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A Review: Manufacturing, and Properties of the D-Fructose Epimer D-Allulose (D-Psicose)

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Abstract

D-allulose, also known by the name D-psicose or just allulose, is a rare keto-sugar epimer of D-fructose in the third carbon (C_3), and naturally occurs in small quantity in fruits such as grapes and figs. It is low calorie sweetener produced enzymatically from D-fructose by enzymes D-ketose 3-epimerase (DKEase) family. D-allulose has a similar taste, texture and functionality as sweetener comparing to high calorie sweetener sugar table sucrose. D-allulose is poorly metabolized in the body with minimal impact on blood sugar levels making it a natural low-calorie sweetener. This property makes D-allulose an attractive sweetener for diabetes and for body weight management. Laboratory studies on D-allulose intake demonstrated its safety with no significant adverse effects. United States Food and Drug Administration (FDA) has granted D-allulose the status of Generally Recognized as Safe (GRAS). Plus, it is considered safe for human consumption by regulatory organizations in other countries except in European Union due to their request for further laboratory testing. Maximum acceptable daily intake of D-allulose is 0.9 grams per kilogram body weight. Excessive intake for more than the recommended daily intake could lead to some side effects such as gastrointestinal discomfort or laxative effects. In general, D-allulose is considered one of the preferred natural low calories sweeteners for those seeking an alternative to table sugar sucrose.

Keywords

D-Allulose, D-Ketose 3-Epimerase (DKEase), D-Tagatose 3-Epimerase (DTEase), D-Allulose 3-Epimerase (DAEase), D-Psicose 3-Epimerase (DPEase)

1. Introduction

D-Allulose (D-Psicose), is a keto-hexose monosaccharide sugar has a molecular

formula of $C_6H_{12}O_6$ and a molecular weight of 180.16. It is an epimer of the keto-hexose sugar D-fructose with only structural difference located at the C_3 atom with invert in the configuration of the hydroxyl group position (**Figure 1**). D-allulose is a rare sugar that naturally occurs in wheat, some fruits, molasses, and maple syrup. It is low calorie sugar with 0.2 - 0.4 calories per gram compared to 4.0 calories per gram for table sugar sucrose [1]. In addition, D-allulose is not metabolized in the body, it is just absorbed by the small intestine, and excreted in the urine [2]. In addition, D-allulose does not elevate blood sugar or insulin level [3]. These properties make D-allulose a good substitute to common sugars such as sucrose, D-glucose, or D-fructose, specially to regulate daily calories intake for people with health conditions such as overweight, obese, and diabetes [4].

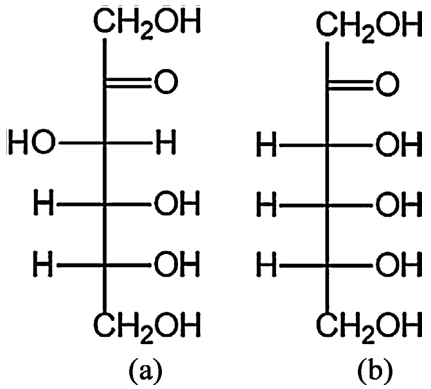

Figure 1. The molecular structures of D-fructose (a) and D-allulose (b) D-allulose is C_3 epimer of D-fructose.

D-allulose can be produced via chemical or biological methods. One of these chemicals methods is the synthesis from D-glucose or from 1,2:4,5-di-O-isopropylidene-β-d-fructo-pyranose under high temperature in the presence of molybdate as a catalyst [5]. The second chemical method is the synthesis from D-fructose by heating in a mixed system of alcohol and triethylamine in a multi-step reaction [6]. These two chemical methods are not suitable for the production of D-allulose on large scale specially for food and pharmaceutical applications, because these chemical methods generate toxic by-products, and cause environmental pollution. In the other hand biological methods are the best approach for D-allulose production with advantages includes the simplicity in production, friendly to environment, and in lower energy production cost. In addition, D-allulose produced by biological methods are recognized as safe for foods, pharmaceuticals and for other applications. There are two biological methods for the production of D-allulose. These biological methods are enzymatic (D-Ketose 3-epimerase enzymes) method and by microbial (bacteria or yeast) fermentation method [7].

Enzymatic method using immobilized D-Ketose 3-epimerase enzymes is the current production method, but it is still costly for D-allulose production, and continuous screening for microorganisms producing ketose 3-epimerase for better enzyme property with higher equilibrium in favor of D-allulose from the

substrate D-fructose is in progress to reduce D-allulose production cost by this enzymatic method. Microbial fermentation method is not yet in production scale due to the limited microbial intracellular enzymes activities for the metabolic pathway to produced D-allulose from hexose or pentose sugars as a carbon source. This lower microbial intracellular enzymatic activity leads into lower conversion rate of carbon sources into D-allulose by microbial fermentation method comparing to enzymatic method. In recent years the application of metabolic engineering technology for overexpression of microbial intracellular enzymes necessary in the pathway for D-allulose production is in progress to improve D-allulose production yield by this microbial fermentation method at lower cost.

2. D-Alllulose Biological Production Methods

2.1. D-Ketose 3-Epimerase Enzymes

Mechanism of these D-keto 3-epimerase (DKEase) enzymes is transforming D-fructose as a substrate into D-allulose without phosphorylation in the presence of Adenine Tri-Phosphate (ATP), epimerization, and dephosphorylation mechanism (**Figure 2**). D-ketose 3-epimerase (DKEase) enzymatic mechanism is based on D-fructose C_3 epimerization in reversable equilibrium reaction. D-Ketose 3-epimerase enzymes family have been isolated and identified from different microorganisms. These D-ketoses 3-epimerases enzymes family are identified according to their optimal sugar substrate. The first D-keto 3-epimerase enzyme was identified in the year 1994, is D-tagatose 3-epimerase (DTEase) by the Japanese scientist Ken Izumori [8] [9], followed by D-psicose 3-epimerase (DPEase) enzyme [10]. D-psicose 3-epimerase (DPEase) is also known in some publications by the name D-allulose 3-epimerase (DAEase) enzyme [11]. The first identified enzyme D-tagatose 3-epimerase (DTEase) has two epimerization reactions (**Figure 3**) epimerization of D-fructose into D-allulose, and the second is the epimerization of its main substrate D-tagatose into D-sorbose [12]. These D-ketoses 3-epimerase (DKEase) enzymes family are intracellular enzymes expressed in both pathogenic and non-pathogenic prokaryotic and eukaryotic microorganisms. The discovery of these D ketose 3-epimerase (DKEase) enzymes family is breakthrough for the large-scale production of D-allulose from D-fructose as a substrate without the need for phosphorylation in the presence the costly Adenine Tri-Phosphate (ATP), followed by epimerization, and finally dephosphorylation by phosphatase enzymes [13].

