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Org. Process Res. Dev., **Just Accepted Manuscript** • DOI: 10.1021/acs.oprd.0c00024 • Publication Date (Web): 04 May 2020

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D-Serine as a Key Building-Block : Enzymatic Process Development & Smart Applications within Cascade Enzymatic Concept

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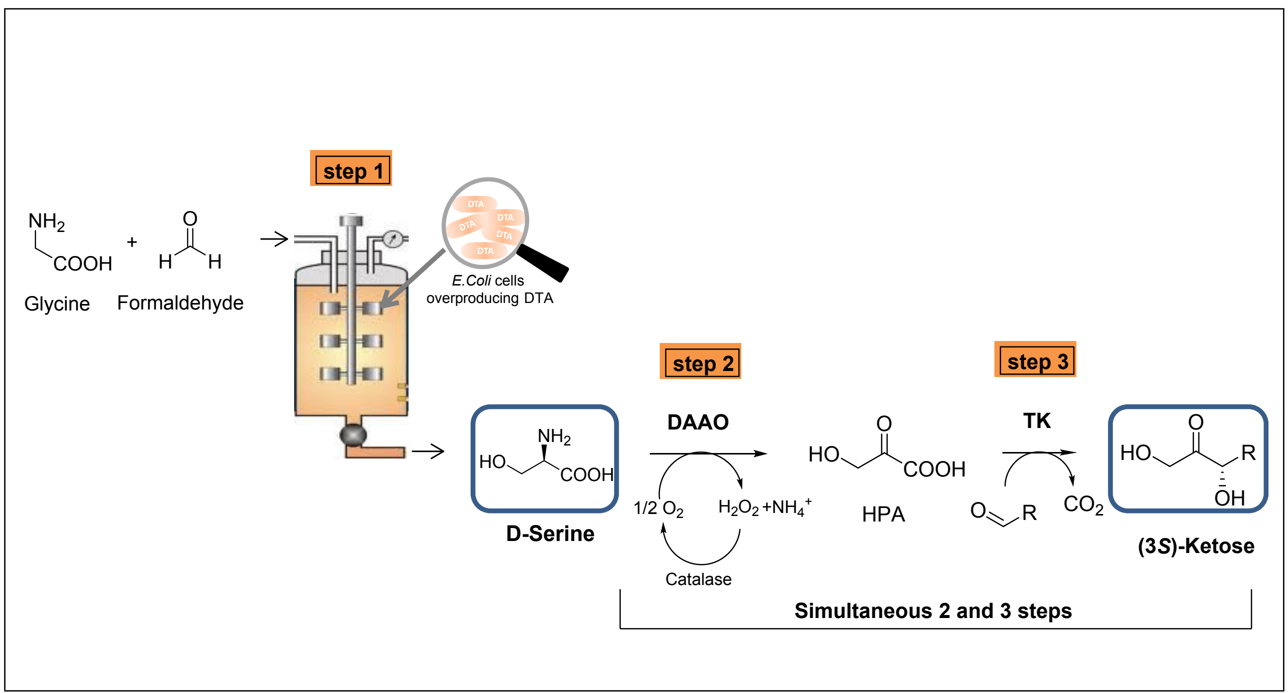

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ABSTRACT. An efficient enzymatic way catalyzed by an enzyme from D-Threonine Aldolase family was developed for D-serine production at industrial scale. This process was used to the synthesis of two valuable ketoses, L-erythrulose and D-fructose, within cascade enzymatic concept involving both other enzymes. Indeed, D-serine was used as a substrate of D-amino acid oxidase for the *in situ* generation of the corresponding ketoacid, hydroxypyruvate, a key donor substrate of Transketolase. This enzyme catalyzed the

irreversible transfer of the ketol group from hydroxypyruvate to an aldehyde acceptor to form a (3*S*)-ketose by stereoselective carbon-carbon bond formation. The compatibility of all enzymes and substrates allowed to perform a sequential three step enzymatic process avoiding the purification of intermediates. This strategy was validated with two TK aldehyde substrates to finally obtain the corresponding (3*S*)-ketoses with a high control of the stereoselectivity and excellent aldehyde conversion rates.

