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Enzymatic Characterisation of a Whole-Cell Biocatalyst Displaying Sucrase A from *Bacillus subtilis* in *Escherichia coli*

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Abstract

In this study, sucrase A (SacA) from *Bacillus subtilis* was successfully displayed on the outer membrane of *Escherichia coli* via fusion with the AIDA-I autotransporter from *E. coli*. The pAIDA-*sacA* plasmid was constructed by fusing *sacA* with the *ctxB* signal sequence and the β-barrel domain of *aida* gene, enabling surface expression under both aerobic and anaerobic conditions. Functional expression of AIDA–SacA was confirmed by the appearance of reducing sugars in enzymatic assays of sucrose hydrolysis and by acid production on phenol red agar. Structural prediction suggested correct localisation of the catalytic domain on the extracellular surface. Enzymatic characterisation revealed that AIDA-SacA exhibits optimal activity at 40 °C and pH 7. The calculated Km for sucrose was 1.18 mM, while the corresponding Vmax was 2.32 U mL^{-1}. Thermal stability assays showed that the enzyme retained over 80% of its activity after 60 min at 45 °C, indicating notable resistance to moderate temperatures. Metal ion assays indicated that K^+ enhanced enzymatic activity, while Zn^{2+}, Cu^{2+}, and Mg^{2+} were inhibitory. SDS-PAGE analysis confirmed the expression of the recombinant fusion protein, with a distinct band at approximately 114 kDa corresponding to the expected size. These results demonstrate the feasibility of employing the AIDA-I system for the surface display of SacA in *E. coli*, providing a functional platform for future applications in whole-cell biocatalysis.

Keywords: sucrose; enzyme immobilisation; *sacA* gene

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1. Introduction

Sucrose is composed of a glucose unit linked to a fructose unit by the glycosidic bond and it is the most abundant disaccharide in nature [1]. This disaccharide can serve as a carbon source for a wide variety of bacteria. Sucrose can be cleaved by sucrose-6-phosphate hydrolases and sucrose phosphorylases after uptake into the bacterial cytoplasm [2]. Recently, attempts for utilisation of sucrose as a cheap and easily degradable feedstock for biochemical production through microbial fermentation have attracted significant interest due to its widespread availability, low cost, and high fermentability, making it an attractive substrate for industrial bioprocesses [3].

In *Bacillus subtilis*, the *sacA* gene encodes sucrase A, a sucrose-6-phosphate hydrolase that is part of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). Through this system, sucrose is internalised and phosphorylated to sucrose-6-phosphate, which is subsequently hydrolysed by SacA into glucose-6-phosphate and fructose [4]. This mechanism enables *B. subtilis* to efficiently utilise sucrose as a carbon source. By contrast,

E. coli lacks the chromosomal sucrose operon, which encodes the PTS transporter and the associated catabolic enzymes; therefore, it is naturally unable to metabolise sucrose.

Enzymatic characterisation of SacA is essential to elucidate its catalytic properties, substrate specificity, and biotechnological potential. Moreover, when SacA is heterologously expressed in *E. coli*, it remains confined into the cytoplasm, where it cannot interact with extracellular sucrose due to the bacterium's lack of a functional uptake system. Therefore, effective hydrolysis requires the enzyme to be secreted or displayed on the outer membrane [3]. To overcome the limitations of intracellular localisation and enhance substrate interaction, surface display strategies have been widely employed, enabling active enzymes to function at the cell surface without disrupting cell integrity [5].

One of the most widely studied systems for surface expression in *Escherichia coli* is the Adhesin Involved in Diffuse Adherence (AIDA-I), a type V secretion system. AIDA-I comprises three main components: an N-terminal signal peptide that directs the protein to the Sec translocon for periplasmic translocation, a central passenger domain that may contain the catalytic activity, and a C-terminal β-barrel domain that anchors the protein to the outer membrane [6]. When a heterologous protein is genetically fused to the β-barrel domain of AIDA-I, the passenger domain can be translocated through the β-barrel pore and exposed on the cell surface. This architecture enables the extracellular localisation of functional enzymes, while the autodisplay system allows the expression of more than 10^5 recombinant molecules per cell and represents a robust tool for displaying active enzymes on the bacterial surface [7].

In this study, SacA from *B. subtilis* was fused to the AIDA-I autotransporter system and expressed in *E. coli* cells. The constructed pAIDA-*sacA* plasmid allowed the generation of strains that exhibited saccharolytic activity, whereas native *E. coli* strains were unable to hydrolyse sucrose due to the absence of a functional sucrose utilisation system. This work demonstrates the feasibility of employing the AIDA-I system for the surface display of SacA, offering a robust platform for future biocatalytic processes using whole cells.

2. Materials and Methods

2.1. Construction of the pAIDA-sacA Plasmid

The construction of the pAIDA-*sacA* plasmid has been previously reported by [3]. Briefly, the *sacA* gene, encoding sucrase A from *Bacillus subtilis* (GenBank accession number CP053102.1), was amplified by PCR using genomic DNA as the template with the primers sacA FW (5′-GGCGCGCCTACAGCACATGACCAGGAG-3′) and sacA RV (5′-CTCGAGCGCATAAGTGTCCAAATTCC-3′). The PCR product was cloned into the pGEM-T vector (Promega, Madison, WI, USA), digested with *Asc*I and *Xho*I, and ligated into the pre-digested pAIDA plasmid, originally developed by [5]. The final construct contains the *ctxB* signal peptide from *Vibrio cholerae*, a flexible linker region, and the β-barrel domain of the AIDA-I autotransporter fused to *sacA*, all under the control of the constitutive gapAP1 promoter. Assembly and sequence verification were performed using MacVector (MacVector Inc., Version 10.1, Apex, NC, USA) and SnapGene software (GSL Biotech LLC, Version 3.3, San Diego, CA, USA). The plasmid was subsequently introduced into *E. coli* strains W3110 and DH5α via heat-shock transformation.