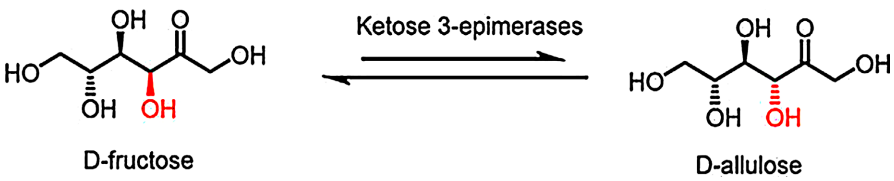

Figure 2. Epimerization of D-fructose by ketose-3-isomerases enzyme into D-allulose. This enzymatic mechanism is based on D-fructose C_3 epimerization in reversable equilibrium reaction.

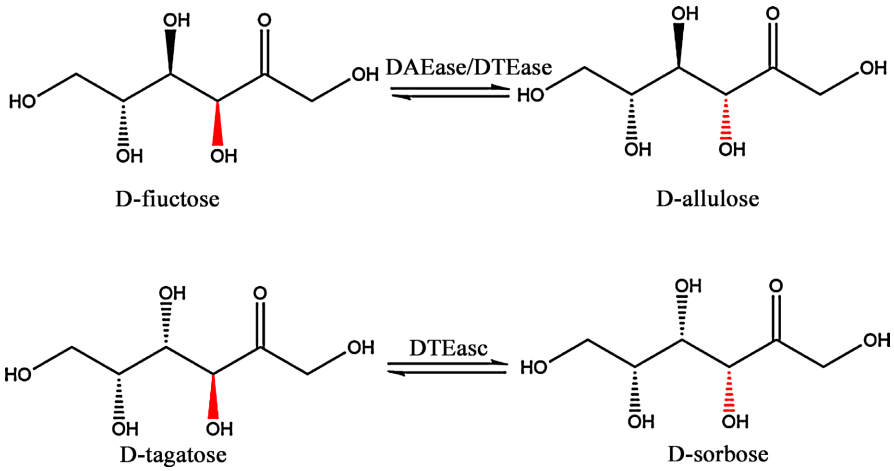

Figure 3. The epimerization reactions on D-fructose into D-allulose and D-tagatose into D-sorbose catalyzed by the enzyme D-tagatose-3-epimerase (DTEase).

Theoretically, these identified D-Ketose 3-epimerase (DKEase) enzymes family epimerize D-fructose into D-allulose in equilibrium 1:1 ratio (D-allulose**:** D-fructose). The actual epimerization ratio was demonstrated to be far below the theoretical epimerization ratio. This is due to the poor thermostability of these D-ketose 3-epimerases (DKEase) enzymes [14]. Improving epimerization ratio of D-fructose into D-allulose by these D-ketose 3-epimerases (KEase) enzymes close to or higher than the theoretical ratio can be achieved by enhancing the thermostability of D-keto 3-epimerase enzymes [15]. This thermostability enhancement can be achieved by microbial mutation or genetic engineering, followed by microbial screening for the selection of microorganisms producing thermostable D-ketose 3-epimerases (DKEase) enzymes at higher optimum temperature (thermostable), and better equilibrium reaction toward D-allulose.

2.2. Microorganisms Producing D-Ketose 3-Epimerase Enzymes

Various microorganisms' genus, and species are naturally identified or genetically manipulated to produce D-ketose 3-epemirase (DKEase) enzymes as intracellular enzymes [16]. Some of these microorganisms includes *Pseudomonas cichorii*, *Rhodobacter sphaeroides*, *Caballeronia fortuita*, *Sinorhizobium sp.* producing D-tagatose 3-epimerase (DTEase) enzyme. *Agrobacterium tumefaciens*, *Bacillus* sp. KCTC 13219, *Clostridium cellulolyticum* H10, *Ruminococcus* sp., *Clostridium* sp., *Clostridium scindens* 35704, *Desmospora* sp. strain 8437, *Clostridium bolteae*, *Dorea* sp. strain CAG317, *Treponema primitia*, *Flavonifractor plautii*, *Arthrobacter globiformis* M30, *Agrobacterium* sp. strain ATCC 31749, *Paenibacillus senegalensis*, and *Staphylococcus aureus* producing D-allulose-3-epimerases (DAEase) enzyme, also known by the name D-psicose 3-epimerase (PAEase) enzyme [17].

Some of these microorganisms have been reported to produce thermostable (thermotolerance) D-ketose 3-epimerase (D-KEase) enzymes such as *C. cellulolyticum* H10 [18], *Agrobacterium sp.* strain ATCC 31749 [19]. These microorganisms

producing thermotolerance D-ketose 3-epimerase (DKEase) enzymes are developed by microbial mutation or by genetically engineering technology, followed by microbial colonies selections for thermotolerance enzyme with high epimerization rate of D-fructose into D-allulose close to the theoretical ratio. There are other approaches to improve D-ketose 3-epimerase (DKEase) enzymes equilibrium toward D-allulose close or higher to the theoretical ratio without microbial mutation or genetic engineering methods, just by increasing molar ratios of borate to D-fructose (up to a ratio of 0.6) for the formation of D-allulose borate complex in order to epimerize more D-fructose into D-allulose [20]. Another approach is continuous D-allulose separation from the substrate D-fructose during enzymatic reaction to drive the enzymatic reaction toward D-allulose close or higher toward the theoretical ratio [21].

