Preparation, characterization and optimization of cross-linked fructosyltransferase aggregates for the production of prebiotic fructooligosaccharides

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Abstract
In this study, the preparation, characterization, and evaluation of the cross-linked enzyme aggregates (CLEAs) of fructosyltransferase isolated from the mutant-type Aureobasidium pullulans NAC8 for the production of fructooligosaccharides (FOS) with prebiotic properties were investigated. The reaction conditions were optimized statistically by using response surface methodology. CLEAs were characterized by using both photo-microscopy and scanning electron microscopy energy-dispersive X-ray spectroscopy (SEM-EDX). The secondary structure of the protein was predicted by using Fourier transform infrared spectroscopy (FTIR). FOSs produced as a result of the biotransformation of sucrose was quantified by using a chemometric analysis of FTIR spectral data. The prepared FOSs were investigated for the prebiotic effects under anaerobic conditions using a standard strain of Lactobacillus sp. Following were the statistically determined, optimum conditions for the production of FOSs: 5% (v/v) glutaraldehyde, pH 5.5, and a temperature of 32°C. The values of $R^2$, F value, adjusted precision, and coefficient of variation were found to be 0.94, 11.50, 9.34, and 35.34%, respectively. Validation of the model resulted in the reusability of the CLEAs in six reaction cycles as it retained 70% of the residual activity. The SEM-EDX and photomicrographic analysis showed that the sizes of CLEAs varied, which ranged between 10 and 40 μm. Aggregation leads to the formation of a higher number of beta sheets in the CLEAs than that in the free enzyme. The total FOSs produced by the CLEAs was around 57% (w/v). A. pullulans FOS supplemented to the medium had a stimulatory effect on the growth of bifidobacteria compared with the control medium. The growth curves of Lactobacillus sp. reached the plateau phase after 24 h of cultivation with FOS. The CLEA of fructosyltransferase catalyzed the biocatalysis of sucrose to FOSs, a process with a biotechnological importance as prebiotics.

Key words: Aureobasidium pullulans NAC8, cross-linked enzyme aggregates (CLEAs), fructosyltransferase, fructooligosaccharides, statistical optimization, SEM-EDX

Introduction
Fructosyltransferases (EC 2.4.1.9) are enzymes that catalyze the transfer of fructose units from a sucrose molecule to another molecule of sucrose or to fructose in a process that yields higher amounts of fructooligosaccharide (FOS) units (Maiorano et al., 2009). FOSs have also been termed as fructose oligomers; they have a terminal glucose residue that is linked to fructose via glycosidic bond ($\beta$-2,1). FOSs are nondigestible carbohydrates that represent one of the major classes of bifidogenic oligosaccharides; they have/show important health benefits as prebiotics (Sabater-Molina et al., 2009). Research in industrial enzymology has resulted in the large-scale production of FOSs by the method of enzymatic transformation of fructosyltransferases and β-fructofuranosidases on sucrose (Flores-Maltos et al., 2014). Industrially, FOS production can be classified under two broad categories: the batch system, where the free enzyme is used and a continuous system, in which immobilized cells and or enzymes are used. Enzyme immobilization, mostly in bioreactors, offers a great potential due to the possibility of the high-enzyme load. This leads to high volumetric productivities and a finite control on the extension of the reaction itself and a simpli-
fication of the downstream process. Furthermore, en-
zyme immobilization offers the possibility of recovering
and reusing the biocatalyst (Fernandez et al., 2005).
It offers numerous advantages such as the prevention of
degradation of enzyme and substrate inhibition, which
often is seen with free enzymes. In addition, it will be
more useful if the process of immobilization is auto-
mated (Sheldon, 2007; Fernandes, 2010).

Traditionally, the technique of enzyme immobiliza-
tion is performed using a myriad of methods, including
entrainment, binding to a solid carrier, and micro-encap-
sulation; these methods are referred to as the carrier-
type immobilization. The carrier-type immobilization
 technique inevitably leads to the dilution of the catalytic
activity resulting from the introduction of a large pro-
portion of noncatalytic mass. This results in a lower volum-
metric yield and a lower productivity of the catalyst (Cao
et al., 2000). The other types of immobilization, where
there is no need to bind to a carrier molecule, are
broadly termed as CLEAs. Immobilization via the cross-
linking of enzyme molecules with a bifunctional cross-
linking agent is a carrier-free method and the resulting
biocatalyst essentially comprises 100% active enzyme.

There are several cross-linking agents that can cross-link
proteins and enzymes, such as glutaraldehyde, dextran
polyaldehyde, diepoxide, p-benzoquinone, L-lysine, pect-
in, and polyethyelyleneimines. Among these, glu-
таралdehyde is the most frequently used cross-linking agent
(Cao et al., 2003; Mateo et al., 2007; Wang et al., 2011;
Ayan et al., 2012; Talekar et al., 2014); however, it has
some drawbacks. For example, glutaraldehyde has been
shown to disrupt the activity of nitrilase as it can easily
penetrate the enzyme due to its small size (0.1 kDa)
(Valdes et al., 2001). In general, the mechanism of ac-
tion of the aforementioned cross-linking agents to form
CLEA is based on the interaction between the protein’s
amino groups and the reactive groups of the cross-linker
resulting in the formation of covalent bonds (Schoevaart
et al., 2004). Glutaraldehyde is the most common cross-
linker; it interacts with the aggregated proteins (ag-
gregation occurs by precipitating the protein using am-
onium sulfate, or other organic solvents).

CLEAs are obtained by precipitating nonpurified
enzymes to form solid particles that bind with each other
via noncovalent bonds (Tischer and Kasche et al., 1999).
They become permanently insoluble after they coval-
ently bind with a cross-linking agent such as glutar-
aldehyde. In a typical reaction model, CLEAs exhibit
greater activity (more than 10-fold) than their correspon-
ding biocatalysts that are immobilized using a carrier.
Comparatively, glutaraldehyde has a negligible molecular
weight compared to the protein of interest which is to be
immobilized; therefore, the resulting immobilized biocata-
lyst is nearly 100% target protein of interest to be cross-
linked with the assumption that the weight of gluteral-
dehyde is negligible. In addition, CLEAs are insoluble and
hence can be easily recovered from the reaction milieu by
performing low-speed centrifugation (Park et al., 2010). In
addition, CLEAs offer an avenue where the proteins’ cata-
lytic features combine readily with the mechanical be-

The advantages offered by the immobilization of FOS-
producing enzyme using CLEAs compared to the free
enzyme are numerous, such as pH and thermal stability,
easy separation of products from biocatalysts, and a con-
dition in which a continuous process can be developed.
Overall, an increased operational stability is envisaged.
For the large-scale production of FOS, the FOS-produ-
cing enzymes have been immobilized on porous glass,
porous silica (Hayashi et al., 1993), ion-exchange resins
(Yun and Song, 1996), gluten (Chien et al., 2001), poly-
methacrylate (Ghazi et al., 2005), macroporous beads
(Tanriseven and Aslan, 2005), and calcium alginate (Jung
et al., 2011).

