RESEARCH ARTICLE

Cooperative enhancement of aldoxime dehydratase stability through whole-cell immobilization and flow reactor integration

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Dedication: This work is dedicated to Ivan Lavandera, a remarkable scientist and man whose kindness and warmth left a lasting impact on all who had the good fortune to know him.

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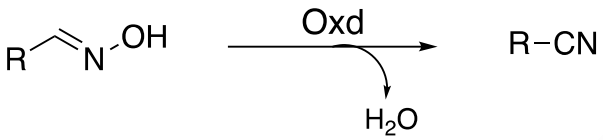
Abstract: This study investigates the synthesis of aromatic nitriles using an evolved variant of OxdF1 (L318F/F306Y), an aldoxime dehydratase from *Pseudomonas putida* F1, engineered for improved catalytic efficiency towards benzaldehyde oxime. The double OxdF1 (L318F/F306Y) mutant effectively catalyses the conversion of various benzaldoxime derivatives to the corresponding nitriles. Due to the enzyme's inherent instability, immobilized whole-cell systems were employed in a flow reactor to improve its stability and broaden its applicability, with the biotransformation of benzaldehyde oxime and 2,6-difluorobenzaldehyde serving as case studies. The enzyme's stability was markedly improved, maintaining a 87% yield even after 8 hours of processing in the preparation of benzonitrile. Production of 2,6-difluorobenzontirile posed additional challenges due to the low water solubility of both the substrate and, even more so, the product, an important intermediate in various chemical applications. To overcome solubility limitations, a segmented liquid-liquid flow system (water/cyclohexane) was implemented, significantly improving the enzyme stability. The process was run continuously for 12 hours, with a conversion of approximately 70% by the end of the operation. Furthermore, 2,6-difluorobenzonitrile was selectively extracted in-line using a liquid-liquid extractor, thus facilitating its efficient recovery and purification.

1. Introduction

Nitriles are key functional groups in organic chemistry, present in both building blocks and final products of numerous important molecules, including herbicides, antimicrobials, antivirals, as well as in the polymer and fragrance industries[1–8]. Their application in perfumery is particularly commercially attractive, as nitriles exhibit similar sensory properties like their corresponding aldehydes while offering enhanced stability, especially in acidic environments[9]. This stability makes certain nitriles particularly suitable for fragrance formulations[10]. Moreover, nitriles are highly versatile since they can be readily converted into a wide range of derivatives, such as amines and aldehydes *via* reduction, imines and ketones through nucleophilic addition, or carboxylic acids and amides *via* hydrolysis[11–18].

The industrial production of nitriles has well-established synthetic methods, such as the direct cyanation of alkenes with hydrogen cyanide[19], the Sandmeyer reaction[20], and ammoxidation[21]. However, these approaches have significant drawbacks, including the need for stoichiometric amounts of hydrogen cyanide, the requirement for high temperatures and pressures, and the generation of toxic byproducts. Although in recent years more sustainable methods have been developed for nitrile synthesis, many still rely on transition metal catalysts, particularly palladium[22] and ruthenium[23]. Cyanide-free methods for preparing nitriles offer a safer and greener alternative. One of the most promising strategies for nitrile production involves dehydration of aldoximes, an attractive process as it produces only water as a byproduct[24,25] (Scheme 1).



Scheme 1. Dehydration of aldoximes catalyzed by aldoxime dehydratases (Oxds).

This reaction is catalyzed by a class of enzymes called aldoxime dehydratases (Oxds) (EC 4.8.1.2-4.8.1.4), which utilize a prosthetic heme group at the enzyme's active site. The heme undergoes a redox-dependent change in the coordination structure of the aldoxime–heme complex, as proposed by Kobayashi et al[26]. Over the past 30 years, numerous Oxd enzymes have been discovered and utilized as recombinant catalysts, primarily for the dehydration of aliphatic oximes[27–29]. However, only a few, such as OxdRE and OxdF1, are capable of efficiently working on aromatic and aryl-aliphatic oximes[30–32]. These Oxds share a narrow optimal pH range (5-8) and temperature range (28-35 °C), with generally low thermostability, resulting in half-lives of only a few hours[33,34].

A common strategy to enhance the stability of enzymes involves their immobilization onto various supports, enabling catalyst reuse across multiple reactions and improving the sustainability of the process[35]. While several approaches have been explored using purified Oxds or raw extracts after sonication[9,36,37], the resulting catalysts have shown only moderate retained activity after reuse and limited stability in the presence of organic (co)solvents[9]. Another approach is the immobilization of whole cells without further manipulation, allowing the enzyme to remain within the native intracellular environment and benefiting from the bacterial membrane as a semipermeable protective layer[36]. Once immobilized, the catalyst could theoretically be used for prolonged reaction times, multiple batch cycles, and, as a heterogeneous catalyst, could be integrated into flow reactors to minimize substrate/product inhibition and mitigate the negative effects of co-solvents[38]. Only a limited number of studies have applied flow reactor systems to Oxds, demonstrating excellent improvements in activity but offering limited insight into their suitability for prolonged reaction times and extended continuous processes..[39,40].