2.3. Microbial Enzyme Method

Free enzyme method for the epimerization of D-fructose into D-allulose is not economical strategy for large scale production due to enzymes high cost and poor D-allulose recovery efficiency. This free enzyme method is currently replaced by immobilized enzyme method [22] or by microbial whole cells immobilization method [23]. These two methods of immobilization, extend the enzyme half-life and reduce production cost. In addition, the presence of Co^{2+} and Mn^{2+} in the enzymatic reaction as co-factors improved the enzyme catalytic activity toward the maximum epimerization of D-fructose into D-allulose [24]. Also, the selection of optimum immobilized enzyme reaction conditions of PH, temperature, and metallic ions concentration are important factors to improve D-fructose epimerization rate toward higher yield of D-allulose [25]. The optimum enzymatic reaction condition for these D-ketos 3-epimerase (DKEase) enzymes demonstrated to be in the range of 7.5 to 9.0 for PH, and 40˚C to 70˚C for temperature at the selected optimum concentrations of co-factors (metallic ions) of Co^{2+} and Mn^{2+} [24]. It is being reported that under these D-ketos 3-epimerase (DKEase) optimum conditions the K_m, k_{cat}, and k_{cat}/K_m for these D-ketose 3-epimerase family toward the substrate D-fructose are in the range from of 24.00 - 549.00 mM, 5.80 - 1059.55 (s^{-1}), and 0.11 - 3.31 $mM^{-1}{\cdot}s^{-1}$, respectively [26].

Enzymes carriers (supports) used for enzymes immobilization are generally reversible mechanism [27] such as adsorption, ionic bonding, and affinity bonding, or irreversible mechanism such covalent binding, entrapment, encapsulation, and cross-linking (**Figure 4**). To maintain the immobilized enzyme activity on these supports with extended half-life, these supports must exploit the effective attachment to only the non-enzymatic active amino acids residues in the enzyme protein [14]. In addition, other factors in the selection of suitable support for enzymes immobilizations for higher epimerization rate and yield includes enzyme loading, and enzyme stability on the support. In general, common support types for enzyme immobilization includes calcium alginate, chitosan spheres, and various resins.

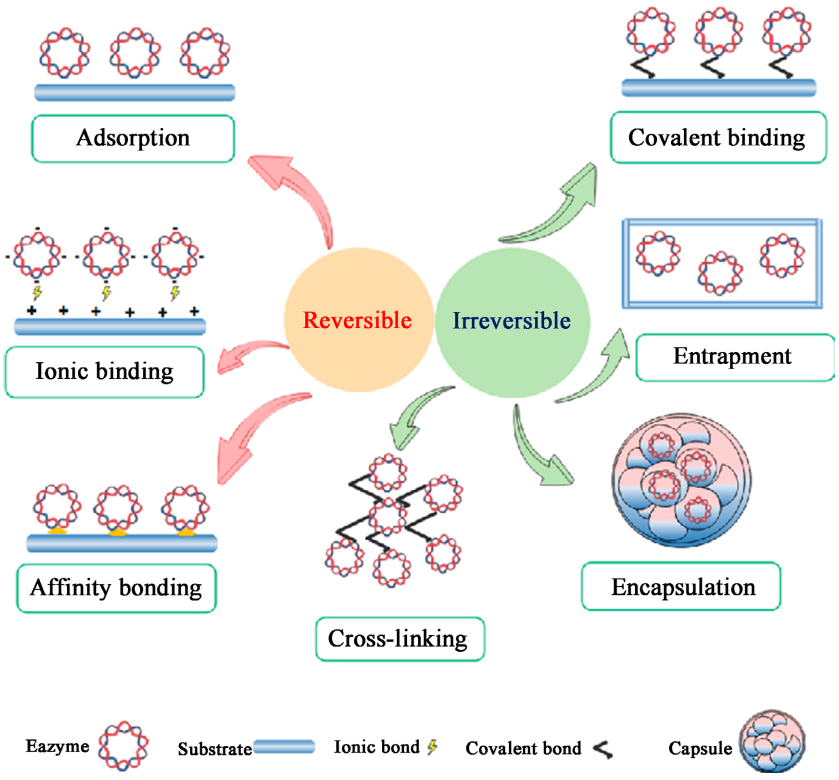

Figure 4. Major enzyme immobilization methods are: reversible (adsorption, ionic bonding, and affinity bonding), and irreversible (covalent binding, entrapment, encapsulation, and cross-linking).

Microbial whole cells immobilization is not suitable for microorganisms producing extracellular enzymes, and is designed for microorganisms producing intracellular enzymes such as D-ketose 3-epimerase (DKEase) enzymes. This microbial whole cell immobilization is less costly method for D-allulose production from the substrate D-fructose comparing to immobilized enzymes method, because there is no need to extract the enzyme from microbial cells before immobilization. Enzyme extraction and purification are important process before immobilized enzymes method. In microbial whole cells immobilization for D-allulose production, the substrate (D-fructose) pass through the microbial cell membrane for the intracellular D-ketose 3-epimerase (DKEase) enzymes interact with the substrate D-fructose to be epimerized into D-allulose. The produced D-allulose is exit from the immobilized microbial cell through microbial cell membrane [28] into the solution for recovery. Other advantage from microbial whole cells immobilization method comparing to immobilized enzyme method is microbial whole cells immobilization is suitable method for multiple intracellular enzymes reactions.

These immobilized methods for the production of D-allulose from the substrate D-fructose by enzyme immobilization or by microbial whole cells immobilization is the current methods for D-allulose production on large scale. These immobilizations technology is the most efficient strategy due to increasing applications frequency, and offers end products separation efficiency with less purification

process comparing to free enzyme's reaction [29]. In the case of D-allulose production by immobilization technology there are still needs for further research to improve D-ketose 3-epimpirase enzymes thermostability, and to improve D-allulose separation efficiency with less purification process in order to reduce D-allulose production cost.