2.2. Structural Prediction of AIDA-SacA

The structure of the AIDA-SacA fusion protein was predicted using the ColabFold v1.5.5 implementation of AlphaFold 2 (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb (accessed on 5 October 2025)) which integrates MMseqs2 for multiple sequence alignment and AlphaFold 2 inference. The complete amino acid sequence, including the signal peptide, SacA domain, linker, and AIDA-I

β-barrel domain, was submitted to generate the model. The resulting structural model was visualised and manipulated using UCSF Chimera software (University of California, San Francisco, CA, USA; Version 1.19) for domain identification and graphical representation.

2.3. Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The presence of the AIDA-SacA fusion protein was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. Cell pellets were resuspended in phosphate buffer and subjected to sonication using short pulses while kept on ice to minimise thermal denaturation. The resulting lysates were mixed with Laemmli buffer (5×) (Bio-Rad Laboratories, Hercules, CA, USA) containing 10% SDS (Sigma-Aldrich, Burlington, MA, USA) and 5% β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA, USA) in a 4:1 (v/v) ratio, and the samples were then boiled at 95 °C for five min. Protein separation was carried out using a 12% polyacrylamide resolving gel with a 5% stacking gel (Bio-Rad Laboratories, Hercules, CA, USA), operated at a constant voltage of 120 V until the tracking dye reached the base of the gel. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) and subsequently destained overnight using a 40% (v/v) methanol–10% (v/v) acetic acid solution (CTR Scientific, Guadalajara, Mexico; reagent grade) until a clear background and well-resolved bands were obtained. Protein molecular weights were estimated using the Prestained PageRuler™ Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA; catalogue no. 266164) as a reference, which was run in parallel to determine the apparent sizes of the expressed proteins.

2.4. Assessment of Saccharolytic Activity Using Phenol Red Agar

The saccharolytic activity of *Escherichia coli* expressing AIDA-SacA was evaluated using a phenol red sucrose fermentation assay. *E. coli* W3110 strains harbouring pAIDA-*sacA* and a control strain without the plasmid were streaked onto phenol red agar plates containing 10 g L^{-1} peptone (Sigma-Aldrich, Burlington, MA, USA), 5 g L^{-1} sodium chloride (Karal, León, Mexico), 0.018 g L^{-1} phenol red (Sigma-Aldrich, Burlington, MA, USA), 5 g L^{-1} sucrose (Materiales y Abastos Especializados, Zapopan, Mexico; ACS grade) as the sole carbon source and ampicillin (Pharmalife, Laboratorios Pisa, Guadalajara, Mexico, ≥99% purity) at a final concentration of 100 μg mL^{-1}. Plates were incubated at 37 °C for 48 h, and colour changes in the medium were recorded as an indicator of acid production from red to yellow resulting from sucrose metabolism.

2.5. Enzymatic Activity Assay

To evaluate the enzymatic activity of AIDA-SacA, assays were performed using whole cells. *Escherichia coli* W3110 carrying pAIDA-*sacA* was cultivated overnight in Luria–Bertani medium (Sigma-Aldrich, Burlington, MA, USA) supplemented with 100 μg mL^{-1} ampicillin (Pharmalife, Laboratorios Pisa, Guadalajara, Mexico; ≥99% purity) at 37 °C with shaking at 200 rpm. Cells were harvested by centrifugation at 8000× g for 10 min at 4 °C, washed twice with 100 mM potassium phosphate buffer (pH 6.5), prepared by mixing appropriate proportions of 100 mM KH_2PO_4 (CTR Scientific, Guadalajara, Mexico, reagent grade) and 100 mM K_2HPO_4 (CTR Scientific, Guadalajara, Mexico; reagent grade) until the desired pH was reached, and resuspended in the same buffer. For whole-cell activity assays, the biomass concentration was adjusted to an optical density at 600 nm (OD_{600}) of 10, and the cells were incubated with 5 mM sucrose at 37 °C for 60 min. A control assay was included in which the cell suspension was heat-inactivated at 95 °C for 5 min before substrate addition. Reactions were stopped by heating at 95 °C for 5 min, and the concentration of reducing sugars was determined using the dinitrosalicylic acid (DNS) method [8]. The concentration of reducing sugars was quantified using a calibration

curve generated with glucose standards (1.0–0.0625 g L^{-1}; R^2 = 0.99). Absorbance was recorded at 540 nm after DNS treatment, and the calibration curve was used to calculate the equivalent amount of reducing sugars in μmol. All assays were performed in 100 mM potassium phosphate buffer (pH 6.5). One unit of sucrase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugars per minute under the specified assay conditions.

2.6. Enzymatic Optimum Temperature

The optimum temperature for AIDA-SacA activity was determined by performing enzymatic assays at 20, 30, 40, 50, 60, and 70 °C. Reactions were conducted in 100 mM potassium phosphate buffer (pH 6.5) with 5 mM sucrose as the substrate. The enzymatic activity was measured by quantifying the reducing sugars released using the DNS method. The enzyme was incubated at each temperature for 60 min, and reactions were stopped by heating at 95 °C for 5 min. The activity at each temperature was expressed as a percentage relative to the maximum observed activity. All experiments were performed in triplicate, and results were reported as the mean ± standard deviation.

2.7. Enzymatic Optimum pH

The optimum pH for AIDA-SacA activity was determined by performing enzymatic assays at pH 3, 4, 5, 6, 7, 8, and 9. Reactions were carried out in 50 mM Robinson's buffer, which consists of 50 mM phosphoric acid (Karal, Guanajuato, Mexico; reagent grade), 50 mM boric acid (CTR Scientific, Guadalajara, Mexico; reagent grade), and 50 mM acetic acid (Karal, Guanajuato, Mexico; reagent grade). The assays were performed at the optimum temperature determined from the temperature experiments, using 5 mM sucrose as the substrate. Enzymatic activity was measured by quantifying the reducing sugars released using the DNS method. The enzyme preparation was incubated at each pH for 60 min, and reactions were stopped by heating at 95 °C for 5 min. Activity at each pH was expressed as a percentage relative to the maximum observed activity. All experiments were performed in triplicate, and results were reported as the mean ± standard deviation.