In this work, we selected the double mutant OxdF1 (L318F/F306Y)[41] as one of the most promising catalysts for the synthesis of benzonitrile derivatives from the corresponding oximes. We further investigated its substrate specificity, evaluating both whole-cell preparations and the isolated enzyme. In this context, we focused on the production of 2,6-difluorobenzonitrile, which is typically synthesized by fluorinating 2,6-dichlorobenzonitrile with potassium fluoride[42–44]. 2,6-difluorobenzonitrile is a valuable and versatile intermediate in the synthesis of herbicides[4], insecticides[45], and serves as a membrane adsorber in uranium extraction and enrichment[46]. Additionally, it is a precursor for 2,6-fluorobenzamide[47,48], a compound with promising potential as a potent antiprotozoal drug candidate[49]. It is also being investigated for novel psoriasis treatments[50] and explored for its potential as an HIV-1 inhibitor[51].

2. Results and Discussion

2.1 Batch reactions with free enzyme and whole cells

The OxdF1 (L318F/F306Y) variant, previously engineered for enhanced activity toward benzaldehyde oxime[32], was expressed using the conventional pET system in BL21 *E. coli* cells. Protein expression was induced with 0.1 mM IPTG for 16 hours at 18 °C yielding up to 20 mg of OxdF1 (L318F/F306Y) per gram of wet cells, after purification by affinity chromatography (see Fig.S1-S3). The activity of OxdF1 (L318F/F306Y) was assessed against a range of aromatic and aryl-aliphatic substrates (Figure 1), some of which had been previously evaluated in cascade reactions[30].

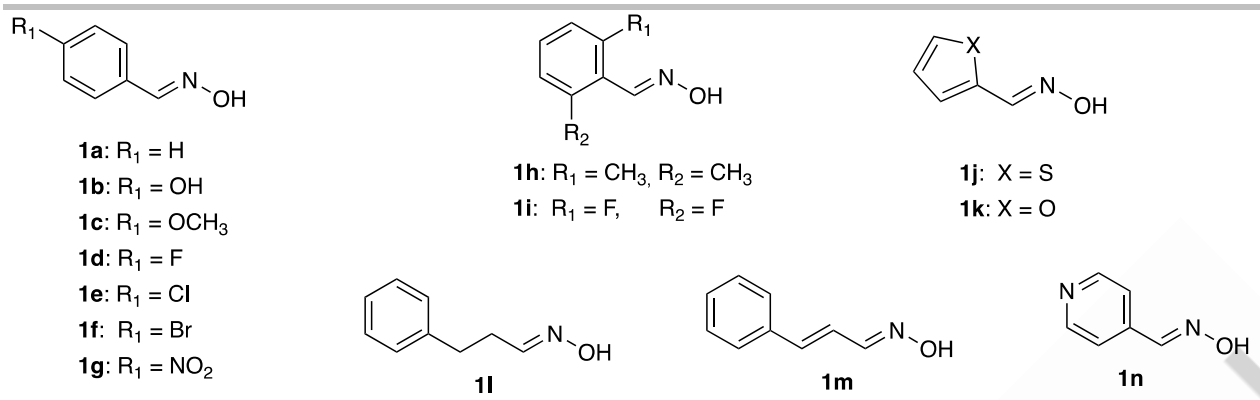


Figure 1. Panel of aldoximes (**1a**-**1n**) tested as substrates.

Biotransformations were carried out using both the purified enzyme and recombinant whole cells (Table 1). Reactions with the free enzyme included 5 mM sodium dithionite as a reducing agent, as purified aldoxime dehydratases are known to exhibit limited stability under oxidizing conditions. For whole cells biocatalysis, 30 mg/mL of wet cells (corresponding to 0.5-0.6 mg/L of enzyme) were used to enable a direct comparison with the results obtained employing the free enzyme.

Table 1. Dehydration of aldoximes **1a**-**1n** with OxdF1 (L318F/F306Y). Reaction with the free enzyme: 0.5 mg/mL of protein, 20 mM of aldoximes in the presence of 10 % ethanol, HEPES buffer pH 7.0, 28 °C, 140 rpm, 5 mM sodium dithionite; reaction with whole cells: 30 mg/mL of wet cells (5.9 $mg_{dry\ weight}$/mL), 20 mM of aldoximes in the presence of 10 % ethanol, HEPES buffer pH 7.0, 28 °C, 140 rpm. Conversion determined by UHPLC.