2.4. Microbial Fermentation Method

Microbial fermentation method is still under investigation and believed to be the future ideal method for D-allulose large-scale production at higher yield and lower cost in replacement to immobilized enzyme methods. This microbial fermentation method is designed for multiple intracellular microbial enzymes pathway, and is based on phosphorylation, epimerization, and dephosphorylation cascades for the bioconversion of D-fructose or other carbon source into D-allulose [30]. This microbial fermentation method is capable to utilize not only D-fructose, but also other carbon sources of D-glucose, D-xylose, sucrose, starch, inulin, lignocellulosic agriculture byproducts, and food industry wastes such as molasses for the production of D-allulose after upfront acid or enzymatic hydrolysis of these disaccharides, and polysaccharides into monosaccharides of D-fructose, D-glucose, and D-xylose before microbial fermentation into D-allulose [31]. This microbial fermentation method requires the intracellular Adenine Tri-Phosphate (ATP), intracellular Nicotinamide adenine dinucleotide phosphate ($NADP^+$) for phosphorylation steps, and the intracellular polyphosphate kinase for dephosphorylation steps in the metabolic pathway for D-allulose production at optimum microbial fermentation conditions of pH, temperature, and microbial nutritional requirements for higher conversion efficiency of these carbon sources into D-allulose.

Advantages from using gram-negative bacteria *Escherichia coli* in this microbial fermentation research comparing to other microorganism, this *Escherichia coli* has fast growth rate, will study at the level of genetic mapping, and easy for genetic manipulation [32]. In addition, *Escherichia coli* is naturally carrying the endogenous enzyme D-allulose 6-phosphate epimerase **(**DAEase) to convert D-fructose-6-phosphate into D-allulose-6-phosphate. These properties making *Escherichia coli* cells a factory to understand the D-allulose pathway at genetic level specially in cell uptake of carbon sources, and in the conversion of D-fructose and other carbon sources into D-allulose in microbial fermentation process. *Escherichia coli* uptake of D-fructose is based on three different phosphorylation/dephosphorylation pathways [33]. One of these pathways is the carbohydrate phosphotransferase system (PTS)**,** the other two are phosphorylation/dephosphorylation pathways belongs to phosphoenolpyruvate (PEP) systems.

In the common phosphotransferase systems (PTS) [34]**,** once D-fructose is uptake by microbial cells**,** it is usually phosphorylated into fructose-1 phosphate (F-1-P), then into fructoae1, 6 Phosphate (F-1,6-P). This (F-1,6-P) generation is undesirable phosphorylation pathway for D-allulose production, and it can be

blocked in *Escherichia coli* cells by mutation or by genetic manipulation for the deletion of undesirable genes and overexpress desirable genes for D-allulose pathway [35]. In the production of D-allulose from D-fructose by microbial fermentation, the Phosphotransferase (PTS)-linked glucose transporter (PtsG) gene should be overexpressed in *Escherichia coli* cells [36]. This PTS-linked glucose transporter (PtsG) gene possesses the ability to transport D-fructose via diffusion into *Escherichia coli* cells where D-fructose can be phosphorylated into the desirable fructose-6-phosphate (F-6-P) [37]. Overexpression of this (PtsG) gene enhances the intracellular enzyme fructose/mannose kinase (*mak*) to phosphorylate D-fructose into fructose-6-phosphate (F-6-P) in the presence, of Adenine Tri-Phosphate (ATP) as a phosphate donor. The generated fructose-6-phosphate (F-6-P) is epimerized in *Escherichia coli* cells into allulose-6 phosphate, also known by the name psicose-6-phosphate (P-6-P), by the overexpressed gene for the intracellular enzyme D-allulose-6-phosphate 3-epimerase (AlsE) for the generation of D-allulose-6-phosphate [38]. The generated D-allulose-6 phosphate inside *Escherichia coli* is dephosphorylated into D-allulose (**Figure 5**) by the overexpressed gene for intracellular enzyme hexitol-phosphatase B (HxpB). The end product D-allulose from this genetically manipulated pathway is excreted from the *Escherichia coli* cells into the microbial fermentation medium Is separated from microbial cells by filtration, followed by purification processes in the