2.8. Enzymatic Thermal Stability

The thermal stability of AIDA-SacA was evaluated by incubating the enzyme at 45, 55, and 65 °C for 15, 30, 45, and 60 min. After each incubation period, aliquots were withdrawn and immediately cooled on ice to halt enzymatic activity. Residual activity was assessed under standard assay conditions using 5 mM sucrose in potassium phosphate buffer (pH 6.5). The amount of reducing sugars released was quantified using the DNS method. Enzymatic activity was expressed as a percentage relative to the initial (non-incubated) enzyme activity. All experiments were performed in triplicate, and results were reported as the mean ± standard deviation.

2.9. Kinetic Parameters Calculation

Enzymatic activity was measured at increasing sucrose concentrations (1, 2, 3, 4, 5, and 6 mM). Reactions were carried out at 40 °C using whole cells of *Escherichia coli* W3110/pAIDA-*sacA* at an optical density (OD600) of 10, suspended in 100 mM potassium phosphate buffer (pH 6.5). An initial-rate time range was established from time-course assays; all kinetic points were collected at a fixed time within this linear interval with low substrate conversion. The amount of reducing sugars released was quantified using the DNS method. The kinetic parameters Km and Vmax of AIDA–SacA were determined by

fitting the experimental data to the Michaelis–Menten equation using GraphPad Software version 8.0.2 (Boston, MA, USA), as follows:

$$v = \frac{Vmax[S]}{Km + [S]} \quad (1)$$

where v represents the initial velocity, $[S]$ the substrate concentration, Km the Michaelis constant, and $Vmax$ the maximum velocity. The data were also linearised using the Lineweaver–Burk representation:

$$\frac{1}{v} = \frac{Km}{Vmax} \cdot \frac{1}{[S]} + \frac{1}{Vmax} \quad (2)$$

confirming agreement between the non-linear regression and linear transformation. All experiments were performed in triplicate, and results are expressed as the mean ± standard deviation.

2.10. Assessment of Metal Ion and Chemical Modulator Effects on AIDA-SacA Activity

To determine the effect of various metals and compounds on the enzymatic activity of AIDA-SacA, enzymatic assays were conducted using whole cells of *Escherichia coli* W3110/pAIDA-*sacA* at an optical density (OD_{600}) of 10, incubated with different treatments. Reactions were carried out in a final volume of 1 mL, containing cell suspension, sucrose at a final concentration of 5 mM, the corresponding metal or additive at a final concentration of 1 mM, and 100 mM potassium phosphate buffer (pH 6.5) as diluent. The tested metal ions included: KCl (J.T. Baker, Phillipsburg, NJ, USA), NaCl (Karal, Nuevo León, Mexico), $ZnCl_2$ (Karal, Nuevo León, Mexico), $CuCl_2$ (Karal, Nuevo León, Mexico), $FeSO_4$ (Fermont, Nuevo León, Mexico; ACS grade), $CaCl_2$ (Karal, Nuevo León, Mexico), and $MgSO_4$ (Fermont, Nuevo León, Mexico). The tested chemical compounds included: EDTA (J.T. Baker, Phillipsburg, NJ, USA), DTT (Bio-Rad, Hercules, CA, USA), and acetic acid (Karal, Guanajuato, Mexico; reagent grade) which was added at a final concentration of 10 mM. Reactions were incubated at 40 °C for 2 h under mild agitation (100 rpm), and glucose release was quantified using the DNS colorimetric method. Relative enzymatic activity was calculated with respect to the control condition without additives (set as 100%).

3. Results and Discussion

3.1. Structural Prediction of AIDA-SacA

The complete amino acid sequence, including the signal peptide, SacA domain, linker, and the AIDA-I β-barrel domain, was submitted to ColabFold v1.5.5 implementation of AlphaFold 2 for de novo structural prediction. The resulting model corresponds to a monomer and exhibited an average pLDDT score > 90, indicating very high confidence in the predicted local structure. Although the construct originally included a signal peptide (Met 1–His 20) to direct secretion, this segment is cleaved during translocation and was therefore not considered in the structural analysis. The SacA catalytic domain (Thr 24–Met 501) was clearly distinguished, followed by a flexible linker region (Leu 505–Arg 626), and the β-barrel autotransporter domain (Gln 627–Phe 951). The linker appeared to provide spatial separation and mobility between the catalytic domain and the membrane anchor. In addition, an extracellular region containing the putative catalytic domain of the SacA sucrase was located on the outer surface of the membrane, suggesting that the enzymatic activity was accessible from the extracellular environment. The structural arrangement of the fusion protein is illustrated in Figure 1.

