

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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ABSTRACT

1, 2, 4-Butanetriol (BTO) is a potential precursor of Cholesterol Lowering Drugs. BTO production is predominantly dependent on chemical conversions at present. Biological route for BTO biosynthesis suffers due to multiple issues like unavailability of efficient biosynthetic pathways, inefficient enzymes, usage of expensive edible feedstock and unavailability of suitable chassis host strains. These issues practically restrict its large scale bio-industrial productions considering lower molar yield, titer and productivity of BTO via biosynthetic route. The main aim of this study is to find out efficient novel biosynthetic pathways which can convert non-edible feedstock (crude glycerol from biodiesel waste) towards BTO biosynthesis. The second aim of this study is to identify putative non-natural enzyme classes predicted to be involved in these biosynthetic pathways. Future perspective will be to functionalize these pathways towards *In-vivo* validation within suitable metabolically engineered microorganisms for improving BTO biosynthesis.

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-butene-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 (NaBH_4) is a widely used industrial method for BTO production (Ikai *et al.*, 1999).

However, this process requires NaBH_4 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).

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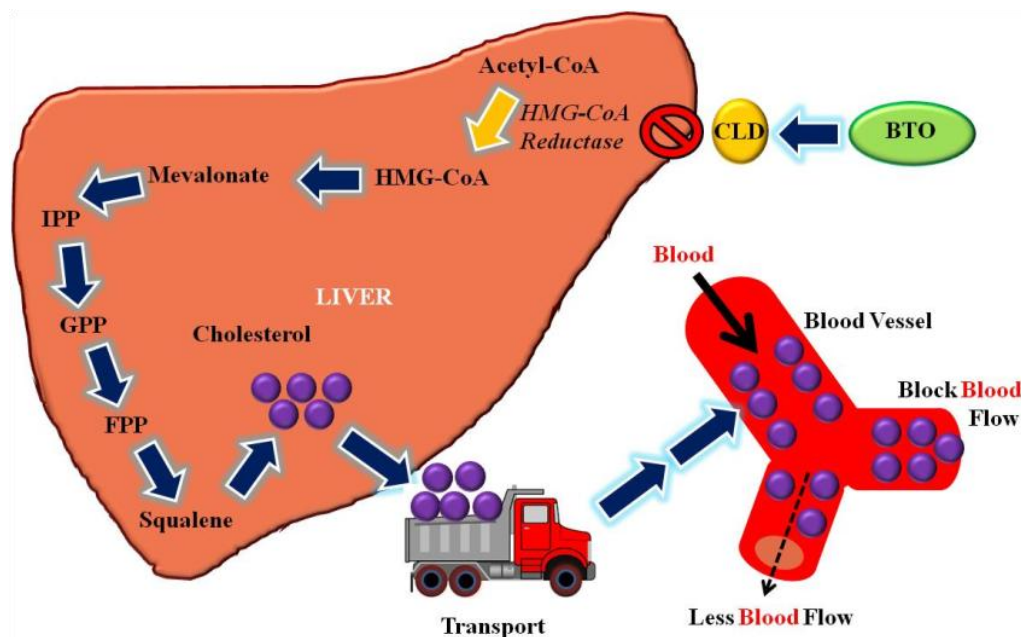


Fig 1: Biological mode of action of BTO as a cholesterol lowering drug.

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 polymorpha*, *Schizoblastosporion kobayashii*, *Stephanoascus ciferrii*, *Sterigmatomyces elviae*, *Sterigmatomyces polyborus*) and R-isomer producers (*Geotricum candidum*, *Geotricum 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*,

Rhodospseudomonas 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.

