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Highlights:

- β-fructofuranosidase and β-fructosyltransferase were immobilized on chitosan spheres.
- FOS and invert sugar were produced continuously on enzymatic reactors.
- Packed bed reactors presented better yelds than fluidized bed reactors.
- Packed bed reactor produced FOS continuously for 40 days without loss of activity.
- FOS composition can be modulated by the flow rate.

Continuous production of fructooligosaccharides and invert sugar by chitosan immobilized enzymes: comparison between in fluidized and packed bed reactors

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Abstract

In this work, β-fructofuranosidase and β-fructosyltransferase were covalently immobilized on chitosan spheres, using glutaraldehyde as a coupling agent, in order to produce invert sugar and fructooligosaccharides (FOS). Maxinvert L was used to make β-fructofuranosidase biocatalyst yielding 7000 HU/g. A partial purified β-fructosyltransferase from Viscozyme L was used to prepare the other biocatalyst yielding 2100 TU/g. The production of invert sugar and FOS was evaluated using different continuous enzymatic reactors: two packed bed reactors (PBR) and two fluidized bed reactors (FBR). The invert sugar production achieved a yield of 98 % (grams of product per grams of initial sucrose) in the PBR and 94 % in the FBR, whereas FOS production achieved a yield of 59 % in the PBR and 54 % in the FBR. It was also observed in both cases that varying the flow rate it is possible to modulate the FOS composition in terms of nystose and kestose concentrations. The operational stability of FOS produced in the PBR was evaluated for 40 days showing no reductions in yields.

Key words: Invert Sugar; Fructooligosaccharides; Packed bed enzyme reactor; Fluidized bed enzyme reactor; Chitosan; Enzyme Immobilization.

1. Introduction

The commercial viability of industrial biotransformations is heavily dependent on the cost of the enzyme. Industrial applications of enzymes are often hampered by the lack of long-term operational stability, difficult recovery and reuse of the enzyme. These drawbacks can be overcome by immobilization of the enzyme [1, 2].

Notwithstanding all these advantages compared to the free enzymes, immobilized enzymes usually have their activities reduced, producing unfavorable effects on their overall catalytic performances. This alteration can result from mass transfer limitations, which may be reduced by applying appropriate reactor designs and immobilization techniques [3, 4]. Among immobilization techniques, the use of chitosan [(1→4)-2-amino-2-deoxy-β-D-glucan] as a support for covalent attachment is widely used for a multiplicity of purposes, mostly in industrial applications. This fact is due to several qualities of chitosan, such as the presence of reactive functional groups for direct reactions and for chemical modifications resulting in high affinity to proteins, hydrophilicity, good biocompatibility, non-toxicity, improved resistance to chemical degradation and ease of preparation in a variety of physical forms [3, 5]. Recently, several works reported the development of packed bed or fluidized bed reactors filled with chitosan biocatalysts for industrial purposes [6-9].

Packed bed reactors (PBR) consist of an immobile stack of particles within a column, through which a reactant solution is pumped. These reactors are preferred over other reactor types because of simpler technology, high bed volume, ease of operation, and scaling up [10, 11]. Fluidized bed reactors (FBR) consist of particles within a column, which are kept in movement (fluidization), by the liquid flow. The particles are retained by a hydrodynamic balance between gravity and drag forces promoted by the upflow substrate stream [12, 13].

Both types of reactors enable continuous production without the need of a prior separation of the enzyme resulting in lower process costs [7, 12]. In comparison to PBR, FBR are less susceptible to column clogging, formation of preferential flow paths and compression due to bed weigh [11, 14]. FBR also present higher axial dispersions than PBR. On the other hand, PBR present lower shear stress on immobilized enzymes, generally leading to long-term enzyme stability [11].

Invertase (or β-fructofuranosidase, E.C. 3.2.1.26) is a suitable enzyme for carriyng out studies concerning enzymatic processes due to its stability, no need for any cofactors and its commercial significance [15, 16]. This enzyme is used for sucrose (β-D-fructofuranosyl(2→1)β-D-glucopyranoside) hydrolysis, resulting in an equimolar mixture of glucose (α-D-glucose) and fructose (β-D-fructose) known as invert sugar. This product is widely used in food and beverage industries due to its higher sweetness and lower susceptibility to crystallization [17]. However, the application of immobilized invertase in enzyme reactors for sucrose hydrolysis, on the industrial scale, is still in the development phase [18].