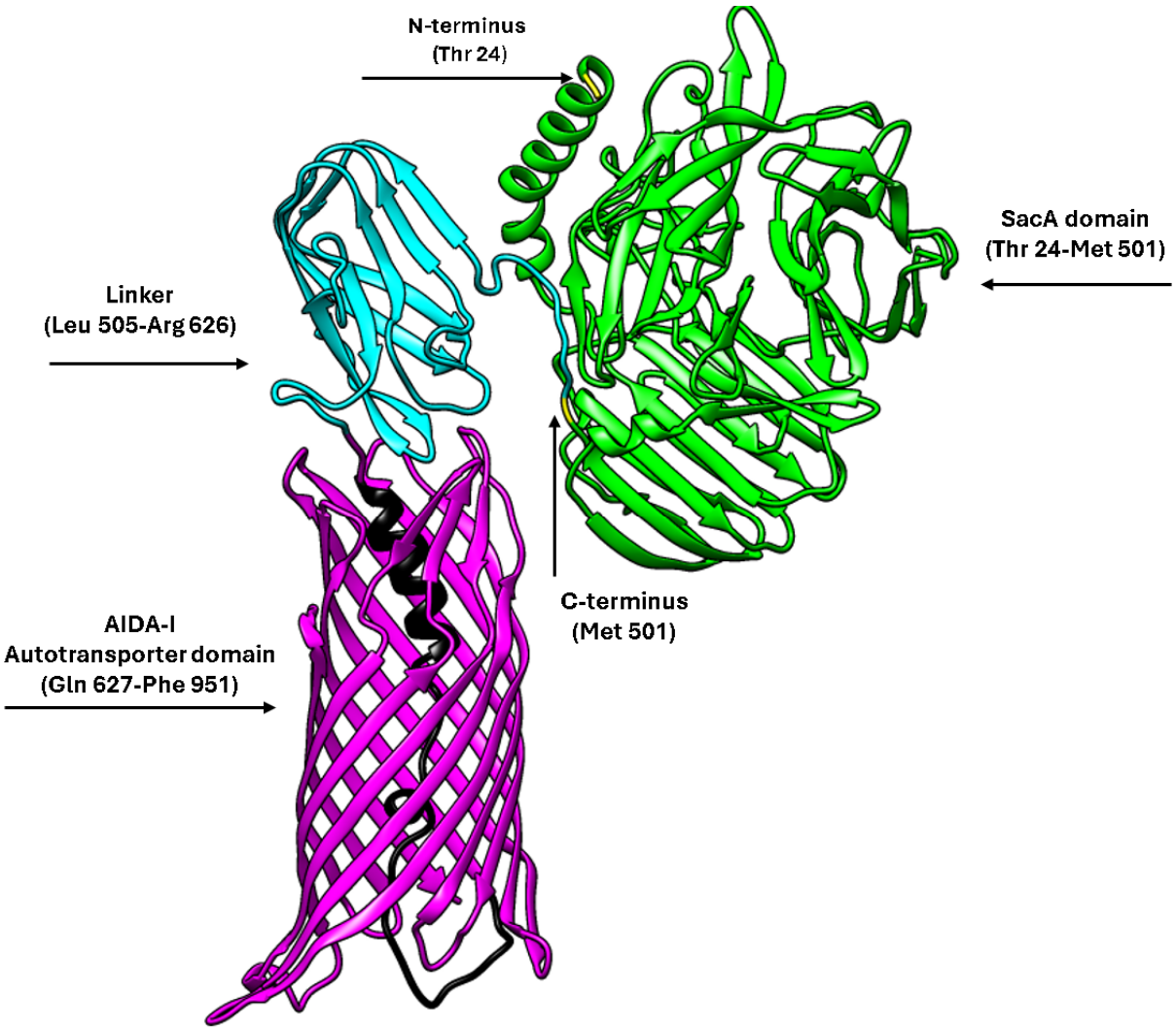


Figure 1. Structural model of the complete AIDA–SacA fusion generated using the ColabFold v1.5.5 implementation of AlphaFold2. The model is based on the full amino acid sequence. The model includes the catalytic domain of sucrase A (Thr 24–Met 501, green), the interconnecting linker region (Leu 505–Arg 626, cyan), and the β-barrel domain of the AIDA-I autotransporter (Gln 627–Phe 951, magenta). The β-barrel anchors the fusion protein to the outer membrane, exposing the SacA catalytic domain to the extracellular environment. The yellow residues mark the initiating amino acids of SacA (Thr 24 and Met 501). The complete amino acid sequence and plasmid vector map used for the AIDA–SacA construct are provided in Supplementary Figure S1.

3.2. SDS-PAGE Analysis of AIDA-SacA Expression

The theoretical molecular mass of the AIDA-SacA fusion protein was estimated based on its individual components. SacA from *Bacillus subtilis* has a reported mass of approximately 55 kDa [2], while the transport unit of AIDA-I, comprising the C-terminal linker and β-barrel domain, contributes approximately 50.9 kDa [9]. The total predicted molecular mass is consistent with the ~114 kDa band observed via SDS-PAGE (Figure 2). This band was absent in the control strain lacking the pAIDA-*sacA* plasmid. The strong intensity and defined migration pattern of the band indicate robust expression and appropriate solubility of the recombinant fusion protein under denaturing conditions.