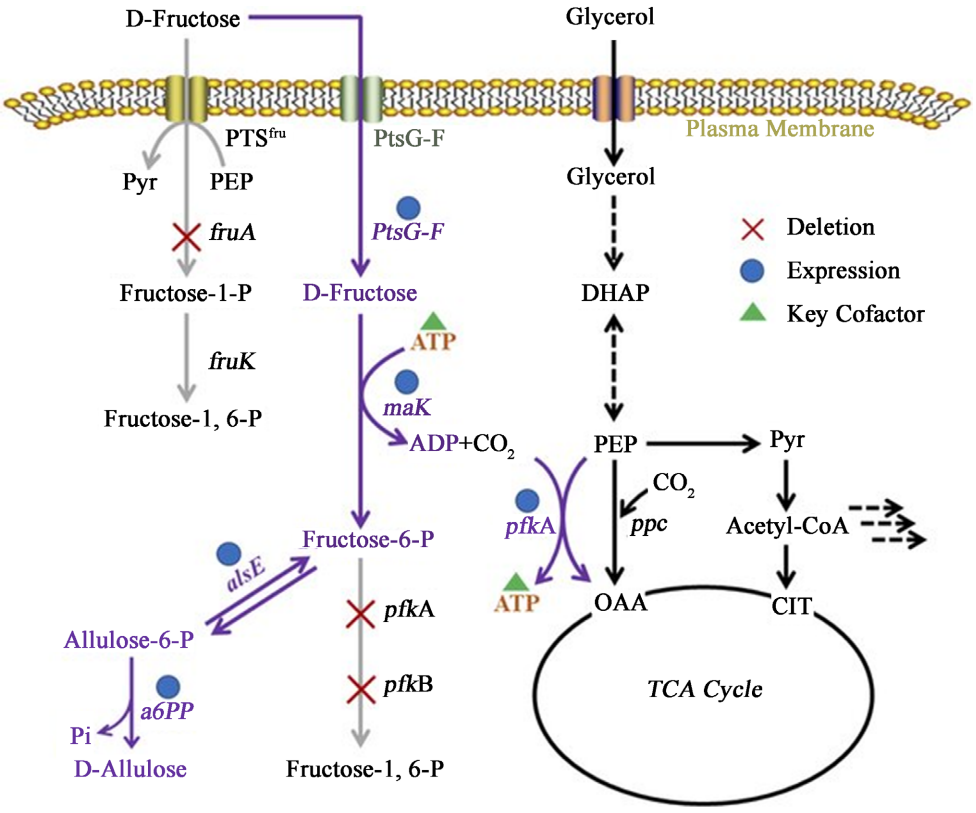

Figure 5. Metabolic pathways engineering in *Escherichia. coli* cell factory for the production of D-allulose from D-fructose via Phosphorylation-dephosphorylation in the presence of ATP as key factor.

fermentation downstream process (recovery process) to produce pure D-allulose in the form of syrup or crystals.

Escherichia coli also can be genetically manipulated to produce D-allulose from the carbon source D-glucose, also via series of phosphorylation, epimerization, and dephosphorylation pathway [39]. In this genetically manipulated pathway, D-glucose in the fermentation medium is first phosphorylated by phosphotransferase system (PTS) into glucose-6-phosphate (G-6-P). The generated G-6-P is transferred (uptake) across the *Escherichia coli* cell membrane into the cell cytoplasm [40] where it is isomerized into fructose-6-phosphate (F-6-P) by the enzyme glucose-6-phosphate isomerase (GPI). In the common glucose pathway, the generated fructose-6-phosphate (F-6-P) is normally directed inside Escherichia *coli* cells toward the glycolysis pathway. This undesirable glycolysis pathway can be diverted towards D-allulose pathway by using static and dynamic carbon flux regulation techniques [41]. This diverted pathway will result in the accumulation of fructose-6-phosphate (F-6-P) inside *Escherichia coli* cells for the epimerization into D-allulose-6-phosphate (D-psicose-6-phosphate) by the overexpressed gene for the intracellular enzyme D-allulose-6-phosphate-3-epimerase (AlsE). The generated D-allulose-6-phosphate is then dephosphorylated to D-allulose by the overexpressed gene for the intracellular enzyme hexitol-phosphatase B (HxpB) (**Figure 6**). The end product of D-allulose produced from D-glucose as carbon source in this genetically manipulated *Escherichia coli* is excreted into the fermentation medium is separated by filtration from microbial cells, followed by purification in the fermentation downstream process (recovery process) to produce pure D-allulose in the form of syrup or crystals.

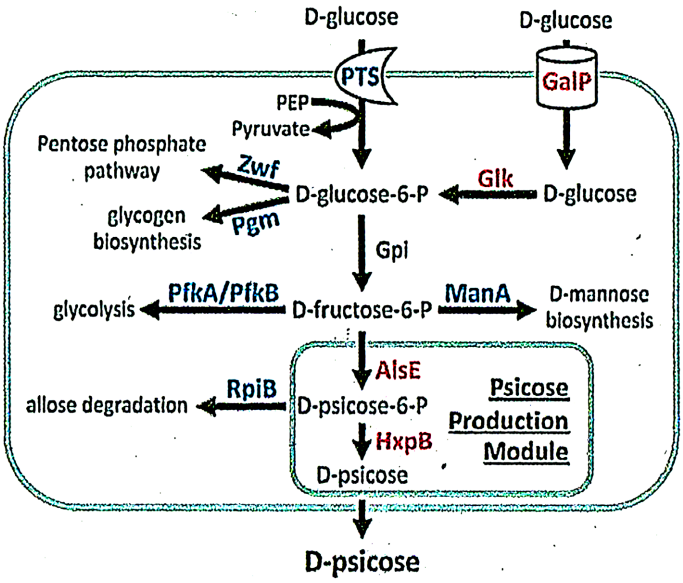

Figure 6. Engineered metabolic pathway of *Escherichia coli* for the production of D-allulose (D-psicose) from D-glucose by fermentation. This modified pathway is based on genes deletion of enzymes responsible to glycolysis and undesirable pathways, and overcrossing genes of enzymes responsible to D-allulose (D-psicose) pathway from D-glucose.

These capabilities of utilizing D-fructose, or D-glucose, as a carbon source in microbial fermentation for the production of D-allulose by genetically manipulated metabolic pathways in *Escherichia coli* cells is promising technology for future D-allulose production by microbial fermentation utilizing disaccharides sugars such as sucrose after acid or enzymatic (invertase) hydrolyses of sucrose into D-fructose/D-glucose, or such as maltose after acid or enzymatic (maltase) into two D-glucose units. These disaccharide hydrolysis methods can be upfront process before microbial fermentation, or can be genetically engineered in industrial microorganisms to produce extracellular enzymes of invertase or maltase to be able to hydrolyze sucrose or maltose into monosaccharides of D-fructose, and D-glucose directly in the microbial fermentation process for D-allulose production.

In addition to utilizing both monosaccharides or disaccharides of hexose sugars as a carbon source for the production of D-allulose by microbial fermentation, there is a potential to utilize pentose sugar D-xylose as well for the production of D-allulose by microbial fermentation. This pentose pathway can be developed by genetically engineered *Escherichia coli* with metabolic pathway manipulation capable to utilize D-xylose–methanol mixture as a caron source in the microbial fermentation method for D-allulose production [42]. Such genetically engineered *Escherichia coli* can be developed by inserting artificial antisense RNA (asRNA) into *Escherichia coli* cells to diminish (block) the flow of normal pentose phosphate (PP) pathway. This can be achieved by knocking down (deletion) the UDP-glucose-4-epimerase (GalE) enzyme to prevent polysaccharides synthesis and other metabolites synthesis as by-products while increase D-allulose yield from D-xylose. This genetically manipulated metabolic pathway for *Escherichia coli* is also designed to regulate the expression of the formaldehyde detoxification operon (FrmRAB) for self-inductive detoxification (**Figure 7**).