β-fructosyltransferase (E.C.2.4.1.9) is another suitable enzyme for studying the reactor design in enzymatic processes. This enzyme is used for short chain fructooligosaccharides (FOS) synthesis from sucrose. FOS are prebiotic food ingredients [19], and short chain FOS present sweet taste and may be used as a sweetener by diabetics [20]. Although the enzymatic synthesis of FOS also requires sucrose as substrate, the concentration of sucrose required is higher than for sucrose hydrolysis. This solution is more viscous, and the use of a highly viscous liquid in PBR may lead to the fatal problem of column clogging [11], therefore this effect must be evaluated.

In this context, the aim of this work is to evaluate the best reactor design to be used for invert sugar and FOS production catalyzed by immobilized invertase and β-fructosyltransferase, respectively. Both enzymes have been recently immobilized in chitosan particles yielding stable biocatalysts [21, 22]. Packed-bed and Fluidized-bed reactors were compared for sucrose hydrolysis and FOS synthesis. Furthermore, it was evaluated the influence of operational conditions on the FOS composition.

2. Experimental

2.1. Materials

Invertase from *Saccharomyces cerevisiae* (Maxinvert L 10000) was kindly donated by DSM Food Specialties (The Netherlands). Commercial enzyme preparation from *Aspergillus aculeatus* with β-fructosyltransferase activity (Viscozyme L), produced by Novozymes, was kindly donated by LNF Latino Americana (Brazil). Chitosan from shrimp shells (>75% deacetylated) was purchased from Sigma–Aldrich (USA). Sucrose and glutaraldehyde 25 % were purchased from Vetec Química Fina Ltda (Brazil). Enzymatic glucose (D-glucose) determination kit was purchased from In Vitro Diagnóstica Ltda (Brazil). All other chemicals were analytical or HPLC grade obtained from readily available commercial sources.

2.2. Enzymatic activities

Maxinvert L (invertase) hydrolytic activity was evaluated using a substrate solution of 100 g/L of sucrose in 50 mM sodium acetate buffer (pH 4.5) at 50 ºC. Samples were taken at regular intervals and the reaction was stopped by the addition of 0.1 M sodium carbonate buffer, pH 10.0. Glucose released was measured with the enzymatic glucose

124 determination kit. One hydrolytic unit of enzymatic activity (HU) was defined as the
125 amount of enzyme that produces 1 μmol of glucose per minute at test conditions.
126 Viscozyme L (β-fructosyltransferase) transfructosylation activity was carried out
127 at 50 ºC using a solution of sucrose 600 g/L in 50 mM sodium acetate buffer pH 5.5.
128 Fifty chitosan particles (17.1 mg) were incubated in 2 mL of sucrose solution. Samples
129 were taken after 15 min and the concentration of saccharides (sucrose, glucose, fructose,
130 kestose and nystose) were measured on HPLC. On unit of transfructosylation activity
131 (TU) was defined as the amount of enzyme that produces 1 μmol of kestose or nystose
132 from sucrose per minute [22].
133 All the activities of immobilized enzymes were carried out under agitation. Protein
134 content of the enzyme solutions was determined by the Lowry assay.
135

136 **2.3. Chitosan spheres synthesis**

137 The chitosan particles were prepared adding dropwise a chitosan solution into an
138 alkaline coagulation solution, previously described by Klein *et al.* [23]. The prepared
139 particles had a spherical shape with a diameter of approximately 2 mm and a dry weight
140 of 0.343 mg ± 0.008 mg per sphere.
141 Activation of chitosan particles was carried out incubating the chitosan particles
142 with glutaraldehyde solution as it was previously described in Lorenzoni *et al*. [22].
143

144 **2.4. Enzyme Immobilization**

145 Invertase immobilization was carried out incubating the particles with a solution of
146 enzyme diluted in activity buffer (50 mM sodium acetate, pH 4.5) at room temperature
147 under gentle shaking for 3 h, in an orbital shaker at 120 rpm with 25 mm of orbital
148 diameter. The amount of enzyme applied to the support was 50 mg per gram of support,

149 in a ratio of enzymatic solution to solid of 146 µL per milligram of support in
150 Erlenmeyer flasks. The amount of support in an immobilization batch varied from 50 to
151 1400 chitosan spheres, *i.e.*, 17.1 mg to 480 mg, with the same results.

