microorganisms. Like carboxylic acid cause membrane damage and change their permeability and change in permeation of cell membrane increase their tolerance. that increasing tolerance and decreased amount of saturated fatty acids can be controlled through metabolically engineering technology that overcome the toxicity effect of carboxylic acid and also enhance the production of the main product.

Acknowledgement

This work done with the help of NSF “National science foundation engineering research center” Award number EEC-0813570.

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