KEYWORDS. D-Serine, D-Threonine Aldolase, D-Amino Acid Oxidase, Transketolase, Ketoses

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INTRODUCTION

Small chiral molecules in the pharmaceutical pipeline becoming more and more complex, their asymmetric synthesis requires multistep processes. The mild conditions and the high specificity of enzymes have led to the development of efficient biocatalytic strategies in the synthesis of bulk chemicals and drugs.[1,2]

Enzymatic one pot synthesis provide competitive strategies to the chemical approaches, by using simultaneously two or more enzymes in cascade avoiding the isolation of intermediates, thus saving time, energy, and limiting waste. The drawbacks of this procedure such as inhibition of the substrate / product / reagent or the incompatibility of the reaction condition, can be circumvented by a sequential one-pot procedure.[3]

For natural and unnatural aminoacids and monosaccharides synthesis, enzymatic reactions are particularly useful to generate asymmetric centers with high

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stereoselectivity, avoiding protection and deprotection steps required by the chemical ways. Enzymes catalyzing a stereospecific carbon-carbon bond formation such as aldolases and transketolases became very useful for obtaining such compounds.[4] Amoung these enzymes, threonine aldolases (TA) have been particularly used for the synthesis of optically pure natural and non-natural amino acids.[5] In the alternative pyridoxal-5′-phosphate (PLP) synthesis pathway, TAs catalyze the retro-aldol cleavage of threonine to form glycine and acetaldehyde.[6] L- or D-TAs are highly selective at the α-carbon of threonine but, only a few native DTA genes have been described.[7] The catalytic mechanism of these enzymes needs PLP and divalent metal ions such as Co^{2+}, Ni^{2+}, Mn^{2+} or Mg^{2+} as a cofactors. For biocatalytic applications, the reversibility of the retro-aldol TAs-catalyzed reaction is of great interest for the synthesis of optically pure natural and non-natural amino acids. The synthetic reactions and the substrate scope of TAs from different microorganisms were recently reported.[8] These enzymes have notably been used for the synthesis of L- and D-β-hydroxy-α-amino acids starting from achiral aldehydes and glycine in creating two chiral centers.[9]

In the area of monosaccharides synthesis, beside enzymatic aldose-ketose isomerization,[10] transketolases (TK EC 2.2.1.1) are also popular.[11] TKs are thiamine diphosphate-dependent enzymes, and catalyze the transfer of a two-carbon unit (ketol group), from a donor to an aldehyde acceptor, leading to a C_{n+2} (3*S*)-ketose by the stereoselective formation of the C2-C3 bond. (Scheme 1) In pentose phosphate metabolic

pathway, TK reversibly transfers a ketol group from a ketose phosphate to an aldose phosphate. Previous *in vitro* studies showed that TK accepts non-phosphorylated substrates leading to the corresponding free α–hydroxyketones. Particularly, hydroxypyruvate (HPA) can be used as donor rendering the reaction irreversible due to carbon dioxide release. This major advantage has been largely applied in the presence of various aldehydes as acceptors for the synthesis of corresponding ketoses in one step. (Scheme 1).[11]

Scheme 1: Cascade reactions involving Transaminase or D-Amino Acid Oxydase-catalyzed reactions coupled with TK for *in situ* generation of HPA.

The main problems of the TK-catalyzed reaction for biocatalytic applications at industrial-scale, are in one hand the HPA synthesis, commonly obtained from toxic bromopyruvic acid in the presence lithium hydroxide, with a moderate yield[12] and in the other hand, its unstability in water at temperature superior at 25°C.[13] To circumvent all these limitations, enzymatic *in situ* generation of HPA has been previously reported, in particular from L-serine using transaminases [14] and more recently, from D-serine using D-amino acid oxidase (DAAO) (Scheme 1).[15] This last way offers major advantages compared to transaminase strategy, such as an irreversible reaction and no requirement of an additional substrate.

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Scheme 2: One pot three-steps enzymatic cascade catalyzed by a crude extract of *E. coli* strain P3005 and purified D-amino acid oxidase from *Rhodotorula gracilis* ($DAAO_{Rg}$) and transketolase from *Geobacillus stearothermophilus* (TK_{gst}) with three different TK aldehyde substrates : glycolaldehyde (R = $-CH_2OH$), and D-erythrose (R= -CHOH(*R*)-CHOH(*R*)-CH_2OH).