To the best of our knowledge, the use of fructosyl-
transferase CLEAs in the synthesis and large-scale pro-
duction of FOSs has not been fully exploited. The only
recent report on the synthesis of FOSs involved the use
of levansucrasc from Bacillus subtilis (Ortiz-Soto et al.,
2009). This implies that the possibility of the synthesis
of FOSs by using carrier-free immobilized fructosyl-
transferase is worth investigating.

Therefore, in this study, we synthesized cross-linked
fructosyltransferase aggregates from an improved mu-
tant strain of Aureobasidium pullulans NAC8 (Adem-
akinwa and Agboola, 2016; Ademakinwa et al., 2017), and
we developed a mathematical model for the industrial
production by optimizing the scale-up conditions using
statistical techniques. The characterization of CLEAs
was performed using photomicroscope and scanning
electron microscopy energy-dispersive X-ray spectro-
scopy (SEM-EDX). The structural changes of CLEAs
after cross-linking with glutaraldehyde were investigated
by way of a peak fitting analysis of the amide I band of
the spectral data obtained by Fourier transform infrared spectroscopy (FTIR). The reusability of (the) CLEAs was also investigated over several reaction cycles. The reaction products of the CLEA biocatalysis of sucrose were purified using activated charcoal, and the sugar content was quantified using multivariate data analysis of the FTIR spectral data. The use of FOSs as prebiotics was investigated under anaerobic conditions using Lactobacillus sp. as the probiotic strain of choice.

Materials and methods

Reagents

Glutaraldehyde was obtained from Fluka, Germany. Sucrose, activated charcoal, potassium bromide, bovine serum albumin, glucose, sucrose, and fructose were obtained from Sigma Aldrich, USA. The commercial FOS standard was purchased from Source Naturals, Santa Cruz, California, USA, and it was purified on activated charcoal (Sigma Aldrich, USA) prior to use. All other reagents were of analytical grade and used as provided without further purification.

Microorganisms and maintenance

The fungus, Aureobasidium pullulans, used in the study was genetically modified using chemical mutagenesis of the wild-type Aureobasidium pullulans NAC8 strain (Accession no. KX023301) (Ademakinwa and Agboola, 2016; Ademakinwa et al., 2017). The fungus was maintained on the malt extract agar slant at 4°C and was routinely subcultured after every 7 days.

Submerged fermentation and isolation of intracellular fructosyltransferase

The medium for enzyme production contained (w/v) the following ingredients: sucrose (20%), yeast extract (0.5%), NaNO₃ (1%), MgSO₄·7H₂O (0.05%), KH₂PO₄ (0.25%), NH₄Cl (0.5%), and NaCl (0.25%) with an initial pH 5.5. After 72 h of incubation, the cultured broth was centrifuged at 6000 × g for 10 min at 4°C using a refrigerated centrifuge. The pellet served as the source of mycelial fructosyltransferase. The mycelia were washed with several rounds of distilled water and blotted to dryness. The intracellular FOS-producing enzymes in the mycelia of the improved mutant strain of A. pullulans were extracted by grinding the mycelia with acid washed sand (pH 7.0) (1:1) in a clean prechilled mortar using 0.1 M acetate buffer, pH 5.0. The material was homogenized at room temperature. The so-obtained homogenate was centrifuged at 4000 × g for 20 min, and the resulting supernatant was used as the source of intracellular enzyme. All operations were performed on the ice at 4°C (Ademakinwa et al., 2017).

Fructosyltransferase assay

Fructosyltransferase activity was determined by incubating an aliquot of the enzyme solution (0.1 ml) with 0.9 ml of 60% sucrose (w/v) in 0.1 M acetate buffer (pH 5.5) at 45°C for 60 min in a water bath. The reaction was stopped by boiling the mixture at 100°C for 20 min. The released glucose was quantified using a glucose oxidase-peroxidase (GOD-POD) kit (Sigma), using a spectrophotometer at 505 nm. One unit of fructosyltransferase was defined as the amount of enzyme activity required to produce 1 μmol of glucose per minute under the described conditions (Ganaie et al., 2014, Ademakinwa et al., 2017).

Synthesis of CLEAs

The optimization of cross-linking reactions was conducted statistically using the response surface methodology (RSM) model (Box-Behnken design (BBD)). Using one factor at a time (OFAT), the determination of the best precipitant from toluene, acetone, ethanol, and ammonium sulfate, at concentrations ranging between 40 and 70% (v/v) in 0.2 M acetate buffer, pH 5.5 was performed (but not included in this report) (Ortiz-Soto et al., 2009). The effect of cross-linking with glutaraldehyde was explored in a concentration ranging between 1 and 20% (v/v). After cross-linking, the mixture was quenched with 900 μl of 0.2 M acetate buffer, pH 5.5. The quenched samples were assayed for enzymatic activity. An aliquot from the suspension, which contained both CLEAs and a residual-free enzyme, was assayed for activity. The activity of the free enzyme fraction was assessed on a sample after removal of CLEAs by centrifugation (Ortiz-Soto et al., 2009). The effect of cross-linking with glutaraldehyde was explored in a concentration ranging between 1 and 20% (v/v). After cross-linking, the mixture was quenched with 900 μl of 0.2 M acetate buffer, pH 5.5. The quenched samples were assayed for enzymatic activity. An aliquot from the suspension, which contained both CLEAs and a residual-free enzyme, was assayed for activity. The activity of the free enzyme fraction was assessed on a sample after removal of CLEAs by centrifugation (Ortiz-Soto et al., 2009). The difference in activities between the two samples was the actual CLEA activity. This approach is the most accurate way of determining the activity of CLEAs, without having to wash and disperse, which can increase clotting of the CLEA and thereby cause limitations in mass-transport (Schooevart et al., 2004). Reactions using sucrose, both as a donor and as an acceptor, were performed using a medium containing 600 g/l of sucrose (pH 6.0), 50 mM sodium phosphate...
Table 1. Selected variables and their assigned levels by Box-Behnken design (BBD)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Component</th>
<th>Unit</th>
<th>Range</th>
<th>Levels of variables studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>pH</td>
<td>–</td>
<td>4–6</td>
<td>-1 0 +1</td>
</tr>
<tr>
<td>B</td>
<td>temperature</td>
<td>°C</td>
<td>4–60</td>
<td>4 32 60</td>
</tr>
<tr>
<td>C</td>
<td>glutaraldehyde %</td>
<td>(v/v)</td>
<td>1–10</td>
<td>1 5.0 10</td>
</tr>
</tbody>
</table>