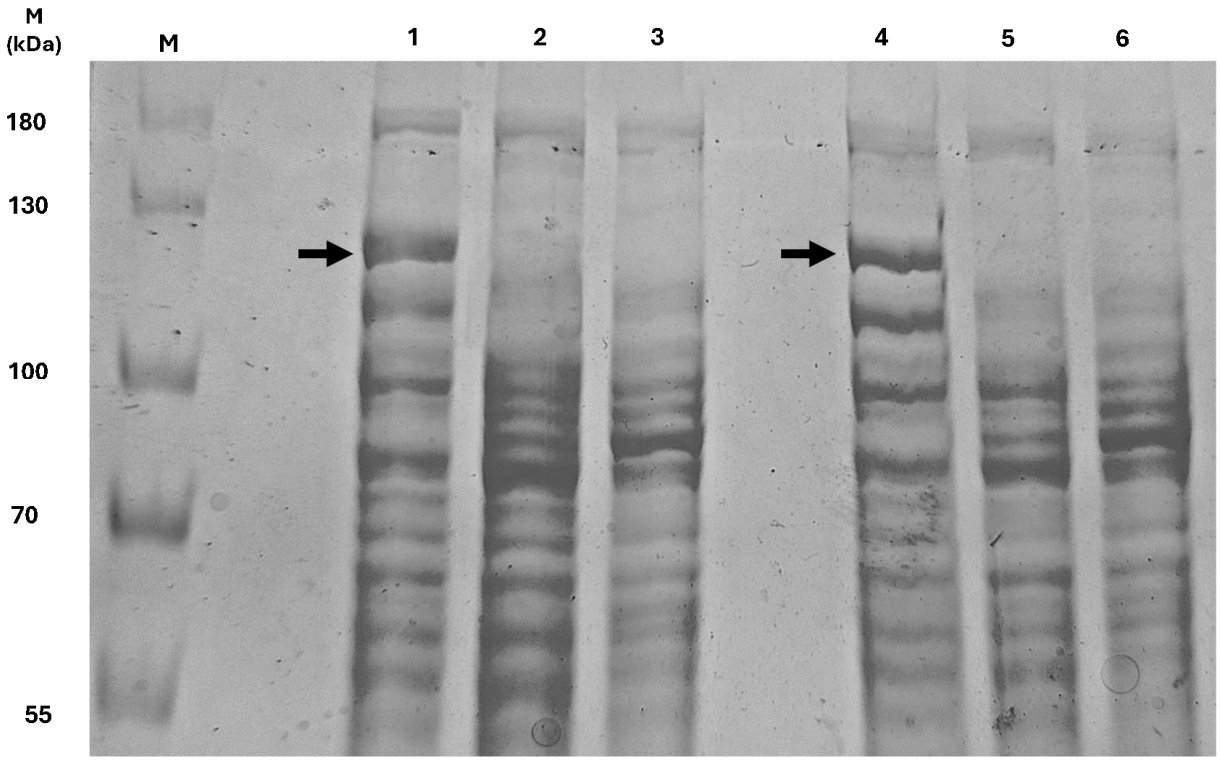


Figure 2. SDS-PAGE verification of AIDA-SacA fusion protein expression in *Escherichia coli*. A prominent band at approximately 114 kDa, highlighted by black arrows, corresponds to the expected molecular weight of the AIDA-SacA fusion. This band is evident in lanes 1 and 4, which represent *E. coli* W3110 transformed with pAIDA-*sacA*. No equivalent band is visible in the control lanes. Lanes 2 and 5 contain untransformed *E. coli* DH5α, while lanes 3 and 6 represent *E.coli* W3110 cells harbouring the empty pAIDA vector.

3.3. Saccharolytic Activity in Phenol Red Agar

To evaluate the saccharolytic activity of *E. coli* W3110 expressing AIDA-SacA, strains with and without the pAIDA-*sacA* plasmid were cultured on phenol red agar supplemented with sucrose as the carbon source. The plates were incubated at 37 °C for 48 h, and colour changes in the medium were recorded as an indicator of acid production resulting from sucrose hydrolysis and fermentation. In a phenol red sucrose broth, *E. coli* typically exhibits a negative reaction, meaning no colour change (remaining red), indicating it does not ferment sucrose [10]. The presence of peptone in the medium allowed bacterial growth in strains lacking pAIDA-*sacA*, but the medium remained red, indicating the absence of acid formation from sucrose. In contrast, strains harbouring pAIDA-*sacA* exhibited a yellow colouration in the medium, indicating acid production because of sucrose hydrolysis and fermentation (Figure 3). Although *sacA* has previously been cloned into *E. coli*, the enzyme remains cryptic in the cytoplasm due to the absence of a functional phosphoenolpyruvate:sugar phosphotransferase (PTS) system, which limits access to intracellular sucrose [2,4]. Therefore, surface anchoring is required to enable extracellular hydrolysis and efficient utilisation of this disaccharide.

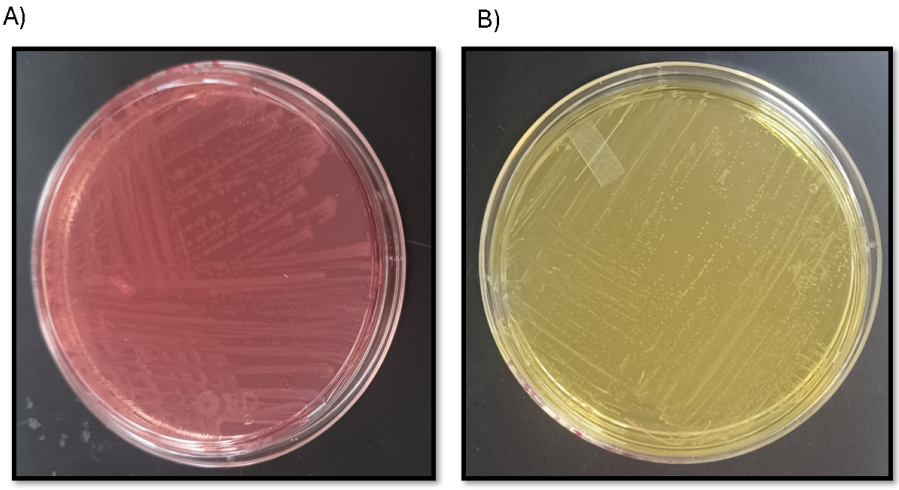


Figure 3. The *E. coli* W3110 strain was grown on phenol red agar supplemented with sucrose and incubated at 37 °C for 48 h. Peptone in the medium supported growth independently of sucrose metabolism. The control strain lacking the pAIDA-*sacA* plasmid (**A**) retained the red colour, indicating no acid production. In contrast, the strain harbouring pAIDA-*sacA* (**B**) hydrolysed sucrose, producing acidic metabolites that lowered the pH and caused a yellow colour change.

3.4. Enzymatic Activity Assay

The enzymatic activity of whole cells expressing AIDA-SacA was evaluated by quantifying the release of reducing sugars over time and expressing the results in enzyme activity units (U), as defined in Section 2.5. The recombinant sucrase displayed an activity of approximately 0.20 U at 10 min, which progressively decreased with longer incubation times, reaching 0.07 U at 80 min (Figure 4). Control assays were conducted using the same *E. coli* W3110/pAIDA-*sacA* cells after heat inactivation, under identical reaction conditions. No reducing sugars were detected in these controls, confirming that sucrose hydrolysis originated exclusively from the enzymatic activity of AIDA-SacA. These findings confirm that *E. coli* successfully expresses functional AIDA-SacA, and that the activity observed in whole cells further supports the notion that the enzyme remains immobilised on the cell surface.