152 β-fructosyltransferase, was partially purified and immobilized as previously
153 described by Lorenzoni *et al.* [22]. The amount of enzyme applied to the support was
154 180 mg per gram of support.

155 After immobilization, both biocatalysts were washed with buffer NaCl (1.0 M) and
156 ethylene glycol (30%, volume fraction) in order to eliminate non-covalently bonded
157 enzymes. The immobilization yield (IY) and immobilization efficiency (IE) were
158 calculated by the equations 1 and 2, respectively, previously described in Valerio *et al.*
159 [21], and Sheldon and Van Pelt [1]:

160 $$IY = \frac{\text{immobilized activity}}{\text{starting activity}} \quad (1)$$

161 $$IE = \frac{\text{observed activity}}{\text{immobilized activity}} \quad (2)$$

162 Immobilized activity is the difference between the activity applied to the support
163 and the activity measured in the washing fractions. Starting activity is the activity of the
164 enzyme added to the support. Observed activity is the activity on the chitosan beads
165 after immobilization.

166

167 **2.5. Reactors setup**

168 The reactors consisted of a water-jacketed glass column, each filled with 930 units
169 of chitosan spheres (280 mg of dry weight). Both enzymatic preparations were
170 evaluated in packed bed and fluidized bed reactors. The reactors were flow rate
171 controlled with adjustable peristaltic pumps.

172 The packed bed column (height, 85 mm; inner diameter, 10 mm; volume, 6.67 mL)
173 has an entrance at the bottom and an exit at the top, which were fitted with a sintered

glass disc to retain the particles within the column. The fluidized bed column (height, 230 mm; inner diameter, 10 mm; volume, 18.0 mL) was equipped with one additional exit at the top and one additional entrance at the bottom for external liquid recirculation, also fitted with sintered glass disc. This design enables fluidization of the biocatalysts independently of the residence time. The bed height was kept constant at 230 mm. Schematics of reactors configurations are illustrated in Figure 1.

2.5.1. Invert sugar production

Production of invert sugar was carried using a solution of sucrose 150 g/L diluted in 50 mM sodium acetate buffer pH 4.5. The solution was pumped at flow rates of (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.7) mL/min in both fluidized and packed bed reactors. Four bed volumes of solution were passed through the column before taking the sample to achieve stationary state. These experiments were carried out in duplicate, changing the column bed at each experiment.

2.5.2. Fructooligosaccharides production

Production of fructooligosaccharides was carried using a solution of sucrose 600 g/L diluted in 20 mM sodium acetate buffer pH 5.5. The solution was pumped at flow rates of (0.052, 0.082, 0.11, 0.17, 0.23, 0.29, 0.44) mL/min in both fluidized and packed bed reactors. Four bed volumes of solution were passed through the column before taking the sample to achieve stationary state and the experiments were carried out in duplicate, changing the column bed at each experiment.

2.5.3. HPLC analysis

All the samples collected in the reactors were analyzed in HPLC system (Shimadzu, Tokyo, Japan) equipped with refractor index and Aminex HPX-87C (300 mm x 7.8 mm) column. Ultra-pure water was used as eluting solvent at a flow rate of 0.6 mL/min, at 85 ºC.

The concentration of saccharides (sucrose, glucose and fructose) was determined in invert sugar samples by interpolation using external standards. In the fructooligosaccharides samples, kestose (β-D-fructofuranosyl(2→1)β-D-fructofuranosyl(2→1)α-D-glucopyranoside) and nystose (β-D-fructofuranosyl(2→1)β-D-fructofuranosyl(2→1)β-D-fructofuranosyl(2→1) α-D-glucopyranoside) concentrations were also determined.