at 37°C. Preparation of CLEA, as well as the enzyme assays, were performed at least thrice.

Aggregation yield

The aggregation yield is defined as the activity recovered in the CLEAs when compared with the total activity of the soluble enzyme in the experiment (Ortiz-Soto et al., 2009).

Statistical optimization for cross-linked fructosyltransferase aggregates

The choice of precipitants (toluene, acetone, and ammonium sulfate), as well as their concentration leading to the formation of fructosyltransferase CLEA, was determined using the OFAT approach. Statistical optimization was performed using the BBD and several factors influencing the formation of CLEA as well as the catalysis, namely, the concentration of cross-linker, temperature, and pH, were investigated.

RSM-based optimization

In this study, the concentration of glutaraldehyde and the optimum pH and temperature were studied at three different levels (-1, 0, and +1) (Table 1). The levels were termed as +1, 0, and -1 which indicates high, central (middle), and low variables, respectively. The variables were set at a central coded value of zero. The minimum and maximum ranges of variables were obtained based on previously conducted independent experiments (data not shown). For each experimental run, the fructosyltransferase activity was measured in triplicate. The aggregation yield was analyzed using a second-order polynomial equation. A multiple regression procedure was used to fit the data into the equation. For the correlation of the relationship that exists between the response (aggregation yield) and the independent variables, the second-order polynomial function was thus fitted. This was used to predict the optimal point. Following is the equation for three factors:

\[
X = \beta_0 + \beta_1 Y_1 + \beta_2 Y_2 + \beta_3 Y_3 + \beta_{12} Y_1 Y_2 + \\
+ \beta_{13} Y_1 Y_3 + \beta_{23} Y_2 Y_3 + \beta_{11} Y_1^2 + \beta_{22} Y_2^2 + \beta_{33} Y_3^2
\]

where \(X\) is the predicted response; \(\beta_0\) is the model constant; \(Y_1, Y_2\), and \(Y_3\) are independent variables; \(\beta_1, \beta_2, \) and \(\beta_3\) are linear coefficients; \(\beta_{12}, \beta_{13}, \) and \(\beta_{23}\) are cross product coefficients; and \(\beta_{11}, \beta_{22}, \) and \(\beta_{33}\) are the quadratic coefficients. The model equation for the analysis is given as representing the constant process effect in total, the linear \((Y_i)\), quadratic effect \((Y_i^2)\), and the effect of interaction between \(Y_i\) and \(Y_j\) for the aggregation yield (Haaland, 1989; Ademakinwa et al., 2017).

Model validation

The model validation for the catalysis by CLEA was performed by preparing the CLEAs using the optimum values (5% (v/v) glutaraldehyde, pH 5.5, and 32°C) predicted by the BBD RSM model. The experimental, as well as the predicted values for the aggregation yield, were confirmed.

Statistical analysis

The RSM model, as well as the analysis of variance (ANOVA), were designed using Design Expert 7.1.5. Positive effects in terms of aggregation yield were considered significant for \(p\)-values lower than 0.05 (Haaland et al., 1989).

Determination of the mass of CLEAs

The CLEAs were dried to constant weight using an incubator (45°C for 4 h) and were then weighed.

Characterization of synthesized CLEAs microscopy

The synthesized CLEAs were analyzed and their size was determined using a camera-enabled light microscope (Accu Scope 3310 series coupled to a Micrometrics Premium SE Software version 4). CLEAs were fixed on microscope slides and viewed using 40 × and 100 × objective lenses in a phase contrast mode (Ortiz-Soto et al.,
Preparation, characterization and optimization of cross-linked fructosyltransferase aggregates for the production of prebiotic (2009). The aggregates formed before, during, and after quenching were observed and captured. In addition, after several rounds of reuse of CLEA (repeated catalysis of sucrose to determine the number of reaction cycles that the CLEA can catalyze), the nature and size (in μm) of the aggregates were also determined.

**SEM-EDX**

The microstructure and chemical analysis of the CLEAs were performed using an SEM model TESCAN equipped with Oxford instrument X-Max (EDS), iThembab LABS, Materials Research Department, Cape Town, South Africa. The CLEAs were dried to a constant weight (45°C for 4 h) prior to the SEM-EDX analysis.

**Analysis of secondary structural features of enzyme in CLEAs and free state by FTIR spectroscopy**

FTIR spectroscopy was used in the analysis of changes in the secondary structure of the enzyme in the CLEAs and free state. Sample preparation involved dispersion of CLEAs (dried pellet) or free enzyme (lyophilized) in potassium bromide (1:10 w/w) followed by homogenization (in a mortar and pestle) before inserting them into disks using the hydraulic gauges. The spectral data were collected in the range of 4000–400 cm⁻¹ using an FTIR spectrophotometer (Thermo Scientific Nicolette iD1, Thermo Electron Corporation, USA). The secondary derivative of peak frequencies in the amide I region (1600–1700 cm⁻¹) (performed using SpectraGryph version 1.2) (Susi and Byler, 1986; Wang et al., 2017) was resolved, identified, and fitted with Gaussian curves. From the best fit, the peak areas (from the full width at half maximum) were observed using the OriginPro 2017 software.