	Free enzyme		Whole cells	
Substrate	Conv. 1 h (%)	Conv. 8 h (%)	Conv. 1 h (%)	Conv. 8 h (%)
1a	> 97	> 97	> 97	> 97
1b	10	12	10	75
1c	10	15	8	38
1d	90	95	70	95
1e	95	> 97	86	> 97

1f	30	45	14	60
1g	0	0	0	0
1h	20	27	10	48
1i	> 97	> 97	> 97	> 97
1j	> 97	> 97	92	> 97
1k	5	10	48	82
1l	75	84	70	90
1m	12	16	30	24
1n	14	15	2	36

All the substrates were transformed at variable rates, except for 4-nitrobenzaldehyde oxime (**1g**). For benzaldehyde oxime derivatives bearing a substituent in *para* position (**1b**-**1g**), the electronic nature of the *para*-group (electron-donating or electron-withdrawing) did not significantly affect reactivity. Instead, substrate-protein interactions played a crucial role in determining conversion efficiency. Arylaliphatic substrates gave excellent (**1l**) to moderate yields (**1m**), showing the versatility of OxdF1 (L318F/F306Y) as catalyst. Notably, 2,6-difluorobenzaldehyde oxime **1i** was fully transformed within 1 h with both systems, whereas the more sterically hindered 2,6-dimethylbenzaldehyde oxime **1h** resulted in a 48% yield after 8 hours with whole cells.

Reactions with the free enzyme typically displayed good activity within the first hour; however, little to no further conversion was observed with extended reaction times. On the other hand, reactions with whole cells showed a continuous progress, achieving higher yields after 8 hours. This difference can be ascribed to the known instability of free aldoxime dehydratases under operational conditions-a limitation that can be partially mitigated by keeping the enzymes within whole cells, providing a more stable catalytic environment.

2.2 Reactions with immobilized systems

To enhance the operational stability of biocatalysts, we focused on immobilizing the free enzyme using **1a** as the current substrate. According to the literature, various immobilization strategies have been employed to graft Oxds onto different supports; however, none of these approaches involve the formation of covalent bonds to stabilize the interaction between the enzyme and the support. In this context, we immobilized OxdF1 (L318F/F306Y) onto activated glyoxyagarose and several epoxy-based resins, following established protocols[35]. Initially, 1 mg of pure enzyme was immobilized onto 1 g of each support. This led to full immobilization yields across the selected matrices; however, the epoxy-based carriers retained almost no residual activity (<1%), whereas the glyoxy-based

support exhibited a relatively low retained activity of 22% (see Figure S3). Further investigations were conducted with varying enzyme loadings (1-10 mg/$g_{agarose}$) on the activated agarose support. The results demonstrated that the immobilization efficiency remained above 97% across all tested enzyme concentrations, with residual activity ranging from 22% to 11% (see figure S4a).

We then assessed the catalyst's reusability over multiple cycles; however, a substantial activity loss (−62%) was observed after the second cycle, with complete enzyme inactivation occurring by the fourth cycle (see figure S4b). A partial explanation for this decline can be attributed to the inherent instability of the enzyme, particularly in the presence of atmospheric oxygen, which leads to the oxidation of the catalytic Fe^{2+} in the active site. Even the use of strong reducing agents, such as sodium dithionite, only partially alleviates this issue.

To address these challenges, maintaining the enzyme within the cellular environment provides an effective strategy to enhance its stability while eliminating the need for the addition of reducing agents. Given the superior operational stability of whole cells, recombinant *E. coli* expressing OxdF1 (L318F/F306Y) was immobilized by entrapment in Ba-alginate beads, offering additional enzyme protection and facilitating separation of the catalyst from the reaction medium. We recently found that whole cells immobilized in Ba-alginate beads showed greater operational and mechanical stability compared to Ca-alginate[52]. Reactions were performed using **1a** (20 mM) dissolved in HEPES buffer (pH 7.0) with 40 mg/mL of alginate beads (containing 40 mg dry weight cells/mL) at 28 °C, achieving 95% conversion within 1 hour. The operational stability of the immobilized cells was assessed through multiple biotransformation cycles revealing that the catalyst retained 65% of its original activity after 8 consecutive reaction cycles.

2.3 Flow reactor

Combining biocatalysis with flow chemistry may enhance reaction efficiency by enabling continuous operation, improved mass transfer, and precise control over reaction conditions. This synergy increases enzyme stability, minimizes byproduct formation, and facilitates scalable, sustainable synthesis[53–56]. In this context, immobilized cells were packed into a column reactor (i.d. 0.78 mm, total volume 7.584 mL) at a concentration of 10 $mg_{dry\ weight}$/mL. Firstly, **1a** (20 mM) in HEPES buffer 50 mM pH=7 was flowed through the reactor at a flow rate of 0.48 mL/min (residence time of 30 minutes). After 8 hours of continuous operation, only a minimal decrease in activity was observed, suggesting that the flow system effectively preserves enzymatic activity by minimizing the mechanical agitation typically utilized in batch systems (Figure 2).

