Synthetic and Systems Biology Approach towards Designing Metabolic Bypass and Identifying Novel Enzymes for Cholesterol Lowering Drug Precursor (BTO) Biosynthesis from Crude Glycerol

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INTRODUCTION

1, 2, 4-Butanetriol (BTO) is a potential precursor of Cholesterol Lowering Drug (CLD) i.e. Crestor and Zetia. Effective target for CLDs is HMG-CoA reductase enzyme to block cholesterol biosynthesis in liver. Biomolecular mode of action of CLDs has already been established (Fig 1). BTO is not only used as a precursor of CLDs but also in synthesis of energetic plasticizer material (D, L-butanetriol trinitrate as a replacement for nitroglycerin), cationic lipids (towards drug delivery), polyurethane foams as natural fiber (having improved elastic properties and similar compression-bending characteristics), 3-hydroxytetrahydrofuran or dehydrated form of BTO (key component for the HIV drug Amprenavir) as well (Ren and Liu, 1999; Lau, 2007; Mandava et al., 2012; Yamaguchi et al., 2008; Gouranlou and Kohsary, 2010). In chemical point of view, BTO is an alcohol having three hydrophilic alcoholic hydroxyl groups and chiral property, with two possible enantiomers. BTO has been mass-produced following few traditional chemical routes utilizing glycidol; 2-buten-1, 4-diol; 3, 4-dihydroxybutanoate or Malate; D-glucose as the initial chemical substrates (Adkins and Billica, 1948; Campbell, 1983; Ikai et al., 2005; Kwak et al., 2005; Molefe, 2005; Monteith et al., 1998; Ritter, 2004). Stoichiometric reduction of esterified malic acid (diethyl D, L-malate) using Sodium borohydride (NaBH₄) is a widely used industrial method for BTO production (Ikai et al., 1999).

However, this process requires NaBH₄ as the reducing agent. For each ton of BTO to be synthesized, multiple tons of borate salts are generated as the by-products, thus resulting in high production costs and severe environmental pollution (Frost and Niu, 2011).
Catalytic hydrogenation of malate over rubidium and carbon is another alternate; though hydrogenation reactions have some physiological constraints like 2900-5000 psi pressure, elevated temperature that results into undesirable toxic by-products (Niu et al., 2003). Moreover, all of these traditional chemical procedures of BTO synthesis suffer from several bottlenecks i.e. extreme reaction conditions, multiple steps, and poor selectivity (Antons et al., 2002). These critical problems associated with traditional industrial synthesis actually hinder further applications of petrochemical-based BTO generation.

Therefore, Biological route for BTO biosynthesis can be a green alternative. There are several promising microbial catalysts in nature that synthesize optically active BTO like S-isomer producers (Bacillus subtilis, Candida succiphila, Comamonas acidovorans, Cryptococcus curiosus, Cryptococcus humicolus, Cryptococcus neoformans, Dekkera custersianus, Eremascus fertilis, Hansenula glucozyma, Hansenula polymorphha, Schizoblastosporon kobayasi, Stephanouscus ciferrii, Sterigmatomyces elviae, Sterigmatomyces polyborus) and R-isomer producers (Geotrichum candidum, Geotrichum fermentans, Sterigmatomyces halophiles, Trichosporon cutaneum, Yarrowia lipolytica) (Yamada-Onodera et al., 2007). The major starting materials for BTO biosynthesis are ligocellulosic biomass, C5 sugars (D-xylose, L-arabinose), and amino acid (malate, a microbial tricarboxylic acid cycle intermediate). The most well established biological de novo or chimerical metabolic pathways are reconstituted and functionalized in Escherichia coli, Arabidopsis thaliana (Fig 2).