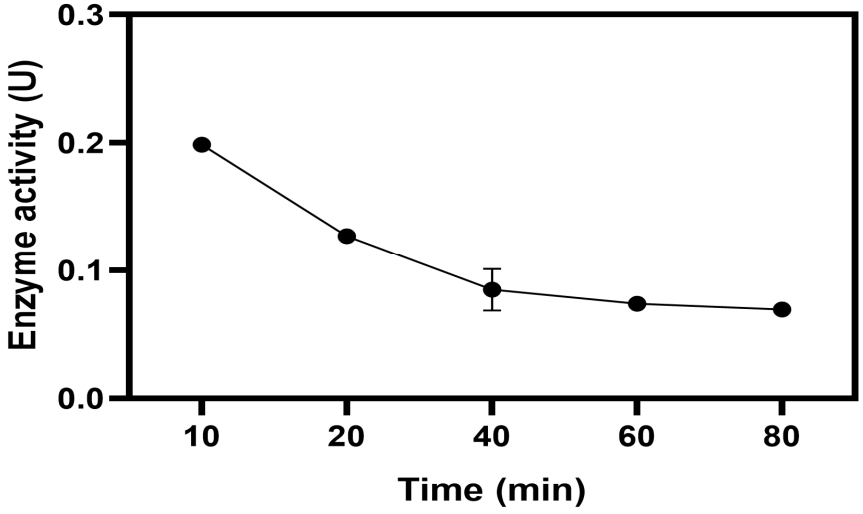


Figure 4. Enzymatic activity of *E. coli* W3110/pAIDA-*sacA* expressing AIDA-SacA in whole cells. A progressive decrease in activity was observed over time, with an initial activity of 0.20 U at 10 min that declined to 0.07 U at 80 min, indicating sustained but diminishing sucrose hydrolysis. Data represent the mean ± standard deviation of at least three independent experiments. At zero time, no enzymatic activity was detected.

3.5. Enzymatic Optimum Temperature

The effect of temperature on AIDA-SacA activity was analysed by measuring enzymatic activity at various temperatures (Figure 5A). The enzyme exhibited maximum activity at 40 °C, with a progressive decline observed at higher temperatures. Beyond 50 °C, enzymatic activity decreased significantly, indicating that AIDA-SacA loses catalytic efficiency under elevated thermal conditions. In *Bacillus subtilis* Marburg 168, an intracellular sucrase was reported to exhibit optimal activity at 37 °C [2], which is slightly lower than the temperature optimum observed for AIDA-SacA. When compared with other bacterial sucrases, AIDA-SacA displays a lower optimal temperature than those derived from thermophilic organisms. For example, the sucrase from *Bacillus cereus* exhibits optimal activity at 50 °C [11], while SurA from *Bacillus stearothermophilus* NUB36 reaches maximum activity at 55 °C [12]. Sucrases from fungi and yeasts, such as *Kluyveromyces marxianus* and *Xanthophyllomyces dendrorhous*, function optimally at even higher temperatures, ranging from 65 to 70 °C, reflecting the thermal adaptation of their respective hosts [13].

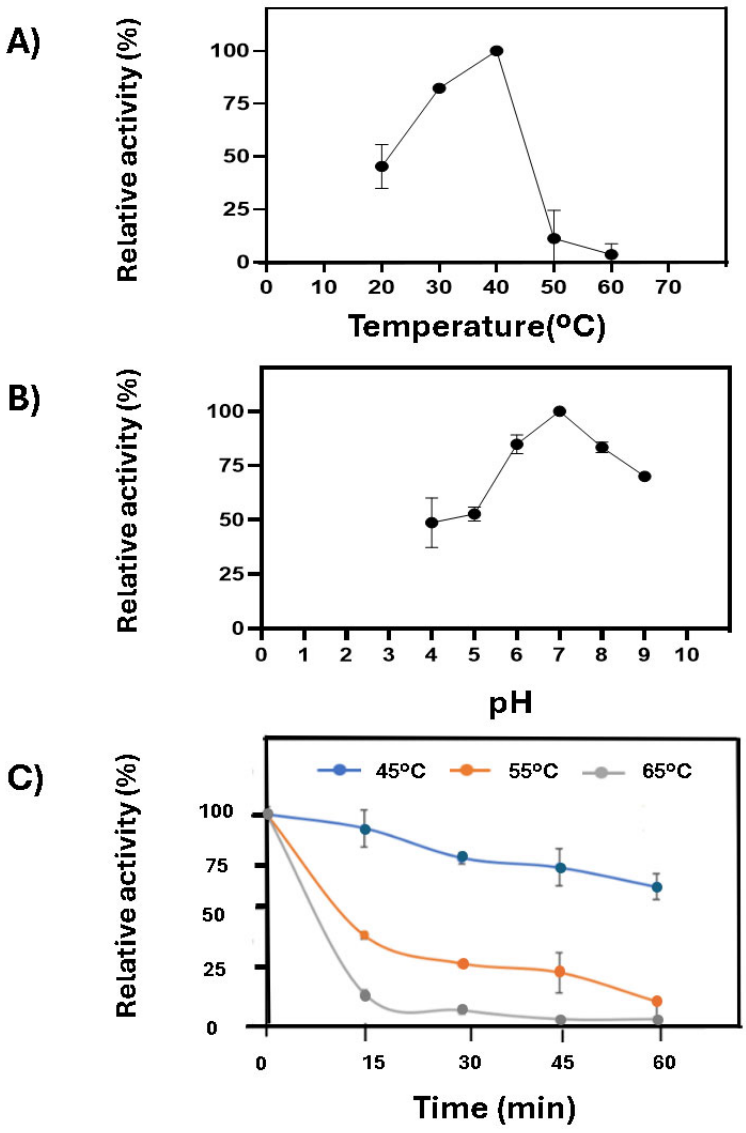


Figure 5. Enzymatic profile of whole cells biocatalyst containing AIDA-SacA. (**A**) Relative activity measured across a temperature range, indicating maximal activity at 40 °C. (**B**) Effect of pH on enzymatic performance, with optimal activity observed at pH 7.0 (**C**) Thermal stability evaluated at 45, 55 and 65 °C; the enzyme maintained over 80% of its initial activity after 60 min at 45 °C, whereas a marked loss of activity was noted at 65 °C.