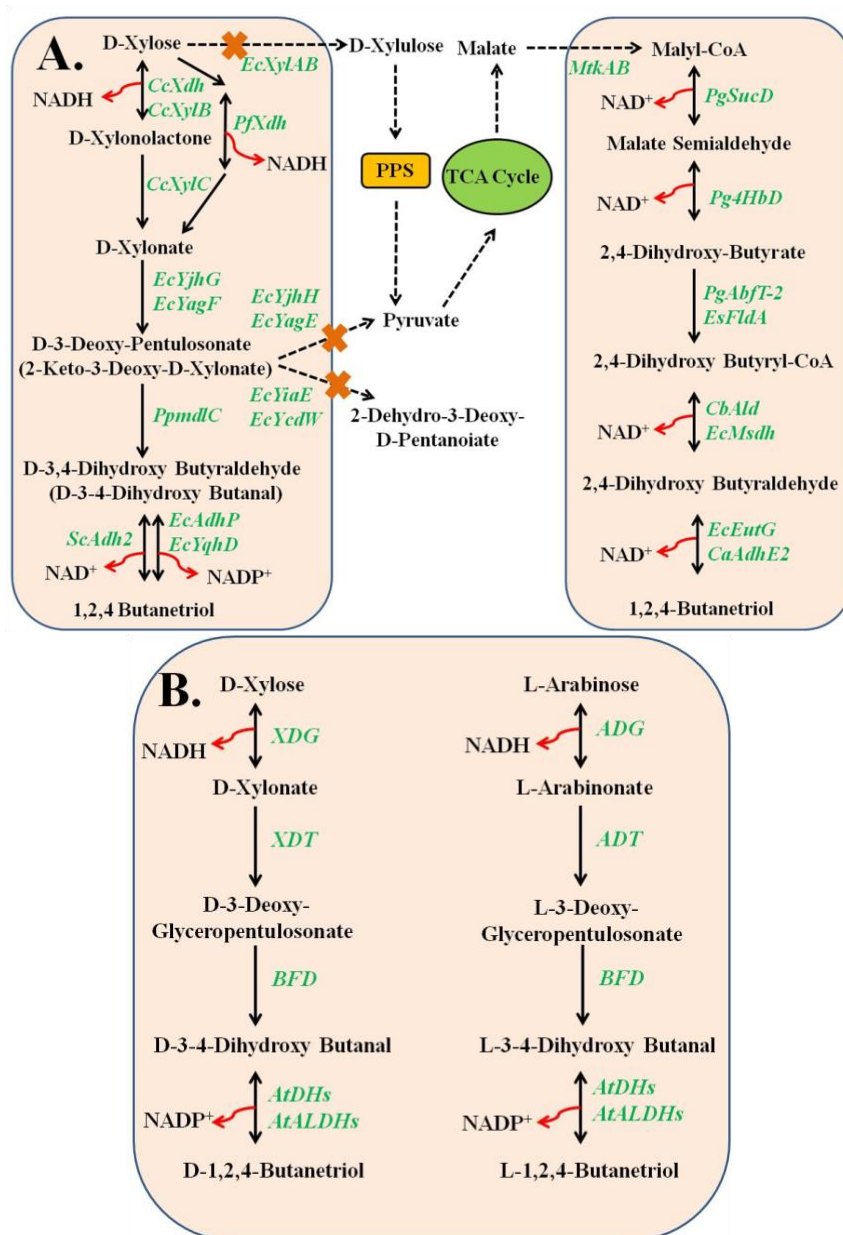


Fig 2: BTO biosynthesis from D-xylose, L-arabinose and malate as feedstock (A) in *Escherichia coli* and (B) in *Arabidopsis thaliana*.

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 from 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

eEquilibrator is a biochemical thermodynamics calculator for individual enzymatic steps involved within the large metabolic networks. eEquilibrator 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.

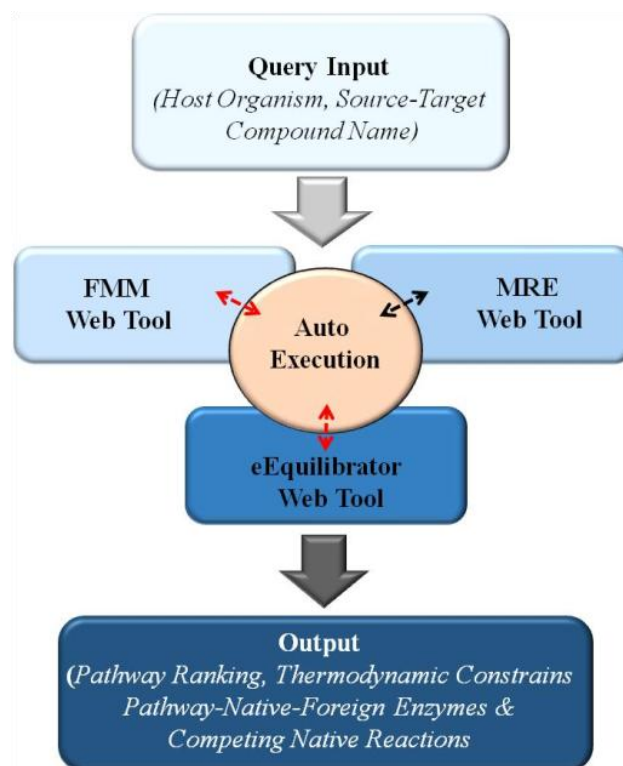


Fig 3: System biology (web based) workflow for prediction and design of biosynthetic metabolic pathways.