**Purification of synthesized sugars**

The granular form of activated charcoal (80 g) (Sigma-Aldrich, USA, G-60, 100–350 mesh) was used as the adsorbent of choice used in the purification experiments (extra pure), with a mean particle diameter of 1.5 mm. Before filling the column, activated charcoal was washed in distilled water and autoclaved to remove any residual particles and air from the pores (Nobre et al., 2012). Next, the charcoal was loaded into a glass column (2.0 × 20 cm) that was previously equilibrated with distilled water. The supernatant (from the CLEA catalysis and the whole cell biocatalysis) was applied to the column and eluted with distilled water to remove the nonadsorbed sugars. The adsorbed sugars (desorption) were eluted using ethanol from 10% (v/v) up to 50% (v/v) in a stepwise manner. The concentration of reducing sugars in fractions was determined using the dinitrosalicylic acid (DNS) reagent (Miller, 1959). The fractions collected in the desorption phase were evaporated at 60°C to remove ethanol and to concentrate the sugars present. Further concentration was performed by freeze-drying and the samples were stored at 4°C until further use (Nobre et al., 2012).

**Analysis of sugars produced from sucrose biocatalysis using the CLEAs**

**Fourier Transform Infrared Spectroscopy of sugars and chemometrics**

All samples (synthesized sugars and standard FOS) were stirred using a stirrer (Sybron/Thermolyne, Iowa, USA) for 1 h at room temperature (25°C) and then incubated for 24 h at 40°C and analyzed using FTIR spectrometer (Thermo Scientific Nicolette iD1). Each FTIR spectrum of both the standard and test samples was recorded in the range of 500–4000 cm⁻¹. To identify the carbohydrate-specific fingerprint region and calibrate the chemometric method for quantification of the sugars, different concentrations (six levels) of standard FOS, glucose, sucrose, and fructose solutions (Table 2) were prepared and then analyzed by using FTIR spectrometer. The FTIR spectra of the reaction products of fructosyltransferase CLEA on sucrose were also recorded in the same conditions (Duarte et al., 2002) (Table 2). The calibration set contained 13 quaternary mixtures, 9 ternary mixtures, 3 double mixtures, and 4 single solutions based on an experimental design using Design Expert software (Table 2). The spectra were processed using an IR solution Software Overview (Shimadzu) and Origin® 7SR1 Software (OriginLab Corporation, Northampton, USA). The synthesized FOSs were characterized and quantified using chemometrics via spectral calibration using partial least squares regression with the aid of Unscrambler X version 10 software (CAMO Software Inc, Oslo, Norway) (Trollope et al., 2015).

**Studies on the synthesized FOSs**

**Preliminary screening for FOS fermenting potential of the Lactobacillus sp.**

A modified method described by Kaplan and Hutckins (2000) was used in the investigation of the utilization of
Table 2. Concentration in % (w/v) of the 4 sugars (glucose, fructose, sucrose and FOS) in the standard solutions of the calibration set

<table>
<thead>
<tr>
<th>S/N</th>
<th>Sugar concentration % (w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>glucose</td>
</tr>
<tr>
<td>1</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>10.0</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>25.0</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
</tr>
<tr>
<td>7</td>
<td>50.0</td>
</tr>
<tr>
<td>8</td>
<td>25.0</td>
</tr>
<tr>
<td>9</td>
<td>10.0</td>
</tr>
<tr>
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<td>5.0</td>
</tr>
<tr>
<td>11</td>
<td>5.0</td>
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<td>12</td>
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</tr>
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</tr>
<tr>
<td>17</td>
<td>5.0</td>
</tr>
<tr>
<td>18</td>
<td>10.0</td>
</tr>
<tr>
<td>19</td>
<td>50.0</td>
</tr>
<tr>
<td>20</td>
<td>50.0</td>
</tr>
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<td>21</td>
<td>75.0</td>
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<tr>
<td>22</td>
<td>10.0</td>
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<tr>
<td>27</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>100.0</td>
</tr>
<tr>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

The sugar concentrations were varied between 5–100% (w/v)

FOS by the probiotic bacteria. Briefly, the potential of the probiotic strain in the fermentation of FOSs was investigated using a bromocresol purple amended Man Rogosa Sharp (MRS) agar medium. An MRS-FOS agar medium was prepared by adding 2% (w/v) purified FOS to MRS agar containing 0.05% L-cysteine, 1.5% agar, and 30 mg of bromocresol purple per liter of medium. The MRS basal medium (i.e., MRS without carbohydrate) was autoclaved and the FOS was filter-sterilized and added to the tempered agar. The probiotic strain was initially grown in MRS broth and then diluted and spread onto the MRS-FOS agar plates.
Table 3. Summary of CLEA of fructosyltransferase aggregation yields for different solvents used for the catalysis over 4 cycles

<table>
<thead>
<tr>
<th>Precipitant</th>
<th>Aggregation yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40%</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st} cycle</td>
<td>156 ± 12</td>
</tr>
<tr>
<td>2\textsuperscript{nd} cycle</td>
<td>158 ± 13</td>
</tr>
<tr>
<td>3\textsuperscript{rd} cycle</td>
<td>97 ± 14</td>
</tr>
<tr>
<td>4\textsuperscript{th} cycle</td>
<td>57 ± 10</td>
</tr>
<tr>
<td>Toluene</td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st} cycle</td>
<td>173 ± 25</td>
</tr>
<tr>
<td>2\textsuperscript{nd} cycle</td>
<td>195 ± 13</td>
</tr>
<tr>
<td>3\textsuperscript{rd} cycle</td>
<td>93 ± 17</td>
</tr>
<tr>
<td>4\textsuperscript{th} cycle</td>
<td>44 ± 7</td>
</tr>
<tr>
<td>Acetone</td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st} cycle</td>
<td>130 ± 11</td>
</tr>
<tr>
<td>2\textsuperscript{nd} cycle</td>
<td>31 ± 9</td>
</tr>
<tr>
<td>3\textsuperscript{rd} cycle</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>4\textsuperscript{th} cycle</td>
<td>3.0 ± 1.0</td>
</tr>
</tbody>
</table>

Table 4. Full experimental Box-Behnken design with coded and actual level of variables and the response function for CLEA aggregation yield