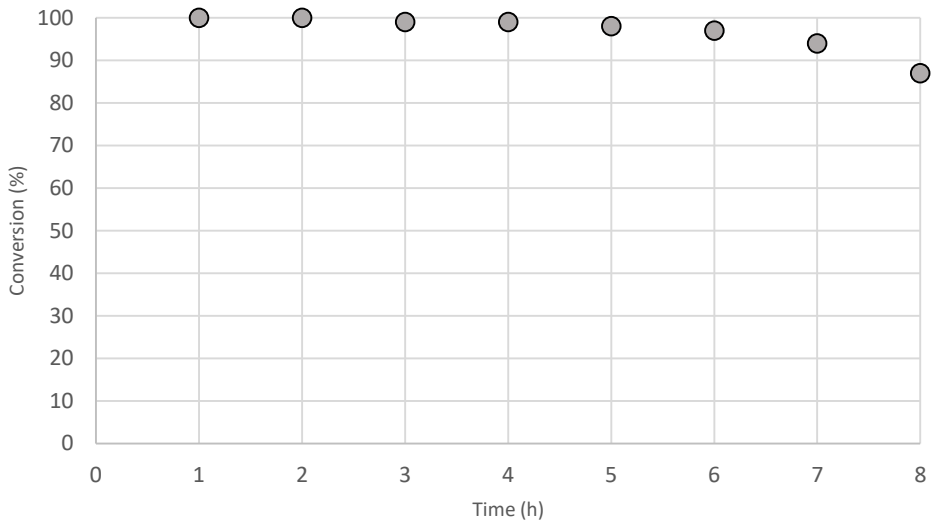


Figure 2. Conversion over time of the continuous flow dehydration of **1a** with OxdF1(L318F/F306Y) (20 mM) with Ba-alginate immobilized cells of *E. coli* expressing OxdF1 (L318F/F306Y) using water as flow solution. Residence time 30 min. Conversion determined by UHPLC. These tests were performed in duplicate.

Since the preparation of 2,6-difluorobenzonitrile deserves interest for different applications, we focused on its synthesis in a continuous flow system starting from **1i**. The main challenge to the development of such a continuous process is the poor solubility of both substrate and product in water. We initially tested aqueous solutions with varying EtOH concentrations. A 10% EtOH solution ensured good enzyme activity while effectively dissolving **1i** up to 10 mM. Notably, batch reactions with Ba-alginate immobilized cells in this medium achieved high yields (>97% after 2 h, Table 1). Thus, a monophasic liquid system (water/EtOH 9:1) was chosen as the medium for the flow-based reaction of **1i** (Table 2).

Table 2. Continuous flow dehydration of **1i** (10 mM) with Ba-alginate immobilized *E. coli* expressing OxdF1 L318F/F306Y). Flow liquid phase: HEPES buffer pH 7.0 containing 10% EtOH. Conversion determined by UHPLC.

Flow rate (mL^{-1} min)	Residence time (min)	Conv. (%)	Rate (μmol $min^{-1}g_{dry\ cells}^{-1}$)
2.88	5	40	88
1.44	10	72	74
0.96	15	83	57
0.48	30	98	33

With a flow rate of 0.5 mL/min, almost total conversion was obtained with a residence time of 30 min (Table 2). Extending the reaction time led to a decline in conversion (40% of residual activity after 4 h of continuous operation). The addition of sodium dithionite to maintain a reducing environment proved incompatible with alginates, as it caused rapid degradation of the beads. The reduced activity was accompanied by a lower recovery of the product in the outflow stream; in fact, a portion of the product was found to be adsorbed onto the immobilized cells that were recovered after the process. To assess the potential negative impact of EtOH on enzymatic stability, a buffer/EtOH solution (9:1) was passed through the reactor for 6 hours in the absence of the substrate. No decrease in activity was observed following this treatment, indicating that ethanol did not negatively affect enzyme stability. Thus, the limited solubility of the product in the flowing solvent emerged as the primary factor contributing to the challenges in sustaining the long-term efficiency of the continuous system.