Glycerol entry point node

In this current study, glycerol to BTO biosynthetic pathways are predicted and enzymatic networks has been designed respectively through two major routes of glycerol entry points likely Methylglyoxal and Lactaldehyde nodes (Fig 4). As far thermodynamic constraints it seems Methylglyoxal and Lactaldehyde nodes are quiet energetically favorable (concerning Gibb's free energy change). Each class of enzyme in these two nodes represents different sub-classes of enzymes from diverse microbial regimes including human and plants (enzyme dataset can be obtained following web servers). In addition, a competing D-fuconate node has also been identified which redirects the flux of Pyruvate and Lactaldehyde towards D-fuconate through 2-dehydro-3deoxy-L fuconate intermediate formation. However, D-fuconate conversion from 2-dehydro-3deoxy-L fuconate is highly energetically unfavorable step. Even though predicted design of this synthetic pathway looks promising but still few reaction steps need to be further re-evaluated as those reactions are energetically unfavorable (i.e. Pyruvate to D-Xylonate and Lactaldehyde to Methylglyoxal reaction steps). The most possible solution space for this issue (having energetically unfavorable enzymatic steps) is to introduce energy coupled enzymatic reaction (i.e. Adenosine triphosphate or ATP hydrolysis along with carboxylation and/or redox reactions) which may turn the overall reaction energetically favorable (Atkinson, 1977). Furthermore, two putative balanced equations (i.e. reaction 1: 2-dehydro-3deoxy-L fuconate to 2-dehydro-3deoxy-D-xylonate and reaction 2: D-fuconate to D-Xylonate) have manually been derived using eEquilibrator tool. It

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-fuconate node). Hence, some empty space still remains towards discovery of novel enzymes in Methylglyoxal, Lactaldehyde and D-fuconate nodes.

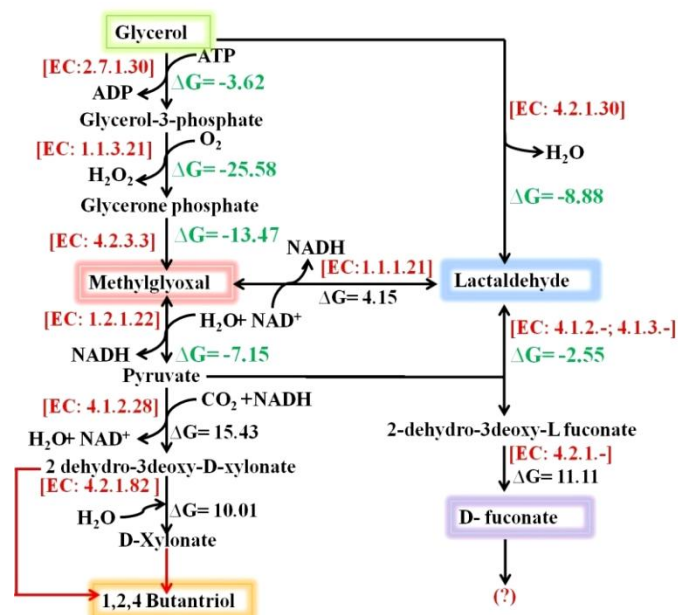


Fig 4: Predicted and designed putative synthetic metabolic network concerning glycerol entry nodes for BTO biosynthesis including thermodynamic constraints and enzyme classes ("ΔG" free energy change value represented in Kcal/mol unit).

Pitfalls of D-Xylonate downstream node

Finally, D-Xylonate 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 (*MdlC*), and thiokinase (*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 catalysis 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*, *FucO*, *EutG*, *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-xylonate 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; EcXylA: *Escherichia coli* Xylose isomerase; CcXdh: *Caulobacter crescentus* Xylose dehydrogenase; CcXylB: *Caulobacter crescentus* Xylonolactonase; PfXdh: *Pseudomonas fragi* Xylose dehydrogenase; CcXylC: *Caulobacter crescentus* Xylonolactonase; EcYjhG: *Escherichia coli* D-Xylonate dehydratase; EcYagF: *Escherichia coli* D-xylonic acid dehydratase; EcYjhH: *Escherichia coli* 2-Keto-3-deoxy-d-xylonate Aldolase; EcYagE: *Escherichia coli* 2-Keto-3-deoxy-d-xylonate Aldolase; PpmdlC: *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 thiokinase; 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; CbAld: *Clostridium beijerinckii* Coenzyme A acylating Aldehyde dehydrogenase; EcMsdh: *Escherichia coli* Methylmalonate-semialdehyde dehydrogenase ; EcEutG: *Escherichia coli* Alcohol dehydrogenase; CaAdhE2: *Clostridium acetobutylicum* Bifunctional aldehyde/alcohol dehydrogenase; XDG: D-xylose dehydrogenase; XDT: D-xylonate dehydratase; BFD: Benzoylformate decarboxylase ; ADG: L-arabinose dehydrogenase ; ADT: L-arabinonate dehydratase ; AtDHs: *Arabopsis thaliana* Dehydrogenases; AtALDHs: *Arabopsis thaliana* aldehyde dehydrogenases).

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