<table>
<thead>
<tr>
<th>Variables</th>
<th>pH std. ord.</th>
<th>Temperature coded</th>
<th>Glutaraldehyde coded</th>
<th>Aggregation yield experimental</th>
<th>predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>coded</td>
<td>real</td>
<td>coded</td>
<td>real</td>
<td>coded</td>
</tr>
<tr>
<td>1</td>
<td>-1</td>
<td>3.0</td>
<td>-1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-1</td>
<td>6.0</td>
<td>-1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>+1</td>
<td>3.0</td>
<td>-1</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>+1</td>
<td>6.0</td>
<td>-1</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>-1</td>
<td>3.0</td>
<td>0</td>
<td>32</td>
<td>-1</td>
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<tr>
<td>6</td>
<td>-1</td>
<td>6.0</td>
<td>0</td>
<td>32</td>
<td>+1</td>
</tr>
<tr>
<td>7</td>
<td>+1</td>
<td>3.0</td>
<td>0</td>
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<tr>
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<td>+1</td>
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<td>0</td>
<td>32</td>
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<td>-1</td>
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<td>60</td>
<td>+1</td>
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<td>+1</td>
<td>4</td>
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<td>12</td>
<td>0</td>
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<td>32</td>
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<td>15</td>
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<tr>
<td>16</td>
<td>0</td>
<td>4.5</td>
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<tr>
<td>17</td>
<td>0</td>
<td>4.5</td>
<td>0</td>
<td>32</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5. Analysis of variance (ANOVA) for the Box-Behnken quadratic model for aggregation yield by the CLEA

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>DF</th>
<th>F-value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>1.072 × 10^5</td>
<td>9</td>
<td>11.50</td>
<td>0.0020</td>
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<tr>
<td>A</td>
<td>5940.50</td>
<td>1</td>
<td>5.74</td>
<td>0.0478</td>
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<tr>
<td>B</td>
<td>780.13</td>
<td>1</td>
<td>0.75</td>
<td>0.4142</td>
</tr>
<tr>
<td>C</td>
<td>10296.13</td>
<td>1</td>
<td>9.94</td>
<td>0.0161</td>
</tr>
<tr>
<td>A^2</td>
<td>6168.32</td>
<td>1</td>
<td>5.96</td>
<td>0.0447</td>
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<tr>
<td>B^2</td>
<td>35270.84</td>
<td>1</td>
<td>34.05</td>
<td>0.0006</td>
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<tr>
<td>C^2</td>
<td>39637.27</td>
<td>1</td>
<td>38.27</td>
<td>0.0005</td>
</tr>
<tr>
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<td>121.00</td>
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<td>0.7425</td>
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<tr>
<td>AC</td>
<td>900</td>
<td>1</td>
<td>0.87</td>
<td>0.3823</td>
</tr>
<tr>
<td>BC</td>
<td>56.25</td>
<td>1</td>
<td>0.054</td>
<td>0.8224</td>
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<tr>
<td>Residual</td>
<td>7250.55</td>
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<tr>
<td>Lack of fit</td>
<td>78.80</td>
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<td>121.35</td>
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<tr>
<td>Pure error</td>
<td>7171.75</td>
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<td></td>
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</tr>
<tr>
<td>Correlation total</td>
<td>1.145 × 10^5</td>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Coefficients of variation = 35.34; R^2 = 0.94; Adjusted R^2 = 0.85; Adequate precision = 9.34; A – pH; B – temperature; C – glutaraldehyde; DF – degree of freedom

Prebiotic effect of Aureobasidium sp. FOS in vitro

The method described by Li et al. (2008) was used in the investigation of the prebiotic properties of the newly synthesized FOSs with minor modifications. Lactobacillus sp. was incubated for 48 h at 37°C and the stock cultures were stored in 10% glycerol at 4°C until needed. MRS broth amended with 0.5 g/l L-cysteine hydrochloride, 0.2 g/l of sodium thioglycolate, and 0.1 g/l of CaCl_2 2H_2O was used as the stock culture medium and as the basal medium. Basal MRS broth supplemented with 0.5%, 1%, and 2% (w/v) of A. pullulan FOS and inoculated with 24 h culture of the probiotic bacteria and incubated at 37°C. The effects of A. pullulan FOS on bacterial growth was studied by cultivating the Lactobacillus sp. for 24 h in the basal medium and further inoculation into the basal medium containing the FOS. The bacteria were then cultured under anaerobic conditions (using anaerobic GasPack, Oxoid, Thermo Fisher) for 24 h at 37°C. Thereafter, the culture broth was centrifuged at 3000 × g for 20 min. The pellets obtained were washed several times with phosphate buffered saline (PBS) (0.1 M phosphate buffer pH 7.4, 0.9% saline) and then were diluted appropriately. The growth of the bacterial cells via turbidity was measured at 600 nm. All measurements were performed in triplicate (Li et al., 2008).

Statistical analysis

The data obtained were analyzed using one-way ANOVA and significant differences were determined by Duncan’s multiple range test. Differences were considered significant at P < 0.05. Statistical analyses were performed using the GraphPad Prism program version 7.0.

Results

Screening of the best precipitant and its concentration for CLEA production

Of the several precipitants (toluene, acetone, and ammonium sulfate) tested, ammonium sulfate was found to be the best as it retained nearly 70% residual activity after four catalytic cycles. Toluene exhibited about 65% residual activity, whereas acetone and ethanol exhibited less than 40% residual activity after four catalytic cycles (Table 3).

Statistical optimization of medium for CLEA production

Following were the optimum conditions obtained after the verification of the RSM model: 5% (v/v) glutaraldehyde, pH 5.5, and 32°C. They corresponded to 190% residual activity after the first reaction cycle. Table 4 shows the full experimental design (BBD) with coded and actual levels of variables and the response function.
Preparation, characterization and optimization of cross-linked fructosyltransferase aggregates for the production of prebiotic

Table 5 shows the ANOVA for the quadratic model for the residual activity of CLEA catalysis. The $R^2$, $F$ value, adjusted precision, and CV were found to be 0.94, 11.50, 9.34, and 35.34 respectively. Figures 1–3 show the response surface plots showing the interaction of these parameters.

**Characterization of CLEA**

The aggregation before and after catalysis has been visualized using photomicrography (Fig. 4). The size of aggregates before catalysis varied from 10 to 20 μm. The SEM-EDX analysis revealed that the size of aggregates (CLEAs) after catalysis was uneven in size, which varied from 10 to 40 μm (Fig. 5). The elemental composition of the CLEAs revealed the presence of phosphorus (30%), sulfur (37.09%), copper (0.17%), zinc (0.94%), oxygen (28.66%), calcium (1.4%), and silver (0.68%) (Fig. 6).