The first microbial biosynthesis approach has shown that 6.2 g/L of D-BTO produces from 20 g/L D-xylose which is 30% molar yield; 8.3 g/L of D-BTO from 28 g of D-xylonic acid or 45% molar yield and L-arabinonic acid (10 g/L) into L-BTO (2.4 g/L) resulting into 35% yield (Niu et al., 2003; Frost and Niu, 2008). Afterwards several microbial engineering techniques have been applied but that did not overcome existing production yield (Valdehuesa et al., 2014; Cao et al., 2015; Zhang et al., 2016). Various chaperone proteins (DnaK–DnaJ–GrpE; GroES–GroEL; Trigger factor) have also been co-expressed in pre-established BTO biosynthesizing E.coli chassis, but there is no improvement obtained (Lu et al., 2016). Moreover, these well established synthetic metabolic pathways were implemented in higher plant Arabidopsis thaliana resulting into low production with 20 mg of BTO per gram of soil-grown plants (Abdel-Ghany et al., 2013). A synthetic bacterial metabolic network has also been reconstituted for improved synthesis of BTO from malate, a Tricarboxylic acid cycle (TCA) cycle metabolic intermediate (In this case, the production was negligible i.e. 180 ng/L) (Li et al., 2014).

In this current scenario, an alternative feedstock “Glycerol” may be tried out towards improving BTO productivity and molar yield. Thus far a very vital question arises “why should we think for an alternative potential feedstock glycerol rather than Xylose or Arabinose?” The first and foremost reason is that different microbial regimes can uptake glycerol as carbon and energy source; mediated by the glycerol diffusion facilitator, an integral membrane protein catalyzing the rapid equilibration of concentration gradients of glycerol across the cytoplasmic membrane under specified physiological growth conditions towards high value added biomolecules generations (Beijer et al., 1993). These potential glycerol utilizing microbes are Klebsiella pneumoniae, Clostridium butyricum, Lactobacillus leuteri, Pseudomonas sp., Cupriavidus sp, Escherichia coli, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Schizosaccharomyces pombe,
Rhodopseudomonas palustris, Chlorella vulgaris, Synechocystis sp. PCC 6803, Synechococcus sp. PCC 7002, Spirulina platensis, Chlamydomonas reinhardtii and Nannochloropsis sp. (Casella et al., 2016; Dharmadi et al., 2006; Ghosh et al., 2012a; Ghosh et al., 2012b; Kong et al., 2013; Lambert and Stevens, 1986; May et al., 1982; Murarka et al., 2008; Narayan et al., 2005; Swinnen et al., 2013; Taghavi et al., 2016). These glycerol utilizing microbes could be the potential hosts for chassis strain development and for functionalization of novel hypothetical biosynthetic pathways towards improving BTO productions. Biodiesel manufacturing generates large quantity of crude glycerol (10 kg/100 L) as a major by-product, which has great impact on environmental pollution concerning disposal issues. First generation biofuel i.e. biodiesel production increases each year with a world total production of about 15 billion liters in 2009. A projection has shown that world biodiesel market possibly would reach 37 billion gallons by 2016, which implies generation of 4 billion gallons of crude glycerol as predominant side product (Kerr et al., 2007). However, crude glycerol price decreases from 0.25 dollar to 0.05 dollar per pound (Thompson and He, 2006; Johnson and Taconi, 2007; Yang et al., 2012). Hence, this current global scenario makes crude glycerol as a potentially relevant and cheap green non-edible feedstock for developing sustainable bioprocess towards high value added biomolecular production.

The major objective of this current study is to design or predict novel hypothetical biosynthetic bypass pathway utilizing glycerol as major feedstock. Moreover, study will be extended towards identification of class of novel enzymes.

Fig 2: BTO biosynthesis from D-xylose, L-arabinose and malate as feedstock (A) in Escherichia coli and (B) in Arabidopsis thaliana.
MATERIALS AND METHODS

Metabolic pathway prediction tools and databases

In this current study, biosynthetic metabolic networks have been designed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map, From Metabolite to Metabolite (FMM) & Metabolic Route Explorer (MRE) pathway finders and eEquilibrator system biology tools. KEGG pathway map represents a high level function of networks of molecular interactions, enzymatic reactions and relations in terms of graphical format. KEGG pathway map tool helps to draw reference pathway maps together with organism specific pathway maps that are computationally generated by matching KEGG Orthology (KO) assignments in the genome with reference pathways.