3.6. Enzymatic Optimum pH

The enzymatic activity of AIDA-SacA was assessed over a pH range from 3.0 to 9.0 (Figure 5B). The enzyme showed highest activity at 7.0, indicating optimal performance under near neutral to slightly alkaline conditions. Activity declined sharply under acidic conditions, with minimal activity at pH 4.0 and negligible activity below this point. The native sucrase from *Bacillus subtilis* Marburg 168 has been reported to exhibit peak activity at pH 6.5 [2]. This behaviour is also consistent with the invertase from *Bacillus cereus*, which operates optimally at pH 7.0 [11]. In contrast, fungal sucrases such as those from *Aspergillus niger* are more active between pH 2.9 and 5.6, while yeast-derived enzymes like those from *Saccharomyces cerevisiae* typically perform best between pH 3.5 and 6.0 [13].

3.7. Enzymatic Thermal Stability

The thermal stability of AIDA-SacA was investigated by pre-incubating the enzyme at 45 55, and 65 °C for various time intervals followed by measurement of residual enzymatic activity (Figure 5C). The enzyme retained more than 80% of its initial activity after 60 min at 45 °C, indicating notable stability at moderate temperatures. In contrast, activity declined progressively at 55 °C, with approximately 30% residual activity remaining after 60 min. At 65 °C, enzymatic activity dropped below 10% within the first 15 min, demonstrating rapid thermal inactivation. AIDA-SacA exhibits a thermal stability profile like that of the intracellular invertase from *Bacillus cereus*, which retains high activity at 40 °C but progressively loses stability between 50–60 °C [11]. In contrast, sucrases from thermophilic organisms, such as *Bacillus stearothermophilus*, maintain activity at 55 °C for extended periods [12]. However, at temperatures exceeding 65 °C, even thermophilic sucrases experience a marked decline in stability, leading to significant enzymatic inactivation and loss of function. These results provide insights into the potential applications of AIDA-SacA in biotechnological processes that operate at moderate temperatures.

3.8. Kinetic Parameters

The kinetic parameters of AIDA–SacA were determined by measuring initial reaction rates at increasing sucrose concentrations (1–6 mM) and fitting the data to the Michaelis–Menten model by non-linear regression. The enzyme exhibited a Km of 1.18 mM and a Vmax of 2.32 U mL^{-1}, with a R^2 value of 0.96 and a 95% confidence level, confirming a good fit to the Michaelis–Menten model. The data were also examined using the Lineweaver–Burk double-reciprocal plot, which produced consistent Km and Vmax values, confirming the reliability of the non-linear regression. The combined presentation of both plots (Figure 6) highlights the agreement between the experimental and fitted values. This Km places AIDA-SacA among bacterial sucrases with efficient substrate binding and compares favourably with several microbial enzymes reported in the literature. For example, *Bacillus cereus* invertase shows a Km of 370 mM [11], while fungal enzymes from *Aspergillus niger* and *Emericella nidulans* present Km values of 117 mM and 4.8 mM, respectively [13]. By contrast, yeast-derived sucrases from *Candida guilliermondii* and *Penicillium janczewskii* display Km values of 0.104 mM and 0.37 mM, respectively [13]. In *Bacillus subtilis* Marburg 168, an endocellular sucrase was described with a Km of 50 mM for sucrose, and a separate activity specific to 6G-phosphorylsucrose with a Km of 1.2 mM. In addition, two distinct native sucrase activities were identified: a levansucrase with a Km of 20 mM and a sucrase-like enzyme with a Km of 40 mM, both characterised from induced bacterial extracts [14]. Compared with these native enzymes, AIDA-SacA exhibits a lower Km under our assay conditions, which may reflect advantages of surface localisation that facilitate direct substrate access in whole-cell systems, highlighting its potential utility in biotechnological applications involving sucrose conversion.