In this work, we first carried out a detailed investigation of D-serine production at industrial scale catalyzed by DTA overexpressed in *Escherichia coli* strain from achiral compounds glycine and formaldehyde. This strategy was then applied in a sequential three step cascade enzymatic concept for the synthesis of two valuable (3*S*)-ketoses (Scheme 2). In a first step, D-TA cell free extract produced D-serine. Then, two simultaneous enzymatic steps were developed at laboratory scale. DAAO from *Rhodotorula gracilis* ($DAAO_{Rg}$) transformed D-serine into the corresponding ketoacid, hydroxypyruvate[16] used by TK from *Geobacillus stearothermophilus* (TK_{gst})[13] in the presence of an aldehyde as acceptor for obtaining the ketose product. The one pot three steps process was validated using three TK aldehyde acceptors, glycolaldehyde and D-erythrose for the synthesis of

corresponding valuable (3*S*)-ketoses, L-erythrulose, a tanning agent in the cosmetics industry,[17] and D-fructose, a sweetener with very low glycemic index.[18]

RESULTS AND DISCUSSION

D-serine Enzymatic Process Development from whole cells

The first step of the cascade reaction consists in the production of D-serine from glycine and formaldehyde as substrates with DTA (P3005) enzyme overproduced in *Escherichia coli strain* (whole cells). This step was developed by Seqens Company and applied to the production of D-serine in an enantiomerically pure form at multiton scale per year. The enzyme candidate DTA (P3005) was selected among off-the-shelf enzymes portfolio of Seqens company[19] which provided high level of enantioselectivity (*ee* >99%) required for fine chemicals application.

HOOC–CH2–NH_2 → (D-TA E3005 (in *E.coli*); HCHO (37% aq.); PLP, $MgCl_2$; 30°C) → HO–CH2–CH(NH_2)–COOH

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Scheme 3: Enzymatic aldolization of glycine with formaldehyde by biocatalyst DTA P3005 expressed in *E. coli* for selectively producing D-serine.

Firstly, the fermentation process of *Escherichia coli* overproducing DTA was developped to ensure production in large fermenters (Figure 1 and SI). The optimal conditions led to 55 g of wet cells per liter of culture medium. The figure below is a representation of successive steps required for the large manufacturing fermentation process.

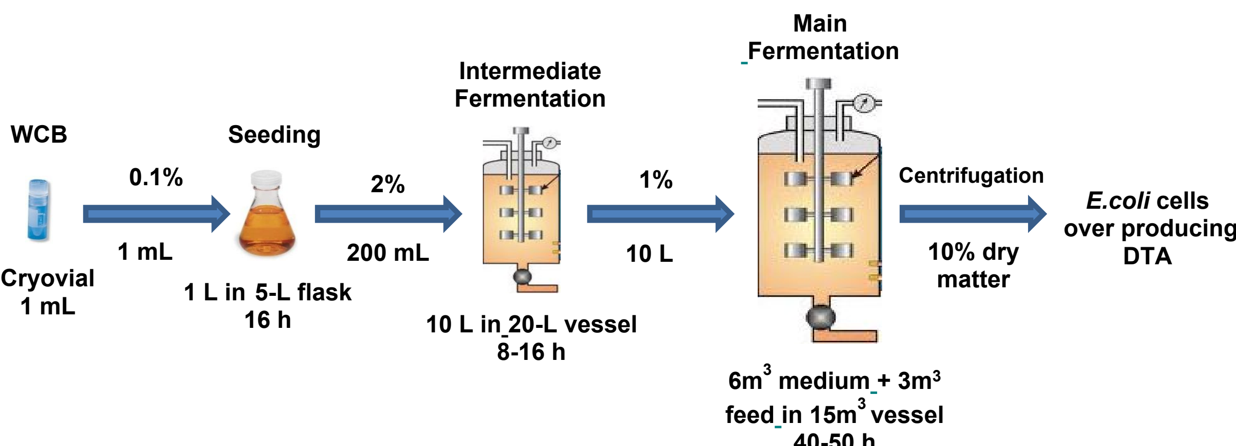

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Figure 1: Different steps of fermentation process flow for the manufacturing production of *E.coli* cells.