3. Results and Discussion

3.1. Enzymatic activity

Maxinvert L contains a specific hydrolytic activity of 3000 HU/mg at 50 ºC and pH 4.5. The enzymatic solution obtained from partial purification of Viscozyme L, contained specific hydrolytic activity of 25.3 HU/mg, at 50 ºC and pH 5.5. It is important to note that the specific activity of the enzyme solution used in this work, after partially purification is much lower compared to Maxinvert L. This difference is due to the fact that Maxinvert L is a commercial enzyme preparation specific for sucrose hydrolysis, whereas Viscozyme L is marketed as endo-β-glucanase that hydrolyzes (1→3)- or (1→4)- linkages in β-D-glucans, and the secondary activity recovered in this preparation is more specific for β-fructosyltransferase activity. Is important to note, despite its high hydrolytic activity, Maxinvert L contains little β-fructosyltransferase activity and therefore is not suitable for FOS synthesis [24, 25]. In the supplementary material, it is presented the HPLC chromatograms of the activity

each enzyme (S.1). For these reasons, Maxinvert L was used for sucrose hydrolysis and its activity measured in HU units, while Viscozyme L was used for FOS synthesis and its activity measured in TU units.

3.2. Enzyme Immobilization

Maxinvert L immobilization resulted in a biocatalyst with 2.4 HU per sphere (7000 HU per gram of dry support), immobilization yield of 42 % and an immobilization efficiency of 12 %. β-fructosyltransferase immobilization resulted in a biocatalyst with 0.73 TU per sphere (2100 TU per gram of dry support), immobilization yield of 82 % and an immobilization efficiency of 42 %. A higher immobilization yield was observed for β-fructosyltransferase than for Maxinvert L, despite the higher protein load applied, but several other factors may cause this result. Different immobilization pH may change immobilization yields when using glutaraldehyde as a coupling agent [26, 27]. In this case, the optimal pH of each enzyme was used for immobilization to avoid protein inactivation during this step. The difference between the enzymes structures can also interfere in enzyme immobilization; Maxinvert L is a highly glycosylated octameric protein with 428 kDa [28], while β-fructosyltransferase from *A. aculeatus* is a dimeric glycoprotein with 135 kDa [29]. However, both enzymes are not completely purified and is not possible to assert something without knowing all other proteins in the mixture.

A β-fructosyltransferase biocatalyst with a better activity than in a previous work was obtained [22]. This fact can be attributed to the smaller size of spheres used in this work, since smaller particles have a greater superficial area and therefore can carry a higher protein load [23]. A remarkable achievement since that biocatalyst was among the highest active found in literature so far [22].

3.3. Invert sugar and Fructooligosaccharides production

Results of yields of invert sugar production (grams of invert sugar per grams of initial sucrose) and FOS production (grams of FOS per grams of initial sucrose) for different flow rates are shown in Figure 2 and 3, respectively.

Packed bed reactors showed higher yields than fluidized bed in both reactions. For invert sugar production, the highest yields were observed at the lowest flow rates due to higher residence times inside the reactor.

For FOS production in PBR, the best yield was 59 %, at 0.08 mL/min, and at 0.05 mL/min the yield decreased to 57 %. In addition, a higher nystose concentration 240 g/L ± 2 g/L and a lower kestose concentration 101 g/L ± 2 g/L were detected in the samples collected at 0.05 mL/min. At 0.08 mL/min, the nystose and kestose concentrations were 218 g/L ± 2 g/L and 133 g/L ± 1 g/L respectively. The maximum concentration of kestose was 198 g/L ± 1 g/L, obtained at 0.29 mL/min. Kestose concentration decreased for higher residence times because kestose serve as a donor for fructosyl moieties to form nystose [30]. Kestose and nystose molar concentrations for the flow rates evaluated are shown in Table 1. In the supplementary material (S.2), it can be seen the HPLC chromatograms that show the variation of nystose and kestose concentrations with the flow rate.

It is possible to observe in Table 1 that fructosyl units transferred from sucrose increased continuously as the residence time increased, mainly because at the lower flow rates, nystose concentration was higher than kestose. However, total FOS concentration in molar units reach a maximum, and remained almost constant at lower flow rates. This fact can be observed when sucrose concentration is close to its chemical equilibrium and only nystose formation from kestose can be observed. These results are

interesting because they indicate that by varying the flow rate it is possible to change the FOS composition in terms of nystose and kestose concentration. This is important because those carbohydrates have different prebiotic and technological properties. Suzuki et al. [31] suggested that kestose has higher and more selective stimulating activity on bifidobacteria when compared to nystose. On the other hand, Stewart et al. [32] pointed out that the long-chain FOS were more readily fermented, which indicates that fermentation would occur over a longer portion of the colon. Additionally, kestose present higher sweetening power than nystose, which could be important in diet products.