To address the solubility limitations of both the substrate and the product, two-liquid systems including water and a hydrophobic solvent at different ratios were evaluated (Table 3). Cyclohexane and toluene were identified as suitable solvents[57], dissolving 2,6-difluorobenzaldoxime (**1i**) up to 10 mM and enabling quantitative extraction of the product from aqueous solutions. Given the low substrate concentration in the flowing phase, maintaining biocatalyst stability is crucial for achieving meaningful productivity. A continuous flow set up was established, where the inlet solution consisted of a buffer and an organic phase (cyclohexane or toluene) containing the substrate. The two phases were mixed in a T-piece, generating a segmented liquid–liquid flow stream through the column.

Table 3. Continuous flow dehydration of 1i (10 mM) with Ba-alginate immobilized cells of *E. coli* expressing OxdF1 (L318F/F306Y) using different two-liquid phase systems. Residence time 15 min. Conversion was determined by UHPLC after 1 hour of operation.

Two liquid phase system	Phase ratio	Conv. (%)
Water/cyclohexane	1:1	65
Water/cyclohexane	75:25	> 97
Water/toluene	1:1	60
Water/toluene	75:25	> 97

The reactions in two liquid systems with a phase ratio of 75:25 (HEPES:organic solvent) proceeded with high conversion with both solvents. For continuous operation, cyclohexane (logP = 3.4) was chosen due to its lower boiling point and greater hydrophobicity, which enhance both product extraction and solvent recycling. An in-line liquid–liquid extractor (Zaiput liquid-liquid separator) was integrated downstream to separate the organic and aqueous phases, ensuring the quantitative recovery of **2i** in cyclohexane (Figure 3).

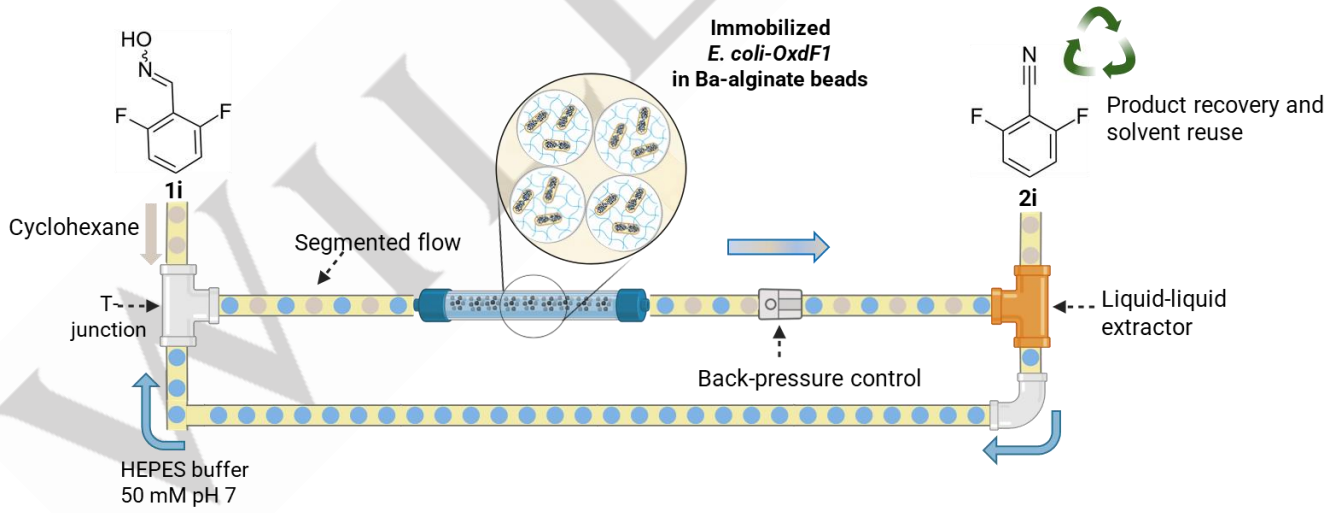


Figure 3. Continuous flow dehydration of **1i** (10 mM) using *E. coli* cells expressing OxdF1 (L318F/F306Y) immobilized in Ba-alginate, with a 75:25 water/cyclohexane flow medium at 28 °C, a residence time of 15 minutes, and a flow rate of 0.48 mL/min."

The developed system demonstrated good stability for up to 12 hours, with conversion remaining around 70% (Figure 3). Biocatalyst productivity (defined as product formed per amount of biocatalyst used) was equal to 22.3 mmolP/$g_{cells\ dry\ weight}$. Significantly, unreacted substrate **1i** was recovered in the aqueous solution, while product **2i** was fully quantified in the organic phase. After the solvent removal, also cyclohexane was recovered and reused. Although the process was conducted at relatively low substrate concentration (10 mM), the biocatalyst demonstrated remarkable long-term stability. (Figure 4).