At first, we developed the fermentation process of the DTA enzyme overexpressed in *Escherichia coli*. To define the fermentation process, a Statistical Experimental Design has been carried out in order to determine the critical and important parameters, such as oxygen concentration, glucose feed and its optimal concentration, and other medium components impact. This approach allowed us to determine a robust domain of operations for producing the overexpressed enzyme. The Fermentation at different scales, from lab scale (1 liter) to several cubic meters volume fermenter size (up to working capacity of 9 m^3), have been implemented successively before ensuring production in large fermenters (Figure 1).

The biomass (whole cells) was then used for the synthesis of D-serine from glycine and formaldehyde. The optimal conditions to ensure high performance, high productivity and robustness for multi-ton manufacturing of D-serine were determined (Table 1).

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Process parameters	Optimal values
pH	7 ± 0,5
$MgCl_2$	41.2 mM
PLP	2.12 mM
Glycine	1.33 M
Formaldehyde	1,2 M
Base	KOH (0.3 M)
Temperature	30°C

Table 1: Optimal conditions for glycine and formaldehyde biotransformation into D-serine by whole cells overproducing DTA.

The reaction was carried out in water with glycine (1.33 M, 300g.L^{-1}) and formaldehyde (37% aq. 1.2 M) with *E. coli* P3005 cells (0.6 g of wet cells.L^{-1}).

PLP was the cofactor required for the catalytic mechanism of DTA and a better conversion rate was observed when 2.12 mM of PLP was added in the medium (SI, chart 1). In addition, the optimal temperature was 30°C because at higher temperature (40-50°C), the conversion rate was rapidly limited by the degradation of formaldehyde and the

formation of significant amounts of by-products (data not shown) yielding to an inhibition of the enzymatic activity (SI, Chart 2).

The effect of pH in the 6-8 range has also been investigated showing that the optimal conversion of substrates was obtained at pH 7 - 7.5 (SI, Chart 3). The pH must be continuously controlled during the reaction because an acidification occurs probably due to the formaldehyde addition. Indeed, typical commercial aqueous solution of formaldehyde 37% has a pH of 3 (certainly due to residual formic acid presence). Finally, in order to avoid local racemization during the feed of the base solution, we determined the best compromise between base nature impact on the reaction rate and the enantiomeric excess of D-serine product. The influence of three different bases was studied (SI, Chart 4). NH_4OH and KOH solutions gave the best conversion rates avoiding potential racemization of D-serine. The conversion in the presence of KOH was significantly better and did not exhibit any side by-products, according to the HPLC analysis.

Using these optimal conditions (Table 1), D-serine was produced at kg scale. After 24 h both substrates were almost fully converted (90 %). The reaction mixture was then concentrated by heating to 40°C under vacuum followed by the elimination of the whole cells by centrifugation. The crude residue containing D-serine was treated with charcoal to remove the salts and then filtered. Finally, a recrystallization in a water/alcohol mixture gave pure D-serine with an isolated yield of 70 %. with an excellent enantiomeric excess of 99% determined by HPLC. This process offers many advantages compared to the other

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microbial procedures[21] such as full conversion of achiral, cheap and affordable substrates, no requirement of enzyme purification, and high stereoselectivity.

Smart Application to ketose production within cascade enzymatic concept

For the cascade enzymatic concept, the first step was the production of D-serine from glycoladehyde and glycine and was conducted with DTA cell free extract from *E.coli* cells obtained previously. This reaction was performed independently because formaldehyde is an inhibitor of DAAO used in the second step and can also be a substrate of TK used in the third step. The DTA (cell free extract)-catalyzed reaction was studied using 50 mM and 100 mM substrate concentrations (Table 2). Lower concentrations were not chosen because we previously showed that the best result for the D-serine conversion catalyzed by $DAAO_{Rg}$ was obtained at 50 mM.[15]

Table 2. Influence of substrate concentrations on D-serine production using DTA (cell free extract,CFE)[a].