Because in packed bed reactor the amount of fructosyl units transferred is higher than in the fluidized bed reactor, it is possible to conclude that this reactor configuration is more efficient for FOS production, and presents potential for scaling up.

Reactors filled with immobilized enzyme systems can be considered as being two-phase systems, thus they present some mass transfer limitations. This limitations may lead to unfavorable effects on their overall catalytic performances [3]. Furthermore, transport phenomena of substrates and products must be addressed by process engineering, optimizing geometry in order to produce a biocatalyst possessing both high activities and stabilities [4]. Because fluidized bed reactors present better axial dispersion rates, it is reasonable to predict that they will result in better yields in comparison to packed bed reactors. However, in this work, the packed bed reactor was kinetically favorable resulting in better yields for both reactions evaluated. A possible reason is the fact that in both reactions glucose is produced, which, in turn, causes product inhibition [30, 33]. In the fluidized bed reactor, part of the product solution is recycled to the entrance of the reactor to allow the bed fluidization. This system leads to higher glucose concentrations in the bottom. Therefore, glucose concentration in the

298 bottom of the fluidized bed is higher than in packed bed, inhibiting enzymatic activity.
299 This fact would not be noted on a non-recirculating fluidized bed, but in such case, a
300 higher flow rate would be needed for particles suspension leading to lower residence
301 times and lower yields.

302 Although presenting higher yields, packed bed reactors can have some drawbacks
303 in comparison to fluidized bed, such as column clogging, creation of preferential flow
304 paths and particle compression [14]. These drawbacks occur after some operation time,
305 thus to evaluate them we measured the operational stability of the packed bed reactor
306 for FOS production. The FOS production medium is also much more viscous than
307 invert sugar production medium as well as the susceptibility to clogging. Operational
308 stability was evaluated for 40 days operating at the flow rate of 0.15 mL/min and no
309 yields reduction were observed, as shown in Figure 4. In the present and in past works
310 [7, 8, 22, 23], we did not observe any kind of breaking or changing in the particles
311 during their uses, which can be indicated by the high operational stability. Nevertheless,
312 the mechanical strength of the particles should be studied, because it is not possible to
313 discard some compressing problems in a scale up process. Although the total FOS
314 concentration remained constant along the operational test, the FOS profile changed
315 over time. In the supplementary material (S.3), it was presented the HPLC
316 chromatograms for the first and last day of the tested operational stability, where it is
317 possible to note that kestose concentration decreased, whereas kestose concentration
318 increased, which could be indicating a loss of activity, because the residence time would
319 not be a sufficient cause to convert kestose in nystose, nor some conformational change
320 in the enzyme structure, which could have affected its activity.
321

4. Conclusions

Packed-bed and fluidized-bed reactors were compared for sucrose hydrolysis and FOS synthesis. It was observed that it is possible to modulate the FOS composition by varying the flow rates in both cases. However, the evaluation of reactor design for enzymatic production of invert sugar and FOS suggested that packed bed reactors are the best choice for the synthesis of those products. Packed bed reactors are not only kinetically favorable but also present a simpler technology and requires smaller volumes. A high operational stability was observed, no column clogging or preferential flows were detected on the packed bed reactor after 40 days of operation for FOS production. The packed bed reactors made in this work presents great potential for industrial production of either fructooligosaccharides or invert sugar from sucrose.

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392
393

Figure Legends:

Figure 1 Schematics of reactors configurations.

Figure 2 Yields of invert sugar production on packed bed (■) and fluidized bed (□) at pH 4.5, 50 ºC and 150 g/L of sucrose.

Figure 3 Yields of FOS production on packed bed (■) and fluidized bed (□) at pH 5.5, 50 ºC and 600 g/L of sucrose.

Figure 4 Yields of FOS production (■), nystose (□), and kestose (Δ) concentrations in the packed bed reactor operated continuously for 40 days at 50 ºC, pH 5.5 and 600 g/L of sucrose.