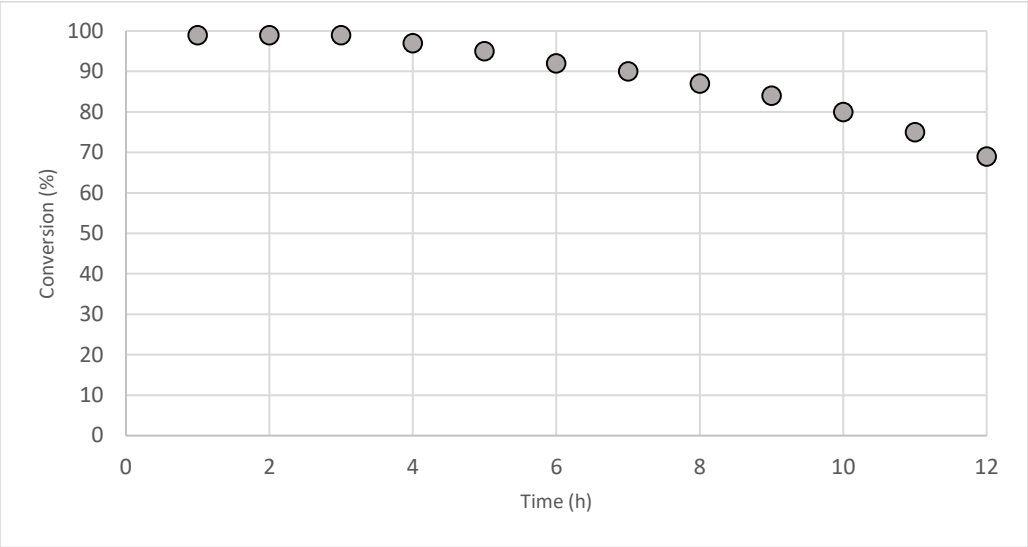


Figure 4. Conversion over time of the continuous flow dehydration of 1i with Ba-alginate immobilized cells of *E. coli* expressing OxdF1n(L318F/F306Y) using water/cyclohexane 75:25 as flow medium. Residence time 15 min. Conversion determined by UHPLC. These tests were performed in duplicate.

3. Conclusion

The synthesis of nitriles from corresponding aldoximes using aldoxime dehydratases (Oxds) offers significant potential. However, the inherent instability of Oxds requires strategies to enhance their long-term stability to ensure a more sustainable process. In this study, the integration of immobilized whole-cell biocatalysis with continuous flow processing successfully stabilized OxdF1 (L318F/F306Y), a double mutant engineered for the efficient production of aromatic nitriles. We selected two case studies: the preparation of benzonitrile and 2,6-difluorobenzonitrile, the latter being noteworthy as a key intermediate in various applications. In the preparation of benzonitrile, where solubility issues are minimal, the flow-based process enabled continuous operation for 8 hours while maintaining high conversion (87%). The synthesis of 2,6-difluorobenzonitrile proved challenging, largely due to the poor aqueous solubility of the substrate and the product. The developed process, utilizing a two-liquid phase system (75:25 water/cyclohexane), addresses substrate and product insolubility while enhancing biocatalyst stability and significantly increasing productivity. Furthermore, the integration of in-line liquid-liquid extraction enhances system automation, while the recovery and reuse of both the aqueous phase containing unreacted oxime and the cyclohexane significantly improve the overall system's sustainability. In conclusion, this approach holds potential for broader applications with other Oxds in nitrile synthesis, providing a safer, automated, and more sustainable alternative to conventional toxic cyanide-based reactions.

4. Experimental section

4.1 Materials

Organic solvents and chemical standards were from Merck (Sigma Aldrich). Resindion™ epoxy-resins EP403/S, EC-HFA/S and HFA403/S was from Resindion (Binasco, Italy). Merck Silica gel 60 F254 (aluminium foil) plates were used for TLC analysis; flash column chromatography was performed on Merck Silica gel (230–400 mesh). Detection of TLC analyses have been performed under UV light at 254 and 365 nm. Reactions were analysed by UHPLC using an Agilent 1290 Infinity system equipped with an Agilent InfinityLab Poroshell 120 EC-C18 (2.1 x 50mm, 1.9 micron) column. ^{1}H-^{13}C NMR spectra were recorded with a Bruker Avance TM NEO 400 MHz (^{1}H 400

MHz) spectrometer. TMS was used as an internal standard, chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hz. Chloroform-*d* was used as deuterated solvents for NMR analysis.