Substrates (mM)	Reaction	Glycine

Glycine	Formaldehyde	time (h)°	conversion rate (%)[b]
50	50	24h	94
100	100	24h	82

[a] DTA CFE (3.5 mg of proteins) ; [b] determined by *in situ* ^{1}HNMR

A concentration of 50 mM for both substrates was used to perform the reaction because an almost complete substrate conversion of both substrates (94%) was obtained after 24h against 82% with 100 mM of both substrates. The reaction mixture was treated with methanol to precipitate proteins and to avoid by-products formation from substrates and reagents used in steps 2 and 3. After filtration and methanol evaporation, D-serine was used in the second part of the process in which both $DAAO_{Rg}$ and TK_{gst} enzymes were coupled for the synthesis of the ketoses.

$DAAO_{Rg}$,[16] overexpressed in *E. coli* and purified by Ni^{2+} chelating affinity column chromatography as reported earlier (SI), was used to convert D-serine into HPA. The choice of this enzyme can be explained by its broad amino acid spectrum including D-serine while the best substrates for L-AAOs are hydrophobic amino acids.[22] In addition,

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$DAAO_{Rg}$ showed much higher turnover number, better stability under different reaction conditions, and a larger active site to accommodate various substrates than the other source of DAAOs.[23] DAAOs (EC 1.4.3.3) are flavin adenine dinucleotide (FAD)-containing flavoenzymes that catalyze the deamination of D-amino acids to their corresponding imino acids. FAD is concomitantly reducted and then re-oxidized by molecular oxygen with generation of hydrogen peroxide. The released imino acid spontaneously led to the corresponding α-keto acid and ammonia (Scheme 2).

The thermostable TK_{gst} was selected for its properties compatible with industrial applications such as high stability and robustness against unusual conditions, reusability after immobilization[24] and evolvability.[25] TK_{gst} was overexpressed in *E. coli* and purified by Ni^{2+} chelating affinity column chromatography as reported earlier (SI).[13]

The strategy was based on the HPA *in situ* generation by $DAAO_{Rg}$ from D-serine (obtained previously with DTA) and its use as donor substrate by TK_{gst}-catalyzed reaction in the presence of an aldehyde as acceptor. To validate this simultaneous two-step cascade reaction, two aldehydes having an increased carbon chain length were chosen, such as

glycolaldehyde C_2 (**1**), and D-erythrose C_4 (**2**) leading to the corresponding valuable C_{n+2} ketoses, L-erythrulose C_4 (**3**), and D-fructose C_6 (**4**) respectively (Table 3).

The influence of enzyme quantity and TK aldehyde substrate concentration were studied and the best conditions are indicated in table 3.

Table 3. Results obtained by coupling $DAAO_{Rg}$ and TK_{gst} for ketoses **3** and **4** synthesis from aldehydes **1** and **2** and D-serine.

TK acceptors Aldehydes[a]	TK products Ketoses	$DAAO_{Rg}/TK_{gst}$ (mg)[a]	Aldehyde conversion rate (%)[b]	Reaction Time (h) [c]	Isolated product yield (%)	*e.e.* or *d.e.* (%)[d]
1	**3**	2/6	82	8	60%	>95%
2	**4**	2/6	>95	8	57%	>95%

[a] Reactions were carried out with $DAAO_{Rg}$ (2 mg, 86 U) and TK_{gst} (6 mg, 42 U), ThDP (0.1 mM), $MgCl_2$ (1 mM), aldose (50 mM), D-serine (50 mM) at pH 7 and 25°C.[b] Alehyde