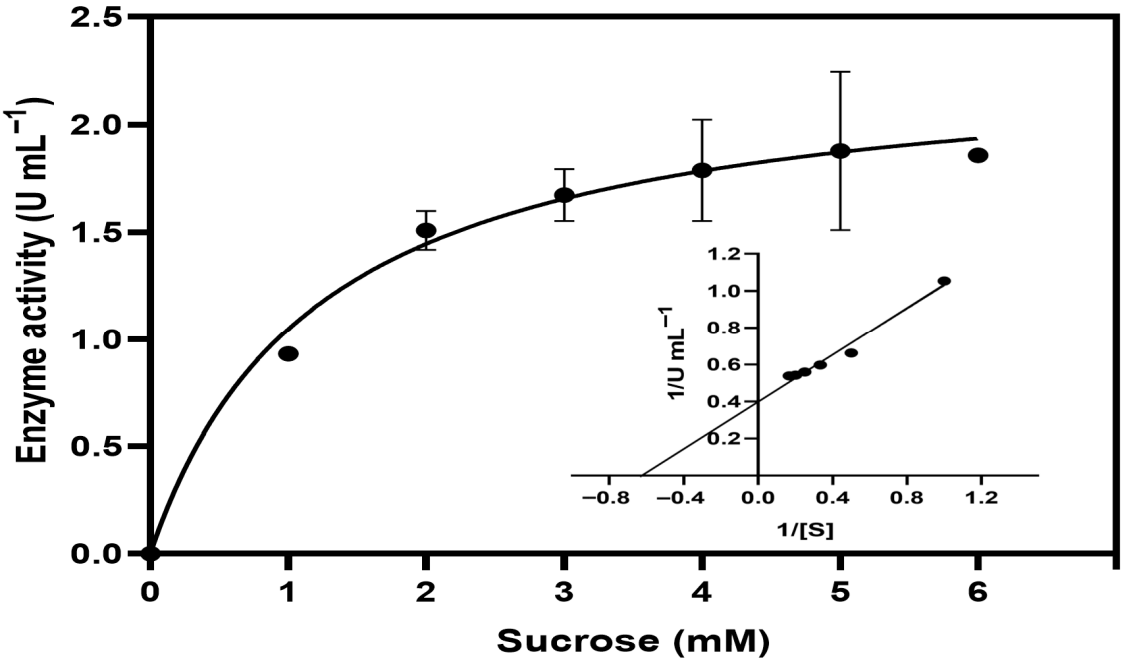


Figure 6. Michaelis–Menten kinetics of whole cell biocatalyst containing AIDA–SacA activity using whole cells of *Escherichia coli* W3110/pAIDA–*sacA*. The enzyme activity was measured at increasing sucrose concentrations (1–6 mM) at 40 °C and pH 6.5. Experimental data (•) were fitted to the Michaelis–Menten model (solid line). The inset shows the corresponding Lineweaver–Burk double-reciprocal plot, confirming the consistency of the kinetic parameters obtained from the non-linear regression.

3.9. Effect of Metals and Chemical Modulators on AIDA-SacA Activity

The effect of various metal ions and chemical compounds on the enzymatic activity of AIDA-SacA was evaluated using whole cells of *E. coli* W3110/pAIDA-*sacA*. All assays were performed with a final concentration of 1 mM for each tested metal ion or compound, unless otherwise indicated. Enzymatic activity was expressed relative to the control condition (without additives), which was set at 100%. Among the tested ions, K^+ was the most potent activator, increasing activity to 155%, followed by Ca^{2+}, and Na^+, which enhanced activity to 134%, and 111%, respectively. EDTA and DTT also moderately increased activity to 120 and 111%, respectively.

In contrast, Zn^{2+}, Cu^{2+}, and Mg^{2+} significantly inhibited enzyme activity, reducing it to 34%, 21%, and 31%, respectively. Fe^{2+} exhibited moderate inhibition, with 74% residual activity. The addition of Ni^{2+} resulted in a substantial reduction in activity to 33%. Similarly, acetic acid at 10 mM led to a moderate decrease in activity to 70% (Table 1). These results reveal a distinct pattern of response to metal ions and chemical modulators, consistent with those reported for other bacterial sucrases.

Notably, the strong activation observed with K^+ aligns with previous reports describing the endocellular sucrase from *Bacillus subtilis* Marburg 168, which is also activated by potassium ions and exhibits similar ionic sensitivity [2]. Similarly, the moderate enhancement observed with Ca^{2+} and Na^+ may reflect general ionic stabilisation, a property also noted for the intracellular sucrase from *B. cereus* [11]. The enhancement of AIDA-SacA activity by EDTA and DTT is consistent with observations for the intracellular sucrase of *Bacillus subtilis* Marburg 168, where both agents were required to preserve enzymatic activity [2].

Table 1. Effect of metal ions and chemical modulators on the enzymatic activity of the AIDA–SacA whole-cell biocatalyst in *Escherichia coli* W3110/pAIDA–*sacA*. Relative activity is expressed as a percentage of the control (set at 100%).

Condition	Relative Activity (%)
K^+	155.3
Ca^{2+}	134.0
EDTA	120.0
DTT	113.2
Na^+	111.0
Fe^{2+}	74.8
Acetic Acid	70.5
Zn^{2+}	34.6
Ni^{2+}	33.8
Mg^{2+}	31.2
Cu^{2+}	21.7
Control	100

DTT also reversed thiol-specific inhibition, suggesting the involvement of redox-sensitive residues. These results support the idea that EDTA prevents metal-mediated inhibition, while DTT protects essential cysteines, contributing to the observed increase in activity. Several metal ions and chemical compounds tested in this study exhibited inhibitory effects on the enzymatic activity of AIDA-SacA. Among the most potent inhibitors were Cu^{2+}, Zn^{2+}, and Ni^{2+}. These metals are known to interact with thiol and imidazole groups, potentially disrupting catalytic residues such as cysteine and histidine [15]. Magnesium also significantly inhibited activity, suggesting that in the case of AIDA-SacA, this divalent cation may interfere with substrate binding or alter protein conformation. Iron caused moderate inhibition, this is consistent with previous findings in bacterial sucrases where Fe^{2+} may act as a redox modulator, particularly in environments with fluctuating oxidative conditions [16]. Additionally, the presence of 10 mM acetic acid suggests that AIDA-SacA retains a degree of functionality under mildly acidic environments, but its activity may be compromised in processes where organic acid accumulation occurs.

4. Conclusions

The *Escherichia coli* strain transformed with the pAIDA–*sacA* plasmid, which expresses the AIDA–SacA fusion protein, exhibits functional activity as indicated by saccharolytic assays, structural modelling, and enzymatic characterisation. The enzyme shows a Km of 1.18 mM, optimal activity at 40 °C and pH 7.0, and retains over 80% of its activity after 60 min at 45 °C, reflecting good thermal stability under mesophilic conditions. Potassium ions enhance enzymatic activity, whereas zinc, copper, and magnesium act as inhibitors. Protein expression is further confirmed by SDS–PAGE, which reveals a distinct band at approximately 114 kDa, consistent with the predicted molecular mass of the AIDA–SacA fusion. Collectively, these findings support the notion that SacA is surface-associated through the AIDA-I system and demonstrate the functional expression of a sucrase on *E. coli* cells. Further studies incorporating biochemical assays such as protease accessibility, immunofluorescence, or cell fractionation are required to provide direct evidence of surface display and to confirm the robustness of this platform.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr13103330/s1, Figure S1. Schematic representation of the pAIDA–*sacA* plasmid construct. The pAIDA–*sacA* plasmid was designed for the surface display of SacA from *Bacillus subtilis* in *Escherichia coli* via the AIDA-I autotransporter system. Figure S2. Sucrose consumption profile of *Escherichia coli* W3110/pAIDA-*sacA* cultivated anaerobically in phosphate buffer medium (medium B) containing 10 g L^{-1} sucrose.

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