**Protein’s secondary structure in the CLEAs and free state**

Figure 7 shows the FTIR spectral data for the CLEAs and free enzyme. Figures 8 and 9 show the curve fitting process for the free and CLEAs. The content of alpha helix, beta sheet, beta turn, and random coils of the free enzyme were 21.3, 5.4, 54.4, and 9.1%, respectively, whereas the content of alpha helix, beta sheet, beta turn, and random coils of the free CLEAs were 3.7, 65.4, 25.3, and 6.7%, respectively (Table 6).

**Chemometric analysis of FTIR spectral data for the biocatalysis of sucrose**

The production of FOSs by the CLEA biotransformation of sucrose was confirmed by FTIR analysis. The FTIR spectra of standards such as glucose, fructose, su-
A.N. Ademakinwa, Z.A. Ayinla, O. Omitogun, F.K. Agboola

Fig. 4. Photomicrography of the CLEA: A) before quenching B) after quenching and C) after several rounds of catalysis

Fig. 5. Scanning electron microscopy image of the CLEA

Fig. 6. Energy dispersive X-ray analysis to estimate the elemental composition of the CLEA

crose, and FOS were compared with the purified reaction products after purification performed using an activated charcoal chromatography. Figure 10 shows the comparative FTIR spectra of the pure component standards and the purified reaction products of sucrose biocatalysis by the CLEAs. Figure 11 shows the principal component analysis. Table 7 shows the summary of the latent variables obtained, as well as the root mean square values for both the raw spectra and the first derivative (Savitsky–Golay). Table 8 shows the quantification of total FOSs, sucrose, glucose, and fructose by the CLEA.

Prebiotic potential of FOSs

Preliminary screening for FOS utilization on bromo cresol purple amended MRS agar

FOSs changed the color of MRS agar plate from purple to light yellow after fermentation by *Lactobacillus* sp. (Fig. 12).

Effect of newly synthesized FOS, commercial FOS, and glucose on growth and pH of probiotic bacterial strain

The bacterial population growth increased when newly synthesized FOS were used, as evidenced by the value of turbidity (optical density (OD at 660 nm) of 0.58. The negative control (bacteria grown on MRS only) and control (bacteria grown on MRS + glucose) had a relatively low-bacterial population (OD660nm of 0 and 0.1, respectively). The commercial FOS showed a comparable bacterial growth with the newly synthesized FOS at 1% (w/v) with an OD660nm of 0.6 (Fig. 13).

Effect of the incubation period on the prebiotic potential of FOS on *Lactobacillus* sp.

A steady decrease in the pH of the medium containing FOS (from 5.6 to 4.3) after 24 h incubation was observed, followed by a steady pH (4.5) up to 48 h. There was a steady increase in the turbidity (OD660nm value of 0.2 to 0.58) after 28 h incubation (plateau phase) followed by a steady increase in the growth rate (OD660nm value of 0.58) for the next 24 h (Fig. 14). In
case of the medium without FOS, the plateau phase was reached at 36 h (Fig. 15).

Discussion

In this study, the CLEAs of fructosyltransferase were prepared and statistically optimized for the biocatalysis of sucrose to yield FOSs. The FOSs were quantified using chemometrics and their potential use as prebiotics was investigated. The statistical approach to optimization is of immense industrial importance, especially for scale-up processes. To the best of our knowledge, there is little or no information regarding the use of a carrier-free immobilization technique for fructosyltransferases. Hence, our study is the first of its kind in that regard.

During the screening of precipitants, we focused on common protein precipitants, namely, ammonium sulfate, acetone, and ethanol at a concentration ranging from 40 to 70% (v/v). During the process of aggregation, ammonium sulfate was found to be the best precipitant; however, toluene was also found to be a good precipitant in comparison with ammonium sulfate. During the conduct of this study, neither toluene nor ethanol could fully precipitate the enzyme prior to the process of cross-linking with glutaraldehyde; therefore, they showed lower recovery than that of ammonium sulfate. Furthermore, under repeated catalysis, the CLEAs produced using ammonium sulfate had a higher recovery after four rounds of reuse than that of toluene and ethanol. This suggests that ammonium sulfate is good as the cross-
Table 6. The content of various structural motifs in predicted protein secondary structures of amide I band in the free enzyme and the cross-linked enzyme aggregates

<table>
<thead>
<tr>
<th>Secondary structures</th>
<th>α-helix [%]</th>
<th>β-sheet [%]</th>
<th>β-turn [%]</th>
<th>random coil [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free enzyme</td>
<td>21.3</td>
<td>15.4</td>
<td>54.4</td>
<td>9.1</td>
</tr>
<tr>
<td>CLEA</td>
<td>3.7</td>
<td>65.4</td>
<td>25.3</td>
<td>6.7</td>
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</table>

Fig. 10. Comparative FTIR spectra of standard sugars (sucrose, glucose, fructose and fructooligosaccharides) and the reaction products of sucrose transfructosylation (crude and activated charcoal-purified)

Fig. 11. Scores scatter plot of the two first principal components, PC1 and PC2 obtained for the set of standard sugar solutions

linker compared to the other organic solvents. There was a gradual loss in the activity of the enzyme as the number of rounds of the catalysis increased. This might have been due to the bioaccumulation of the products of sucrose catalysis. The ammonium sulfate-CLEA was able to maintain its 100% residual activity over four rounds of catalysis. This is quite remarkable as other precipitants such as toluene and ethanol showed a loss of more than 50% and 90% activity, respectively. In addition, in the initial choice of the concentration of cross-linker, we found that a high concentration of glutaraldehyde (up to 10%) had very a negligible adverse effect on CLEA catalysis. The amount of glutaraldehyde used for CLEA production reported in the recent literature was 0.125% v/v (Ortiz-Soto et al., 2009). According to our results, we noted that at this concentration, there was no formation of CLEA and biocatalysis was found to be very poor (result not shown). High amounts of glutaraldehyde have
Table 7. The application of partial least regression square to the four sets of data for calibration of the sugars

<table>
<thead>
<tr>
<th></th>
<th>Fructooligosaccharide</th>
<th>Fructose</th>
<th>Glucose</th>
<th>Sucrose</th>
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</thead>
<tbody>
<tr>
<td>Raw Spectra RMSEP [%]</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>7</td>
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<tr>
<td></td>
<td>8.4</td>
<td>11.2</td>
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<td>11.4</td>
</tr>
<tr>
<td>First Derivative RMSEP [%]</td>
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<td>4</td>
<td>5</td>
<td>6</td>
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<tr>
<td></td>
<td>3.2</td>
<td>2.9</td>
<td>3.2</td>
<td>6.2</td>
</tr>
</tbody>
</table>