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4 acceptor conversion determined by *in situ* ^{1}H NMR analysis. [c] ^{1}H NMR of reaction mixture
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6 (SI).[d] Enantiomeric excess (*e.e.*), diastereoisomeric excess (*d.e.*) determined by ^{1}H NMR.
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9 According our previous studies, D-serine and aldehyde **1** or **2** were used at a
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12 concentration of 50 mM. A minimum amount of $DAAO_{Rg}$ and TK_{gst} was used and the ratio
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16 of both enzymes allowed a total conversion of D-serine avoiding the accumulation of HPA.
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19 Hence, the bi-enzymatic cascade led to an almost total (82 % and 95 % for **1** and **2**
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23 respectively) and rapid (8 h) conversion of both substrates as observed by *in situ* ^{1}H NMR
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26 (Table 2). The purification and the characterization of ketoses **1** and **2** showed high
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30 enantio- and diastereoselectivity (> 95 %) and good isolated yields (57-60 %).
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36 CONCLUSIONS
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40 An efficient enzymatic way catalyzed by D-TA overproduced in *Escherichia coli* was first
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42 developed in whole cells for D-serine production at industrial scale offering many advantages
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44 compared to the other microbial procedures[21] such as full conversion of achiral, cheap and
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47 affordable substrates, no requirement of enzyme purification, and high stereoselectivity. Then, a
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50 smart application of DTA-catalyzed process using crude extract was applied to ketose
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54 production within cascade enzymatic concept involving both other enzymes, DAAO and
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TK. The three enzymatic steps were performed with cell free extracts for the synthesis of two valuable (3*S*)-ketoses, L-erythrulose **3** and D-fructose **4** with good yields and excellent stereoselectivities. This process allowed to resolve the major drawback of TK-catalyzed reaction for potential industrial applications. Indeed, HPA the key TK donor substrate rendering the reaction irreversible was *in situ* generated by DTA and then DAAO from achiral glycine and formaldehyde according anenvironmentally friendly procedure. This strategy offers a competitive way to the commonly used chemical synthesis of HPA from bromopyruvate[12] and to the *in situ* HPA generation by transaminase-catalyzed reaction.[14c,d] Since the three enzymes are active under similar conditions, this cascade synthesis avoided the isolation of intermediates and reduced waste.

The entire process could be further optimized, especially by co-immobilization of all enzymes to ensure their reusability and by performing the conversion in a continuous reactor for higher scale production of ketoses. The procedure could also be extended to TK variants or to other HPA-dependent enzymes in place of wild-type TK for obtaining a wide range of (3*S*)-ketoses and analogs.

EXPERIMENTAL SECTION

General. All chemicals were purchased from Sigma-Aldrich, Alfa-Aesar and CarboSynth. Bradford reagent was from Bio-Rad. Ni-NTA resin was obtained from QIAGEN. Lyophilization was carried out with Triad LABCONCO dryer. UV-visible absorbance was measured using a Spark control 10 microplate reader from TECAN and an Agilent Technologies, Cary 300 UV-Vis spectrophotometer enabling Peltier temperature control. Liquid Flash Chromatography were performed with MARCHEREY-NAGEL GmbH & Co KG 60/40-63 mesh silica gel and for Thin Layer Chromatography MARCHEREY-NAGEL GmbH & Co KG 60 F254 silica gel TLC plates with anisaldehyde stain for detection was used. A pH autotitrator (TitroLine 7000) allowed to continuously adjust the pH of the reaction. NMR spectra were recorded in D_2O or DMSO on a 400MHz Bruker Avance III HD spectrometer. Chemical shifts are referenced to the residual solvent peak. The following multiplicity abbreviations were used: (s) singlet, (d) doublet, (t) triplet, (m) multiplet. DTA-catalyzed reaction was monitored with HPLC Agilent with a Spherisorb column (CN – 250 x 0.46 – 5 µm – 80 Å) and a flow at 0.2 mL . min^{-1} (eluent: aqueous formic acid 0.05%). The enantiomeric excess of serine produced with DTA from glycine and formaldehyde was determined with HPLC (waters 2487) with a Crownpack column (Cr(-), 5 µm , 150 x 4 mm), using a flow at 0.2 mL . min^{-1} (MeOH/$HClO_4$ aq (pH 1) : 10/90), at 30°C, and a detection at 200 nm.

***In situ* ^{1}H NMR measurements.** Progress of preparative scale enzymatic synthesis were monitored by using quantitative *in situ* ^{1}HNMR relative to 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP-d_4) as internal standard. Aliquots of the reaction mixture were removed overtime (450 µL) and mixed with 50 µL of TSP-d_4 (50 mM in D_2O).