402

403 **Table 1: Results of FOS synthesis in continuous reactors**

	Flow Rate (mL/min)	Nystose concentration (mM)	Kestose Concentration (mM)	Total FOS (mM)	Fructosyl Units Transferred (mM)
Fixed bed	0.05	359 ± 4	201 ± 2	560 ± 6	920 ± 10
	0.08	327 ± 3	264 ± 2	592 ± 5	919 ± 7
	0.11	291 ± 1	310 ± 1	601 ± 3	892 ± 4
	0.17	243 ± 6	355 ± 3	598 ± 9	841 ± 15
	0.23	199 ± 1	382 ± 1	581 ± 1	780 ± 2
	0.29	174 ± 10	393 ± 2	568 ± 12	742 ± 21
	0.44	118 ± 1	374 ± 1	492 ± 2	610 ± 3
Fluidized bed	0.05	304 ± 1	242 ± 0	547 ± 1	851 ± 2
	0.08	278 ± 0	272 ± 0	550 ± 0	828 ± 0
	0.11	248 ± 1	296 ± 0	544 ± 1	793 ± 2
	0.17	209 ± 0	321 ± 1	531 ± 1	740 ± 1
	0.23	175 ± 1	331 ± 1	505 ± 1	680 ± 2
	0.29	151 ± 2	324 ± 1	475 ± 3	626 ± 5
	0.44	121 ± 1	316 ± 1	438 ± 2	559 ± 2

404

*Graphical Abstract (for review)