KOs refers to sequence similarity groups as well as functional orthologs. Three major segments i.e. metabolic pathways, gene clusters, and phylogeny have always been considered while defining KOs (Kanehisa et al., 2017). FMM reconstructs metabolic pathways form one metabolite to another metabolite among diverse groups of species depending on the KEGG database and other integrated biological databases (UniprotKB, Swissprot, dbPTM etc) (Chou et al., 2009). MRE is another system biology tool used in this study to suggest foreign enzymes for the biosynthesis pathway design with competing endogenous reactions.

It is an effective tool to guide the design and optimization of heterologous biosynthesis pathways providing pathway ranking scores and competing naive reactions. MRE workflow functions considering KEGG databases for biochemical transformations and Boltzmann factor for thermodynamic considerations (Kuwahara et al., 2016) within the web based interface.

Thermodynamic constraint prediction tool

eQuilibrator is a biochemical thermodynamics calculator for individual enzymatic steps involved within the large metabolic networks. eQuilibrator is effective online system biology search tool which connects a comprehensive and accurate database of thermodynamic properties of biochemical compounds and enzymatic reactions. It empowers easy Gibb’s energies (in Kcal/mol units) calculation of compounds and reactions considering arbitrary pH, ionic strength and metabolite concentrations in an online interactive interface (Flamholz et al., 2012).

RESULTS AND DISCUSSION

All in-silico results have been generated and designed based on a simple workflow (Fig 3). The workflow consists of query input, auto execution (using different system biology web tools), and probable outputs considering thermodynamic constraints and enzymatic reaction network logistics.
shows that both reactions (reaction 1 & 2) are highly energetically unfavorable redox reactions having Gibb’s free energy change values 35.4 kcal/mol, 30.8 kcal/mol, respectively with undefined enzyme classes (D-fucanate node). Hence, some empty space still remains towards discovery of novel enzymes in Methylglyoxal, Lactaldehyde and D-fucanate nodes.

**Fig 4:** Predicted and designed putative synthetic metabolic network concerning glycerol entry nodes for BTO biosynthesis including thermodynamic constraints and enzyme classes (“ΔG” free energy change value represented in Kcal/mol unit).

**Pitfalls of D-Xylolate downstream node**

Finally, D-Xylolate is converted into BTO following pre-existing biocatalytic reactions in downstream metabolic networks. In downstream pre-existing metabolic networks also suffer from different bottlenecks at present. The first and foremost issues are intermediate aldehyde toxicity due to untunable 2-keto acid decarboxylase (MdIC), and thio kinase (PgSucD) enzyme activities, imbalance in cellular redox, unfavorable physiological conditions (unavailability to redox equivalents i.e. NADH or NADPH and ATP concentration) (Sun et al., 2016), non-natural enzymatic reactions (Van Dien, 2013; Yim et al., 2011), and flux diversion due to competing pathways (Zhang et al., 2016).

**Solution space for BTO production**

To this end, there are plenty solution spaces still remain for further studies with careful outlook specially (a) Finding novel efficient enzymes for better catalyst and toxic intermediate clearing off, (b) Protein engineering of existing enzyme to make non-natural reactions thermodynamically-kinetically favorable, (c) Knocking out competing pathways to maximize flux towards BTO biosynthesis, (d) Implementation of micro aerobic growth condition to improve redox pool i.e. NADH, excess ATP generations for cell growth, maintenance and product transport and (e) Identification and protein engineering of specific alcohol dehydrogenases (AdhP, FacO, EatG, AdhE, YjgB, and YiaY) for prevalence of NADH over NADPH.