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Obtention of *E.coli* cells overproducing DTA. *Escherichia coli* strain BL21DE3 was used for DTA overexpression with the plasmid pET26b+. This strain was incubated at 37°C, 180 rpm for 16h in LB medium containing kanamycin at 30 µg/mL (PC1). 0.2 liter of the pre-culture was used to inoculate 1 liter of culture medium containing 30 µg . mL^{-1} of kanamycin and grown at 37°C, 180 rpm. Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.15 mM was added when the OD_{600nm} was 0.7-0.8. The cells were then grown overnight at 37°C, under stirring at 180 rpm, and harvested by centrifugation at 3600 g at 4°C for 30 min. Bacterial pellets were frozen and stored at -80°C (200 g of wet cells . L^{-1}).

D-serine production from whole cells at industrial scale. Glycine (4.4 M, 13.3 mol, 1 kg), formaldehyde (4 M, 12 mol, 0.9 kg), PLP (6.7 mM, 0.02 mol, 5 g), $MgCl_2$ (13.3 mM, 0.04 mol, 0.08 kg) were introduced in a suspension of *E.coli* cells (0.02 kg cells . L^{-1}) at 30°C. The pH was continuously adjusted at 7-7.5 with KOH (1 M). After 24 hours, the reaction mixture was then acidified at pH 6 with a solution of phosphoric acid (0.7 M) and then concentrated (at 40°C max) by reaching a final volume of 4 liters. The whole cells were then eliminated by filtration on charcoal adjuvant. The crude residue containing D-serine was treated with charcoal to remove the salts and then filtered. Finally, a recrystallization in a water/alcohol mixture gave pure D-serine as a white powder with an isolated yield of 70 % and with an excellent enantiomeric excess of 99% determined by HPLC.

Obtention of cell free extract from *E.coli* whole cells. Harvested recombinant cells from 1 liter of culture were resuspended in 50 mL of phosphate buffer (0.2 M) at pH 7. The cells were disrupted

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with a French Press at 2 kbar. After sonication of the lysate (3 cycles of 30 seconds each at 60W on ice), cellular debris were removed by centrifugation during 30 min at 20 000 g. The supernatant was centrifuged a second time in the same conditions.

D-serine synthesis catalyzed by cell free extract D-TA P3005. To the solution containing glycine and formaldehyde (50 mM, 1 mmol, 30 mg) or (100 mM, 2 mmol, 60 mg) were added PLP (0.05 mM, 0.001 mmol, 0.25 mg), $MgCl_2$ (10 mM, 0.2 mmol, 19 mg) and cell free extract of D-TA P3005 (133µL of CFE containing 3.5 mg of proteins) in H_2O. The pH was adjusted at 7.3 with 0.1M NaOH. After 24 h, methanol (twice the reaction mixture volume) was added. After 16 h at 4°C, the solution was filtrated and evaporated under reduced pressure. D-serine was characterized by HPLC and ^1H NMR (SI) and was obtained as a white solid with 97% yield.

Synthesis of ketoses 3 and 4 catalyzed by purified TK_{gst} and $DAAO_{Rg}$. ThDP (0.1 mM, 0.002 mmol, 0.85 mg), $MgCl_2$ (1 mM, 0.02 mmol, 1.9 mg), D-serine previously produced using DTA (50 mM, 1 mmol, 105 mg) and glycolaldehyde (50 mM, 1 mmol, 60 mg) or D-erythrose (50 mM, 1 mmol, 120 mg) were dissolved in H_2O and the pH was adjusted to 7 with 0.1 M NaOH. In this reaction mixture were introduced catalase (1 mg / 2 h, 1998-4995 U / 2 h), $DAAO_{Rg}$ (2 mg, 86 U). TK_{gst} was then added (6 mg, 42 U) in three portions (2 mg, 14 U at initial time, 2mg, 14 U at 2 h 30 and finally 2 mg, 14 U at 5 h) giving a final volume of 20 mL. Oxygen was bubbled into the reactor (10 mL · min^{-1} at atmospheric pressure). The reaction mixture was stirred (100 rpm) at 25°C and the pH was automatically maintained at 7 by adding 0.1 M HCl using a pH stat. D-serine and aldehydes consumption were monitored by *in situ* ^1H NMR and TLC. The reactions were stopped after total conversion aldehyde and enzymes were removed by precipitation and centrifugation. Silica was added to the supernatant and the suspension was concentrated to dryness

under reduced pressure. The powder was loaded onto a flash silica column. Products **3** and **4** were purified using eluent CH_3-$COO(C_2H_5)$/CH_3OH (9/1) and CH_2Cl_2/CH_3OH (9:1) respectively.