RMSEP – root mean square error

Table 8. Partial least square regression model for the predicted and actual concentrations (% w/v) of the four sugars present in the reaction products of sucrose biocatalysis

<table>
<thead>
<tr>
<th></th>
<th>Total fructooligosaccharides</th>
<th>Fructose</th>
<th>Glucose</th>
<th>Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>predicted % (w/v)</td>
<td>56 ± 2.1</td>
<td>12 ± 1.8</td>
<td>23 ± 2.1</td>
<td>13 ± 4.1</td>
</tr>
<tr>
<td>actual % (w/v)</td>
<td>57 ± 0.3</td>
<td>10 ± 1.8</td>
<td>22 ± 2.1</td>
<td>11 ± 4.1</td>
</tr>
</tbody>
</table>

been reported to inhibit the catalysis by CLEA because of the adverse effect of excessive cross-linking (Ortiz-Soto et al., 2011). However, we postulate that because glutaraldehyde reacts primarily with the ε-amino groups of lysine and other groups such as hydroxyl, secondary amino, and alpha-amino groups (Migneault et al., 2004), the A. pullulans fructosyltransferase might contain fewer lysine residues than that of other enzymes used so far to prepare CLEAs; therefore, there is a need for a higher amount of glutaraldehyde to prepare CLEAs.

In this study, a statistical method was employed to optimize the operational conditions for CLEA biocatalysis, as well as to optimize the product release. The effects of pH, the concentration of glutaraldehyde, and the effective temperature were statistically optimized using the RSM model. In the BBD model, we observed that reactions with low concentrations of glutaraldehyde showed a low aggregation yield irrespective of the pH and the temperature (Table 3). ANOVA revealed that the model was statistically significant, and the lack of fit was not significant (Table 4). The CV, $R^2$, adequate precision, predicted-$R^2$ were 35.34, 0.94, 9.34, and -0.0035, respectively. The regression equation showing this interaction is given in coded units as follows:

\[
\text{Aggregation yield} = +197.80 + 27.25 \times A + 9.87 \times B + 35.88 \times C - 38.27 \times A^2 - 91.53 \times B^2 - 97.03 \times C^2 + 5.50 \times A \times B + 15.00 \times A \times C + 3.75 \times B \times C
\]

where $A$, $B$, and $C$ represents pH, temperature, and concentration of glutaraldehyde, respectively.

The three-dimensional (3D) surface plots showed varying degrees of interaction between the variables considered. The responses obtained from the 3D response surface plots indicate how the numerous interactions that occur between the studied variables affect the aggregation yield, which is very useful in characterizing the responses (Ademakinwa et al., 2017). The optimum conditions statistically derived from the RSM model were found to be 5% glutaraldehyde, pH 5.5, and 32°C.

In the response plots of the effects of pH and temperature, the shape of the curves shows that an increase in the pH led to an increase in the aggregation yield up to 200% prior to a decrease. A similar observation was observed for temperature; as the temperature was simultaneously increased, there was an increase in the aggregation yield. At temperature higher than 45°C, the concomitant effects on the aggregation yield were adverse.

The effects of the concentration of glutaraldehyde and pH on the aggregation yield resulted in a bell-shaped curve indicating the effects of an increase in either of the variables on the aggregation yield. An initial increase in the concentration of glutaraldehyde (1–2.5% v/v) increased the aggregation yield, a further increase of over 5% resulted in a loss in the aggregation yield. The same was observed with respect to the effects of pH, in which an increase in the pH resulted in the initial increase in
the aggregation yield, but a further increase did not lead to a significant loss in the aggregation yield (Fig. 1–3). The effect of pH showed a moderate effect on the aggregation yield as the pH increased. Although few reports exist on the effects of statistically optimized conditions on the potentials of CLEA biocatalysis, some authors have reported that CLEA catalysis can be performed under mild conditions of temperature and pH. Vrsanska et al. (2017) reported that 4°C and pH 4.0 were optimum conditions of catalysis for laccase CLEA. It is noteworthy that the effects of pH on the biocatalysis by CLEA is caused due to the ionic interactions between the enzyme and the cross-linking agent. This often leads to a change in the hydrogen ion concentration, which shifts the optimum pH between acidic and alkaline conditions (Fernandez-Fernandez et al., 2013).

The effect of concentration of glutaraldehyde (1–10% v/v) and temperature (4–60°C) on the aggregation yield showed varied results when compared with the combined variables discussed above. An initial increase in the central values (pH – 5.5, temperature – 32°C and glutaraldehyde – 5.0% v/v) led to an increase in the aggregation yield but a further increase above these central values led to a significant decrease in the aggregation yield.

The combined effect of temperature of up to 60°C and increased concentration of glutaraldehyde (up to 5% v/v) resulted in an increase in the aggregation yield; this might be attributed to the stabilization of linkages that keep the conformation of the catalytic active site of the enzyme (Wilson et al., 2004). Thus, the increased aggregation yield is often a result of a better thermal stability of the CLEAs provided by the coaggregation and cross-linking by the glutaraldehyde. In addition, it is observed that extremes of temperatures might lead to thermal denaturation of CLEAs leading to a loss of their activity (Broun, 1976). The use of the CLEA under the optimized conditions resulted in the repeated reuse of CLEA for catalysis in over six catalytic cycles while still retaining about 70% of their residual activity. This is albeit less visible with the 10 catalytic cycles obtained when Bacillus subtilis levansucrase (Ortiz-Soto et al., 2009).

The results of SEM-EDX experiments of CLEA showed the morphology, as well as the elemental composition of the CLEA. The sizes of the CLEAs ranged from 10 to 40 μm, and they were not evenly shaped. The elemental composition of CLEA revealed that they had a higher composition of phosphorus (30.6%), sulfur (37.7%), and oxygen (28.7%) than that of calcium (1.5%), copper (0.17%), zinc (1.0%) and silver (0.7%). This indicates that CLEA is proteinaceous in nature. This is the first-ever report of the determination of the elemental composition of CLEA using EDX analysis. The size of the CLEA increased after quenching and repeated catalytic cycles. Initially, the microscopic analysis revealed that the size of CLEAs was around 10 μm, whereas after
quenching, the size increased to around 40 μm. This increase in size might be due to the increased aggregation caused by the process of centrifugation and/or due to the presence of some residual glutaraldehyde which had not been washed off completely (Ortiz-Soto et al., 2009). Ortiz-Soto et al. (2009) reported the effect of quenching on the sizes of the CLEA. Increased sizes (from 20 to 50 μm) were also noted by the levansucrase CLEA after quenching and repeated catalysis (Ortiz-Soto et al., 2009).