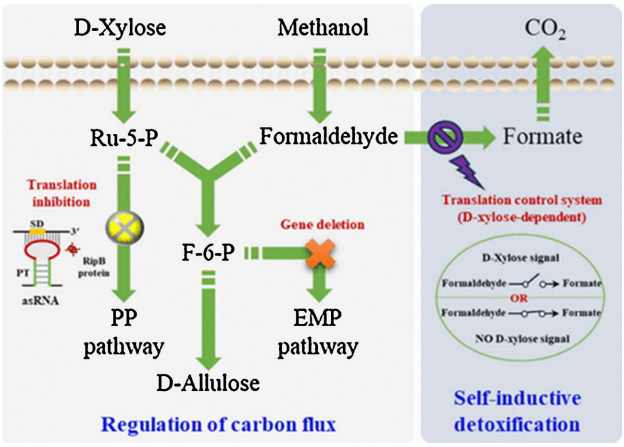

Figure 7. Genetically engineered *Escherichia coli,* capable to utilize D-xylose-methanol mixture as a caron source in fermentation for D-allulose production. This is based on genetically diminishing pentose phosphate (PP) pathway, and genes for UDP-glucose-4-epimerase (GalE) enzyme to increase D-allulose (D-psicose) yield. This genetically manipulated pathway is also designed to regulate the expression of the formaldehyde detoxification operon (FrmRAB) for self-inductive detoxification.

This genetically manipulated D-xylose pathway in genetically engineered *Escherichia coli* cells for D-allulose production by microbial fermentation showed the potential for the future production of D-allulose from polysaccharides by microbial fermentation [43] [44]. These polysaccharides such as dextrin, amylose starch, amylopectin starch, inulin, and lignocellulosic biomass (cellulose, and hemicellulose) for D-allulose production [45]. These polysaccharides for D-allulose production by fermentation require upfront pre-treatments of these polysaccharides before microbial fermentation. These upfront pre-treatments can be enzymatic or acidic hydrolysis to hydrolyze (breakdown) these polysaccharides into monosaccharides of D-fructose, or D-glucose, or D-xylose before microbial fermentation into D-allulose by these genetically manipulated microorganisms.

The gram-negative bacteria *Escherichia coli* is a food-borne pathogen bacteria and is not Generally Recognized as Safe (GRAS) by United States (U.S.) FDA, or by other overseas regulatory organizations. This genetically modified *Escherichia coli* is not acceptable to be used for D-ketose 3-epimirase enzymes (DKEase) production for enzyme immobilization, or for microbial cells immobilization, to produce food grade D-allulose. Also, this genetically modified *Escherichia coli* is not acceptable in microbial fermentation method for the production of food grade D-allulose. That said, all published research on genetic manipulated *Escherichia coli* for D-allulose production is just learning experiments by the known genetic sequence *Escherichia coli* just to understand metabolic pathway manipulation methods for future apply of these genetically manipulation techniques in Generally Recognized as Safe (GRAS) microorganisms for food grade D-allulose production on large scale acceptable for foods and other applications. In fact, some publications are currently utilized *Escherichia coli* genes manipulations techniques for the application in Generally Recognized as Safe (GRAS) microorganisms such as the published *Bacillus subtilis* harboring the gene for D-tagatose 3-epimerase (*DTEase)* for D-allulose (D-psicose) production by immobilized enzyme or by whole cells immobilization methods [46].

3. Downstream (Recovery) Process

Researchers paid highly intention on the separation and purification of D-**a**llulose in downstream (recovery) process, after the upstream (immobilization or fermentation) methods to improve D-allulose recovery efficiency and yield with acceptable standard specifications at lower production cost. These recovery process methods include separation, decolorization, desalination, crystallization, and drying. There are two main separation methods for the isolation of D-allulose from carbon sources and from impurities (intermediate metabolites). These separation methods are ion exchange resin method, and biological method.

Ion exchange resin method playing a key role in bioprocess technology for both capturing and purification steps of target molecules. There are many ion exchange resins that are available in the market, and selecting the suitable one for the separation of D-allulose from the substrate carbon sourced and from impurities is

important factor to obtain highly separation efficiency, of purified and accepted D-allulose in the form of syrup or crystals [47]. In the case of immobilized enzymes or whole cells immobilization, the separated substrate of D-fructose from end product D-allulose by ion exchange resin method can be recycled as substrate for D-allulose production. Recycling separated D-fructose is a way to improve the epimerization efficiency of D-fructose into D-allulose.

Biological method theory is based on the D-allulose is non-fermentable while the substrate D-fructose and other carbohydrates are fermentable by yeasts into ethanol [48]. In this biological method the D-fructose residue after epimerization of D-fructose into D-allulose by immobilized enzyme or whole cells immobilization is fermented under anaerobic conditions by yeasts such as *Saccharomyces cerevisiae* into ethanol that can be separated from the produced D-allulose as a valuable byproduct using pervaporation technology [49]. This biological method for D-allulose separation from D-fructose demonstrated to be the more environmentally friendly method than ion exchange resin method.

D-allulose produced is marketed in the form of syrup or crystals. Produced D-allulose syrup (**Table 1**) is about 80 %, solid at pH range from 2.5 - 8.0 and the syrup might contain additive such as antioxidant to improve the syrup shelf-life. Produced D-allulose crystal or powder (**Table 2**) usually has specific particle size distribution according to the type of applications to enhance taste when is used as a replacement to table sugar or other sweeteners in foods or other applications.

Table 1. D-Allulose (D-psicose) Syrup standard specifications.