4.2 Expression and purification of OxdF (L318F/F306Y)

The His-tagged OxdF1 (L318F/F306Y) variant from Xiao et al. [32] was cloned into the pET-28a(+) expression system by Officinae Bio Srl (Venice, Italy). The plasmid was transformed into One Shot™ BL21(DE3) STAR chemically competent *E. coli* following the supplier's protocol. A single colony of OxdF1-expressing *E. coli* was inoculated into 20 mL of 2XYT medium (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl) supplemented with 50 mg/L kanamycin and grown overnight. Cells were then inoculated at an initial OD_{600} of 0.05 into a 1 L baffled flask containing 200 mL of 2XYT medium with 50 mg/L kanamycin and cultured for 4 hours (OD_{600} = 0.8–1.0). The culture was placed in ice for 10 minutes, followed by induction with 0.1 mM IPTG under orbital agitation at 150 rpm for 16 hours at 18 °C. Cells were harvested by centrifugation at 5000 rpm for 30 minutes at 4 °C. From a 1 L culture, up to 12 g of wet cell mass was obtained and stored at 4 °C until further use. For the purification, the protocol developed by Chen at al. [58] was followed with minor modifications. After centrifugation, 2 g of wet cells were suspended in 10 mL of potassium phosphate buffer (50 mM pH=7.0) and 1 mg/ml of lysozyme (Merck) was added; the suspension was kept under agitation (120 rpm) at 28°C for 30 minutes. Then, the cells disrupted by sonication (12000 amplitude, 7 cycles of 2 min each, in ice). Cell debris were harvested by centrifugation (45 min, 15000 rpm, 4 °C). The enzyme was purified by affinity chromatography with His-Nickel Affinity Gel. The column was equilibrated with loading buffer (20 mM potassium phosphate buffer pH 7.0, 20 mM imidazole) and the crude extract was loaded in the loading buffer; finally, the adsorbed enzyme was eluted with elution buffer (20 mM potassium phosphate buffer pH 7.0, 500 mM NaCl, 250 mM imidazole) with a characteristic brown colour due to the heme group (fig. S1) Purified protein was dialyzed against 50 mM potassium phosphate buffer at 4 °C for 18 hours. Dialysing buffer was changed twice. Bradford assay has been used to quantify protein concentration. Purity has been assessed using standard SDS-page gel (see S2).

4.3 Enzyme immobilization

Glyoxyl agarose has been prepared according to previously available protocol.[60] Then adequate amount of Oxd*F1* has been added to 1 mL of 50 mM Potassium Carbonate buffer pH=10 with 250 mg of activated Glyoxy-agarose to immobilise 1-10 $mg_{OxdF1}/g_{support}$. The suspension is kept under mild agitation at 4°C for 18 hours. Immobilization efficiency is assessed by standard Bradford assay. Then 2mg of $NaBH_4$ is added and kept under gentle agitation for 30 minutes. Finally, the immobilized enzyme was rinsed three times with Milli-Q water, once with phosphate buffer solution 50 mM pH 5.0 and stored in Buffer phosphate 50 mM pH 7 at 4 °C. Immobilization on Resindion™ epoxy-resins EP403/S, EC-HFA/S and HFA403/S has been performed following protocol developed[60] using 1 $mg_{OxdF1}/g_{support}$. Briefly, 1 g of epoxy-support was incubated with 2 mL of modification buffer (0.1 M sodium borate, 2 M iminodiacetic acid pH 8.0) for 2 h. After filtration and washing steps, the resin was incubated with 4 mL of metal Buffer (30 mg/mL of $CoCl_2$). After filtration and washing with milli-Q water, 1.5 mL of Oxd*F1*(0,66 mg/mL) was added to the resin and the suspension was incubated for 18-24h under gentle shaking at 4°C. Then supernatant was tested to detect unbounded enzyme, and the resins were washed with 3 mL of desorption buffer (50 mM EDTA, 0.5 M NaCl in 50 mM phosphate buffer, pH 7.2). Finally, the remaining epoxy groups were blocked by incubation with 4 mL of 3 M glycine pH 8.5 overnight and afterwards the resin was washed with 50 mM phosphate buffer pH 7 and stored at 4°C.

4.4 Whole-cell immobilization in alginate beads

Barium alginate beads were prepared as described in a previously published protocol.[52] Centrifuged cells were resuspended in 20 mM sodium acetate buffer (pH 7.0) at 400 OD (80$mg_{dry\ weight\ cells}$/mL) and mixed with an equal volume of 4% sodium alginate solution. The resulting mixture was then dropped using a peristaltic pump into a 0.2 M $BaCl_2$ solution under continuous agitation. Alginate beads were allowed to cure for 30 minutes at 4 °C under agitation, followed by thorough washing with distilled water for immediate use. The beads were stored in 20 mM sodium acetate buffer (pH 7.0) containing 0.02 M $BaCl_2$ at 4 °C and were washed again prior to use.