To further ascertain the structural features between the free enzyme and CLEAs, the secondary structure of all CLEAs was observed by FTIR spectroscopy, as it is considered an important technique to monitor the conformational changes in proteins (Jung, 2000). The structural variation in the proteins’ conformation is mostly studied in the range that constitutes the amide I band, as this is the most intense absorption band for polypeptides (Susi and Byler, 1986). It was observed that there was an increased content of the beta sheet component in the CLEAs compared to the free enzyme. This is the first report in which the secondary structure of fructosyltransferase CLEA is explained using the FTIR spectral data. Wang et al. (2017), predicted the changes in the secondary structure of lipase CLEA; they observed a higher number of beta sheets after aggregation. Thus, we can conclude that cross-linking with glutaraldehyde allowed for the transformation of helical structures and beta sheet to beta turns due to protein aggregation.

The transfructosylation of sucrose catalyzed by fructosyltransferase CLEA resulted in the production of FOS. Figure 8 shows the FTIR spectra of the aqueous solution of each pure sugar component as well as the purified reaction products. The characteristic regions of carbohydrate fingerprint from the FTIR spectra for glucose, sucrose, commercial FOS, fructose, and purified reaction products of FOS had peaked at regions that varied from 1007 to 1193 cm$^{-1}$, 995–1047 cm$^{-1}$, 969–1039 cm$^{-1}$, 956–1171 cm$^{-1}$, and 968–1271 cm$^{-1}$. The crude reaction products had peaked at 969, 997.2, 1078, and 1095 cm$^{-1}$. It was observed that the spectral signatures of each sugar were distinguishable. Sugars are known to have exocyclic and endocyclic C-O bonds located at 995 cm$^{-1}$ and 1080 cm$^{-1}$ for sucrose and glucose/fructose, respectively (Adina et al., 2010). The broad band observed around 3700–3000 cm$^{-1}$ are fingerprint regions for H$_2$O and OH absorption frequencies. There are other fingerprint regions for other functional groups such as peaks that correspond to stretching vibrations of –C-H inside CH$_3$ or CH$_2$ groups. Characteristically, a unique peak that exists for the FOS was found in the 965 cm$^{-1}$ region. This peak was found in the FTIR spectra of the commercial FOS, crude, and purified reaction products, and it was noticeably absent in sucrose and glucose. This is a nondestructive and non-toxic confirmation of the transfructosylation of sucrose to FOS by the CLEA.

The physiological and biotechnological importance of FOS necessitated the investigations of the use of FOS
from *A. pullulans* NAC8 as a prebiotic source. There is an increasing trend in the addition of FOS in various food products because they possess prebiotic properties (Bali et al., 2015, Flores-Maltos et al., 2015). Their consumption increases fecal bolus and the frequency of depositions and reduces constipation, which is a problem existing in the modern world in humans and in infants (early months) (Sabater-Molina et al., 2009).

In this study, bacterial strains that were able to perform the process of fermentation of FOS (and possibly produced acid end products) grew as colonies surrounded by yellow zones against a purple background. The use of bromocresol purple as an indicator of utilization of FOS by probiotic strains served as a necessary yardstick needed for quick preliminary screening of these strains. As reported in this study, the newly synthesized FOS, as well as the commercially obtained FOS, had colonies that existed as a yellow zone against a purple background. This agrees with the report of Kaplan and Hutckins (2000), where several *Lactobacillus* sp. were found to utilize FOS concomitantly forming yellow colonies.

Investigations into the prebiotic effectiveness (i.e., how the growth of beneficial bacteria is enhanced) of *A. pullulans* FOS on *Lactobacillus* sp. revealed that 1% (w/v) FOS had a slight stimulatory effect on the growth of biobacteria compared with the controlled medium. In addition, the pH values of the media dropped with an increase in the mass of biobacteria. The growth curves of *Lactobacillus* sp. reached a plateau phase after 24 h cultivation with FOS, whereas it took 36 h to reach the plateau phase without FOS. Therefore, the growth rate of the bacteria in FOS-containing medium was faster than that in the medium without FOS. Moreover, the nal bacterial mass in the medium with FOS was found to be greater than that in the medium without FOS. Furthermore, the pH values of the media dropped along with the increases of beneficial bacterial populations. The findings of our study agree with those of Li et al. (2008) where the 1% (w/v) FOS had a stimulatory effect on the lactobacilli and bifidobacteria used. In addition, it was observed that as the growth rate increased, the pH decreased simultaneously.

During the fermentation of fructose oligomers by probiotic bacteria, authors have observed a gradual decrease in the pH which is indicative of the production of lactic acid (Rastall et al., 2000). The decrease in pH values after incubation with FOS suggests that the biobacteria were able to utilize FOS. These results indicate that FOS potently stimulated the growth of biobacteria, which supported the potential prebiotic effect of FOS.

In this study, the FOS produced by *A. pullulans* was able to enhance the growth of prebiotic bacteria that are native to the gastrointestinal microbiota; therefore, the use of these FOS as food additives is advised.

**Conclusion**

In this study, we synthesized and evaluated the cross-linked fructosyltransferase aggregates. The conditions for the CLEA preparation were statistically optimized, a step necessary for a scale-up process for industrial/biotechnological biocatalytic applications such as the mass production of the prebiotic FOSs. The structural characterization of the CLEAs revealed nonuniform morphological features while sulfur, oxygen, and phosphorus was established to be present in high amounts. Our report is the first to use a statistical approach for the optimization of fructosyltransferase CLEA production. Based on CLEA reusability, the fructosyltransferase CLEAs can be applied as industrial biocatalysts in the production of FOS. The produced FOS had prebiotic properties comparable to those obtained from a commercially obtained FOS, hence *A. pullulans* NAC8 FOSs could be a novel source for its production.

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**Disclosure**

All authors declare no conflict of interest.

**References**