Physical Tests	**Unit**	**Specification Data**
Appearance	–	Liquid
Color	–	Light yellow
Odor	–	Neutral
Solid Substance	%	≥70
pH value	–	3.0 - 7.0
Ash	%	≤0.5
Chemical Tests	**Unit**	**Specification Data**
D-Allulose	%	≥95
Arsenic (As)	mg/kg	≤0.5
Lead (Pb)	mg/kg	≤0.5
Microbiological Tests	**Unit**	**Specification Data**
Total Plate Count	cfu/g	≤1500
E. coli	MPN/100g	≤30
Staph aureus	–	Negative

Table 2. D-Allulose (D-psicose) Powder standard specifications.

Physical Tests	Unit	Specification Data
Appearance	–	Powder
Color	–	White
Odor	–	Neutral
pH value	–	3.0 - 7.0
Ash	%	≤0.1
Chemical Tests	**Unit**	**Specification Data**
D-Allulose	%	≥98.5
Arsenic (As)	mg/kg	≤0.5
Lead (Pb)	mg/kg	≤1.0
Microbiological Tests	**Unit**	**Specification Data**
Total Plate Count	cfu/g	≤1000
E. coli	MPN/100g	≤30
Pathogen	–	Negative

4. Analytical Methods

Enzymatic activity of D-Ketose 3-epimerase (DKEase) enzymes is usually calculated based on units per milliliter or grams of enzyme sample. This enzymatic activity assay is performed in 50 mM Tris buffer at pH 6.0 - 8.0 containing 100 mM D-fructose as substrate, 50 μM test enzyme, or standard enzyme, or blank (distilled water) sample at 30˚C - 55˚C incubation for 5 min. The enzymatic reaction is terminated by heating at 100˚C for 10 min. to deactivate the enzyme, and stop the enzymatic reaction. The amount of D-allulose produced in this enzymatic reaction can be determined using High Performance Liquid Chromatography (HPLC). Enzyme activity of one unit of D-Ketose-3-epimerase (DKEase) activity is defined as the amount of enzyme that catalyzes the production of 1 μmol. of D-allulose per minute at the optimum standard enzyme reaction conditions [50]. All enzymatic assay must be performed in triplicate and the average reading is represented with standard deviation.

In addition, analytical methods for monitoring production process (immobilized enzymes, whole cells immobilization, or microbial fermentation) is also performed by using High Performance Liquid Chromatography (HPLC) for the analysis of D-fructose, D-glucose, D-xylose, D-allulose, and other intermediate metabolites in D-allulose production processes.

5. Safety Status of D-Allulose for Human Consumption

When D-allulose is ingested by human it is absorbed from the small intestine into blood circulation where it is excreted in the urine. Some of ingested D-allulose reached the colon where it is excreted in the feces [31]. The LD_{50} of D-allulose in

laboratory rats was in the range of 16 gm/kg., and its maximum toxic concentration to human was in the range of 0.55 gm/kg. body weight. D-allulose intake demonstrated to be beneficial to human with minimum adverse effects including gastrointestinal discomfort or diarrhea. D-allulose benefits to human includes have anti-obesity, and does not affect blood sugar or insulin levels. These two benefits making D-allulose a good alternative low calories natural sweetener comparing to high calories sweeteners, such as sucrose, D-glucose, D-fructose, and high fructose corn syrup (HFCS). In addition, D-allulose demonstrated to has therapeutic effects against atherosclerosis, and inflammatory symptoms [51].

United States Food and Drug Administration (FDA), has granted D-allulose the status of Generally Recognized as Safe (GRAS). Also, D-allulose received similar status by regulatory bodies from most other countries. In European Union, Canada, and Australia D-allulose is not yest received such status, but it is regarded as a 'novel food'. meaning it must be authorized for market by these countries' food safety authorities after further testing. Currently D-allulose is approved for sale in United States of America (USA) and in other countries includes, South America, Singapore, and Korea [52].

6. Applications of D-Allulose

D-allulose is low calorie keto-sugar, has mild sweetness, with good solubility. It is rare natural sugar with low glycemic index, and does not raise blood sugar or insulin levels. It has Millard reaction property that give browning characteristic to foods making it a suitable sweetener in baked goods. Maillard reaction is non enzymatic chemical reaction in which reducing aldehyde or keto group in sugar reacts with amino acids in foods to form amid chemical compounds responsible for browned foods with desirable flavor and aroma characteristics [53].

In addition to, D-allulose is a natural sweetness with browning property for baked goods it is also, suitable for the applications in beverages, confectionery, nutritional bars, energy bars, ketchup, low calorie sweeteners desserts, functional foods, milk beverages and other food products. D-allulose has stronger water holding capacity in food making it a good replacement to high calorie sweeteners such as sucrose, D-glucose, and D-fructose [54]. For health benefits D-allulose has potential applications in anti-obesity [55], dental caries, pharmaceuticals formulations and in cosmetics industries. Also, this rare keto-sugar of D-allulose has potential applications in chemical industry as a precursor for the production of the rare monosaccharide aldohexose sugar D-allose [56], and also for the production of the rare monosaccharide sugar alcohol D-allitol [57].

7. D-Allulose Market Size

Estimated D-allulose global market size for the year 2022 was about $10 billion and the expected D-allulose market size by the end of the year 2029 is estimated to reach $17 billion, with Compound Annual Growth Rate (CAGR) close to 4.7%. (**Figure 8**).