(3*S*)-1, 3, 4-trihydroxy-butan-2-one 2 (L-erythrulose) 3. Compound **3** was isolated as a yellow oil; yield : 160 mg (44%). $[\alpha]_D^{25}$= +6.3 (c 1.48, H_2O), lit. $[\alpha]_D^{25}$= +6.3 (c 1.1, H_2O),[28] $[\alpha]_D^{25}$= +6.7 (c 1.48, H_2O).[29] NMR spectra were identical to those previously described.[28] ^{1}H NMR (400 MHz, MeOD): d=4.52 (d, J=19.3 Hz, 1H, 1-HA), 4.45 (d, J=19.3 Hz, 1H, 1-HB), 4.26 (t, J=4.2 Hz, 1H, 3-H), 3.80 (dd, J=11.6, 4.5 Hz, 1H, 4-H), 3.76 (dd, J=11.6, 4.0 Hz, 1H, 4-H); ^{13}C NMR (101 MHz, MeOD): d=212.98 (C-2), 77.82 (C-3), 67.75 (C-1), 64.86 (C-4). m/z HRMS found $[M+HCOO]^-$ 165.0405, $C_5H_9O_6$ requires 165.0396.

(3*S*,4*R*,5*R*)-1,3,4,5,6-pentahydroxyhexan-2-one (D-fructose) 4. Compound **4** was isolated as colorless oil (118 mg, 65% yield). NMR data were identical to those previously described (ratio :73/22/5; lit. Ratio : 75/21/4).[30,31] ^{1}H NMR (400 MHz, D_2O) : δ (ppm) β-D-fructo-2,6-pyranose 4.09-4.04 (m, 1H, H6a); 4.04-4.02 (m, 1H, H5); 3.93 (dd, *J*=10.0Hz, *J*=3.5Hz, 1H, H4); 3.83 (d, *J*=10.0Hz, 1H, H3); 3.76-3.72 (m, 2H, H6b, H1a); 3.60 (d, *J*=11.7Hz, 1H, H1b), β-D-fructo-2,5-furanose 4.17-4.13 (m, 2H, H3, H4); 3.84 (m, 1H, H5); 3.83 (m, 1H, H6a); 3.69 (m, 1H, H6b); 3.63 (d, *J*=12.1Hz, 1H, H1a); 3.58 (d, *J*=12.1Hz, 1H, H1b), α-D-fructo-2,5-furanose 4.17-4.13 (m, 2H, H3, H5); 4.05-4.01 (m, 1H, H4); 3.86-3.82 (m, 1H, H6a); 3.74-3.70 (m, 1H, H6b); 3.72-3.68 (m, 2H,H1a/b). ^{13}C NMR (101 MHz, D_2O): δ (ppm) β-D-fructo-2,6-pyranose 99.12 (C2); 70.72 (C4); 70.25 (C5); 68.61 (C3); 64.94 (C1); 64.42 (C6), β-D-fructo-2,5-furanose 102.54 (C2); 81.72 (C5); 76.44 (C3); 75.49 (C4); 63.74 (C1); 63.43 (C6), α-D-fructo-2,5-furanose 105.49 (C2); 83.0 (C3); 82.37 (C5); 77.09 (C4); 63.96 (C1); 62.15 (C6). m/z HRMS found $[M+Cl]^-$ 215.0317, $C_6H_{12}O_6Cl$ requires 215.0321.

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ASSOCIATED CONTENT

Supporting Information.

Production of DTA, $DAAO_{Rg}$ and TK_{gst}, ^{1}H NMR, ^{13}C NMR spectra of ketoses **3** and **4**.

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ACKNOWLEDGMENTS

Funding Sources

This work was funded by the Fonds Régional Innovation Laboratoire (grant DOS00494484/00, to L.H.).

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