4.5 Biotransformations

Biotransformations with the free enzyme were performed by adding 20 mM aldoximes to a 0.5 mg/mL solution of OxdF1(L318F/F306Y) in 20 mM HEPES buffer (pH 7.0) containing 10% ethanol and 5 mM sodium dithionite; the reaction mixture was kept at 28°C, under agitation (140 rpm). Reactions with whole cells were performed by adding 20 mM aldoximes to a suspension containing 30 mg/mL of wet cells (equivalent to 5.9 mg dry weight/mL) in the

presence of 10% ethanol; the reaction mixture was incubated at 28 °C under agitation at 140 rpm. Batch reactions using the immobilized enzyme were performed using different amount of the immobilized system in 20 mM HEPES buffer (pH 7.0), containing 20 mM aldoximes; the reaction mixture was incubated at 28 °C under agitation at 140 rpm. Immobilized cells (40 mg/ml) were suspended in in 20 mM HEPES buffer (pH 7.0) containing 20 mM solution of substrate and the suspension was incubated at 28 °C under agitation at 140 rpm. For recycle assessment, the heterogeneous reaction mixture was filtered at the end of the reaction, and the beads were washed twice with water. The recovered beads were added to fresh buffer containing the substrate for the next cycle.

4.6 Flow reactor

A Vapourtec series E flow reactor equipped with an Omnifit column of 0.78 cm of internal diameter (final volume 7.584 ml) was packed with 6 g of immobilized barium alginate-recombinant *E. coli* cells. For monophasic liquid reactions, a solution consisting of HEPES buffer (pH 7.0) and 10% ethanol was used, and reactions were carried out at varying flow rates. For two-phase liquid reactions, HEPES buffer (pH 7.0, 20 mM) and the substrate in organic solvent (cyclohexane/toluene) were independently pumped and mixed in a T-piece, generating a segmented flow stream with varying phase ratios, which was directed into the column packed with the biocatalyst. The product was collected at steady state and analyzed by UHPLC (See figure S5-S18). After the selection of the best system (water/cyclohexane 75:25, 15 min residence time, 30 °C) the reactor was left to work for 12 hours collecting samples every hour for the determination of product conversion. The organic phase was continuously separated using a Zaiput liquid/liquid separator. The collected organic phase dried over Na_2SO_4, filtered, and evaporated under vacuum giving the desired product (650 mg). The product was characterized via NMR.

4.7 UHPLC conditions

Reactions were analysed by UHPLC using an Agilent 1290 Infinity system equipped with an Agilent InfinityLab Poroshell 120 EC-C18 (2.1 x 50mm, 1.9 micron) column, using Sol A: Milli-Q with 0.15% TFA and Sol B: acetonitrile as elution solvents. Detector is a 1260 Infinity III Diode Array Detector HS at 254 or 220 nm. A flux of 0.3 ml/minute, 7: 3 Sol A/Sol B ratio in isocratic mode has been used for all the tested substrates except 1b where 8:2 ratio was used. For 1i, 7:3 Sol A/Sol B and a flux of 0.4 ml/min was used. Retention times are reported in Table S1. Substrate and product concentrations were determined using calibration curves generated by plotting absorbance values (at 220 nm or 254 nm) against the concentrations of corresponding pure standards.

Supporting Information

Acknowledgements

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Keywords: Aldoxime Dehydratase (Oxd) • Nitrile synthesis • Whole-cell immobilization • Flow reactor • Enzyme stabilization.

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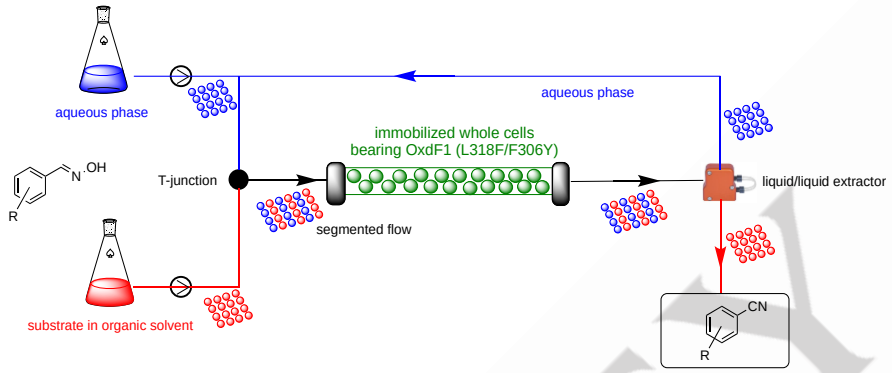

This work presents a synergistic approach to enhance the stability of the engineered aldoxime dehydratase OxdF1 (L318F/F306Y) by combining whole-cell immobilization in Ba-alginate with continuous flow processing. Applied to challenging substrates like 2,6-difluorobenzaldoxime, the system enables efficient, stable nitrile synthesis with in-line product extraction and improved sustainability.