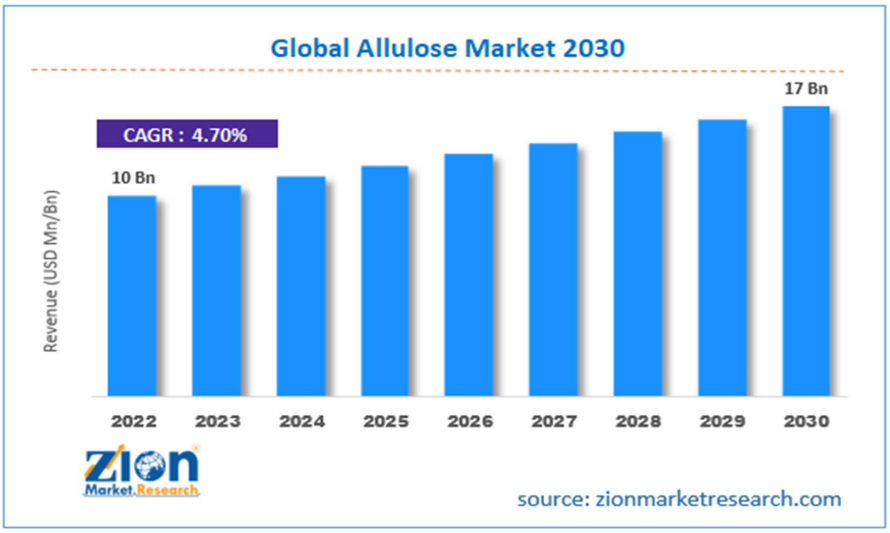

Figure 8. The global D-allulose (D-psicose) market size was evaluated in year 2022 at about $10 billion and is expected to reach about $17 billion by the end of the year 2030, with a CAGR of nearly 4.7% between 2023 and 2030.

Key players in D-allulose market includes but not limited to Tate & Lyle, Quest Nutrition, McNeil Nutritional, Matsutani Chemical Industry Co. Ltd., Ingredion Incorporated, CJ CheilJedang, Cargill Incorporated, Bonumose LLC, Apura Ingredients, and Anderson Global Group.

8. Discussion

D-allulose (D-psicose) production Is currently produced by Izumoring strategy, utilizing microbial enzymes D-ketose-3-epimerase (DKEase) enzymes family for the epimerization of keto-sugar D-fructose into D-allulose. These D-ketose 3-epimerase family are D-tagatose 3-epimerase (DTEase), and D-allulose 3-epimerase (DAEase), also known by the named D-psicose-3-epimerase (DPEase). The equilibrium mechanism for these D-ketose 3-epimerase family in immobilization method for the epimerization of D-fructose into D-allulose disfavors D-allulose with less than 40% conversion rate. Other negative factors that lower D-allulose production yield by enzyme immobilization method includes poor thermostability of D-ketose 3-epimerase enzymes causing short half-life for these enzymes' activity result in increase the cost of enzymes consumption. The second negative factor is non-enzymatic browning of D-allulose produced by this enzyme immobilization method. This browning complicates D-allulose purification process and lower its recovery efficiency. These negative factors for D-allulose production by enzyme immobilization method are the main reasons for high D-allulose production cost. Improving D-ketose 3-epemirase enzymes activities, thermostability, and long half-life of enzyme activity are important research to reduce D-allulose production cost by enzymes immobilization method. This research requires advance technologies of microbial mutation, gene expression, and enzyme (protein) engineering to improve the expression of D-ketose-3-epimerase as intracellular

enzyme from selected Generally Recognized as Safe (GRAS) microorganisms with desired properties for enzyme immobilization method. These are important researches that will give higher epimerization ratio favor D-allulose and higher yield to lower D-allulose production cost. This enzyme immobilization method requires upfront microbial fermentation process for D-ketose 3-epimaese enzymes production, followed by extraction process of intracellular D-ketose 3-epimerae enzymes from microbial cells, and purification process before enzyme immobilization on selected support for D-allulose production. This upfront for D-ketose 3-epimorase enzymes production are extra processes and extra cost for D-allulose production from D-fructose by enzyme immobilization method.

Microbial whole cells immobilization method is quite similar to enzymes immobilization method in operation, but it is designed only for immobilize cells from microorganisms producing intracellular enzyme, as the case for this intracellular enzyme of D-ketose 3-epimerases family. The application of these microorganisms producing intracellular enzyme in whole cells immobilization method require less upfront microbial fermentation process for D-ketose 3-epimaese enzymes production, because it does not require enzyme extraction and purification from microbial cells. This making microbial whole cells immobilization method for intracellular enzymes is less costly comparing to enzyme immobilization method.

Microbial fermentation for the production of D-allulose considered to be the future ideal route to replace the current enzyme immobilization, and microbial whole cells immobilization methods. Microbial fermentation method is not based on D-ketose 3-epimerase enzyme family for epimerization of D-fructose into D-allulose, but it is based on microbial metabolic pathways with multiple overexpressed enzymes directed to utilize D-fructose or other carbohydrates such as D-glucose, D-xylose, and polysaccharides that are rich in these monosaccharides for D-allulose biosynthesis via phosphorylation, epimerization, and dephosphorylation pathway without the concern of equilibrium mechanism. Polysaccharides as a carbon source in microbial fermentation for D-allulose biosynthesis requires upfront acid or enzymatic treatment to release D-fructose, D-glucose, or D-xylose from these polysaccharides before microbial fermentation process.

Metabolic pathways of selected microorganisms for the biosynthesis of D-allulose require genetically engineering based on understanding the actual metabolic pathways for these selected microorganisms to alter these microorganisms metabolic pathways by genes manipulations techniques for the deletion of unnecessary enzymes genes, and for overexpression necessary enzymes genes to direct the selected microorganism metabolic pathway toward the biosynthesis of D-allulose with less intermediate metabolites as impurities in the microbial fermentation process. This advance research of metabolic pathway engineering for microbial cells for D-allulose biosynthesis showing promising results for future high D-allulose production yield at lower cost by microbial fermentation method.

9. Conclusion

The current method for D-allulose production is based on the Izumoring strategy for the epimerization of D-fructose into D-allulose by immobilized D-ketose-3-epimerase enzymes family or by immobilized whole cells producing these intracellular D-ketose-3-epimerase enzymes family. These immobilization methods still show high D-allulose production costs. Ongoing research to Improve enzymes activity and properties for higher D-allulose production yield by these immobilization methods is very important to reduce D-allulose production cost. The advantage of microbial fermentation method research for D-allulose production, is the potential for future utilizing cheap carbohydrates byproducts from agriculture and food industry as a carbon source for D-allulose production. This research on microbial fermentation method for D-allulose production is promising and considered to be the ideal route for future D-allulose production due to its simplicity and lower production cost.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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