**CONCLUSION**

In general, biosynthetic pathway prediction and designing through systems biology tools are really very useful (Ghosh, 2016). It reduces time for experimentation (i.e. trial and error conventional approach for biosynthetic pathway design & novel enzyme finding), expenses of laboratory, and manual efforts. In this study, predicted and designed synthetic metabolic pathways for D-xylolate from glycerol feedstock seems to be promising towards BTO biosynthesis, though this entire metabolic pathway needs to be validated through in vivo experimentation in suitable chassis microorganisms. These model microorganisms could be heterotrophic (Escherichia coli), and/or mixotrophic cyanobacteria (Synechococcus sp. PCC 7002, Synechococcus elongatus PCC 7942 and Synechococcus sp. PCC 6803) and green algae (Chlamydomonas reinhardtii) which can utilize crude glycerol as a carbon feedstock (towards biodiesel waste bioremediation) and sunlight as energy source (by capturing photons). Furthermore, current systems biology pathway prediction and design tools could be improved by plugging in enzyme regulations, enzyme promiscuity phenomena in near future.

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**ABBREVIATION**

HMG-CoA: Hydroxymethylglutaryl-coenzyme A; IPP: Isopentenyl pyrophosphate; GPP: Geranyl pyrophosphate; FPP: Farnesyl pyrophosphate. PPS: Pentose phosphate shunt; NADPH: Nicotinamide adenine dinucleotide phosphate Reduced; NADH: Nicotinamide adenine dinucleotide Reduced; NADP+: Nicotinamide adenine dinucleotide phosphate oxidised; NAD+: Nicotinamide adenine dinucleotide Oxidised; EcYjxA: *Escherichia coli* Xylose isomerase; CcXdh: *Caulobacter crescentus* Xylose dehydrogenase; CcXylB: *Caulobacter crescentus* Xylonolactonase; PtXdh: *Pseudomonas fragi* Xylo dehydrogenase; CcXylC: *Caulobacter crescentus* Xylonolactonase; EcYjhG: *Escherichia coli* D-Xylolate dehydrogenase; EcYagF: *Escherichia coli* D-xylonic acid dehydrogenase; EcYjhH: *Escherichia coli* 2-Keto-3-deoxy-d-xylonate Aldolase; EcYagE: *Escherichia coli* 2-Keto-3-deoxy-d-xylonate Aldolase; PpmdIC: *Pseudomonas putida* Benzoylformate
decarboxylase; EcYiaE: *Escherichia coli* 2-Keto acid dehydrogenase; EcYcdW: *Escherichia coli* 2-Keto acid dehydrogenase; EcAdhP: *Escherichia coli* Aldehyde reductase; EcYqhD: *Escherichia coli* Alcohol dehydrogenase; ScAdh2: *Saccharomyces cerevisiae* Alcohol dehydrogenase 2; MtkAB: *Mesorhizobium loti* Malate thiolase; PgSucD: *Porphyromonas gingivalis* Succinate-semialdehyde dehydrogenase; Pg4Hbd: *Porphyromonas gingivalis* 4-Hydroxybutyrate dehydrogenase; PgAbfT-2: *Porphyromonas gingivalis* 4-Hydroxybutyrate CoA-transferase; EsFldA: *Eggerthella* sp. Cinnamoyl-CoA: phenyl lactate CoA-transferase; CaAdh: *Closstridium beijerinckii* Coenzyme A acylating Aldehyde dehydrogenase; EcMsdh: *Escherichia coli* Methylmalonate-semialdehyde dehydrogenase; EcEutG: *Escherichia coli* Alcohol dehydrogenase; CaAdhE2: *Closstridium acetobutylicum* Bifunctional aldehyde/alcohol dehydrogenase; XDG: D-xylene dehydrogenase; XDT: D-xylulose dehydratase; BFD: Benzyloformate decarboxylase; ADG: L-arabinose dehydrogenase; ADT: L-arabinonate dehydratase; AtDHs: *Arabipsosis thaliana* Dehydrogenases; AtALDHs: *Arabipsosis thaliana* aldehyde dehydrogenases).

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