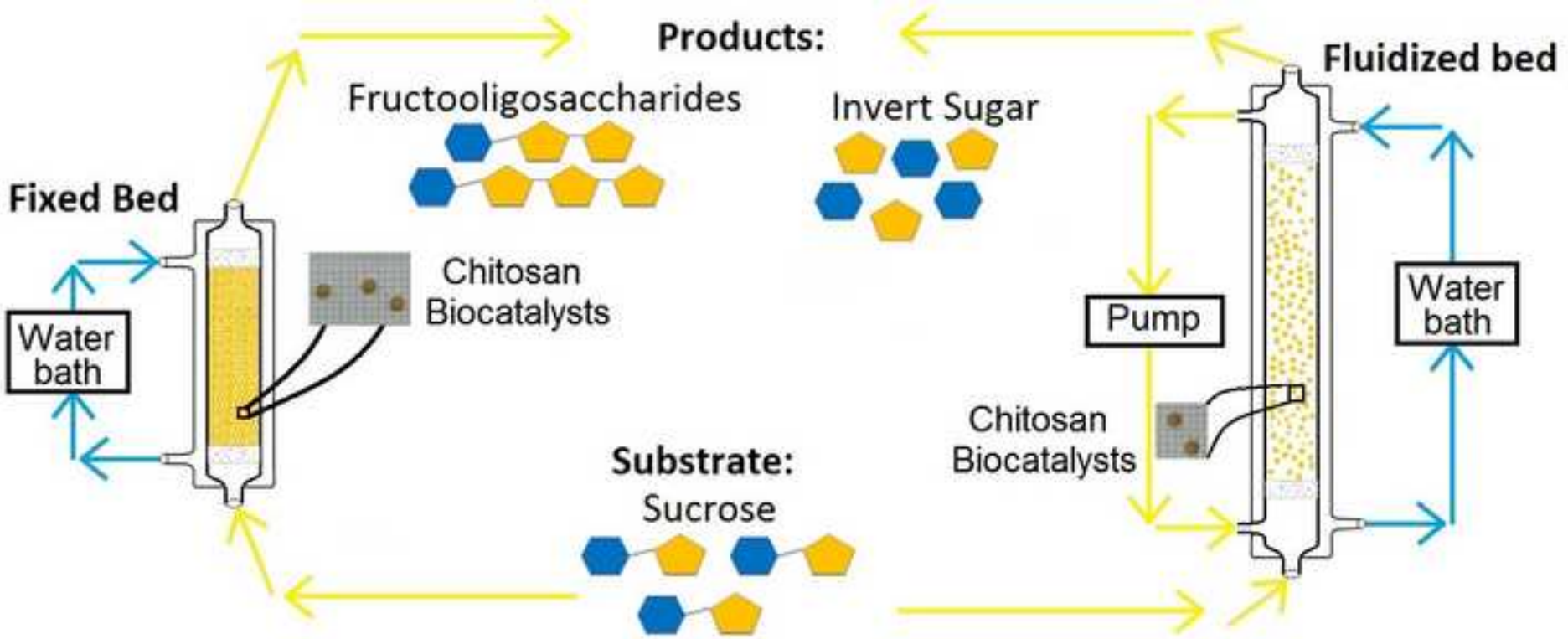


Figure 1

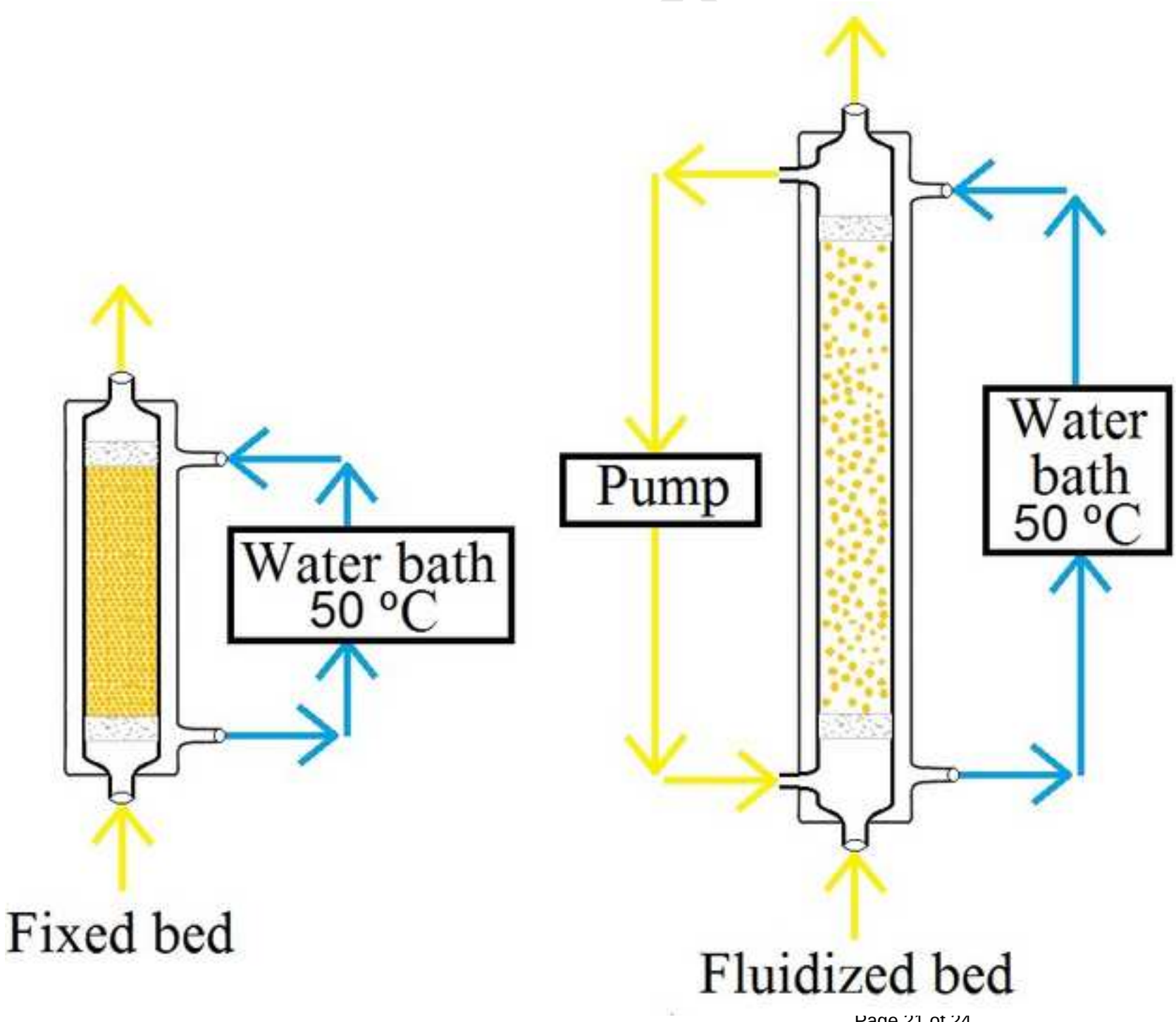


Figure 2

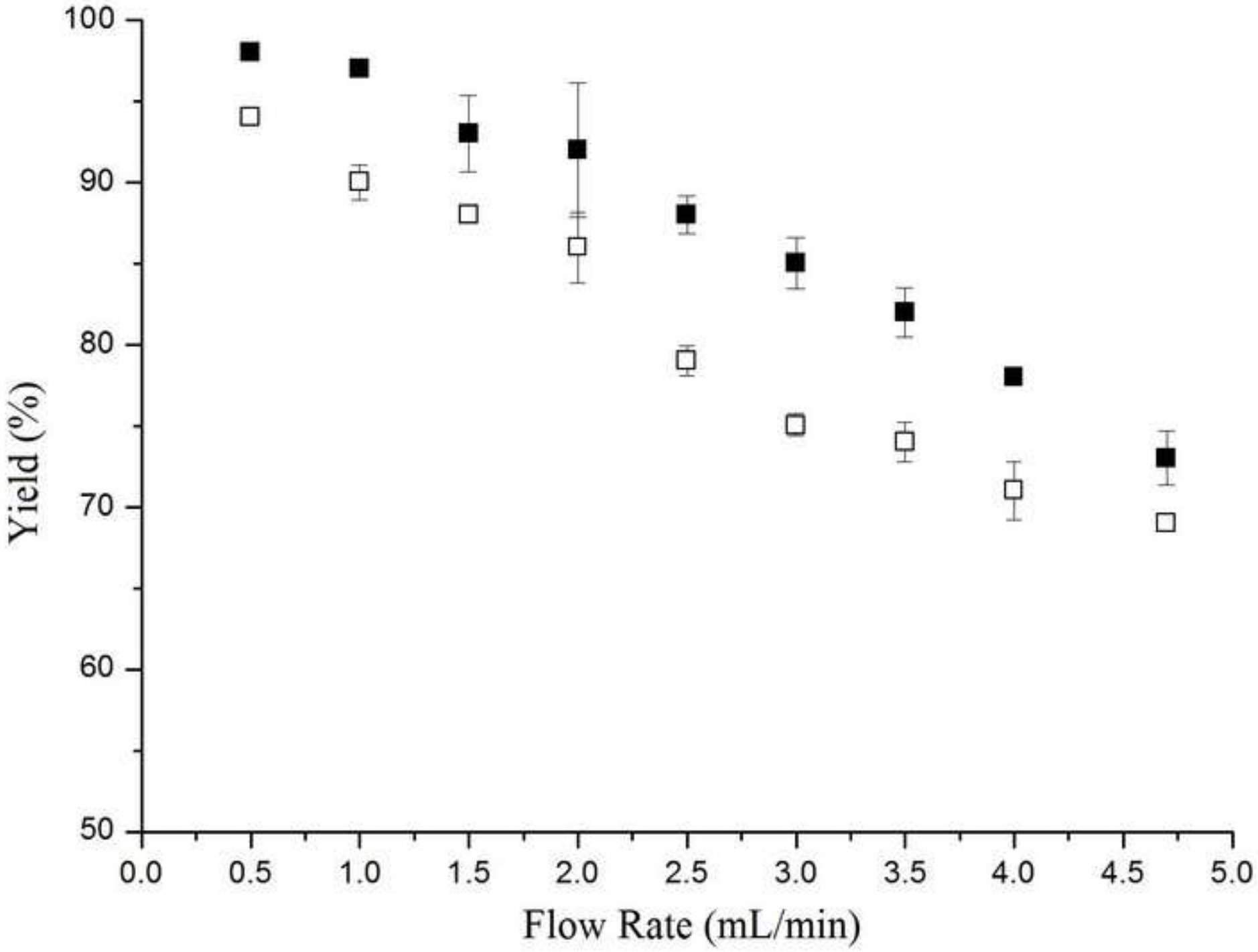

100
90
80
70
60
50
Yield (%)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Flow Rate (mL/min)

Figure 3

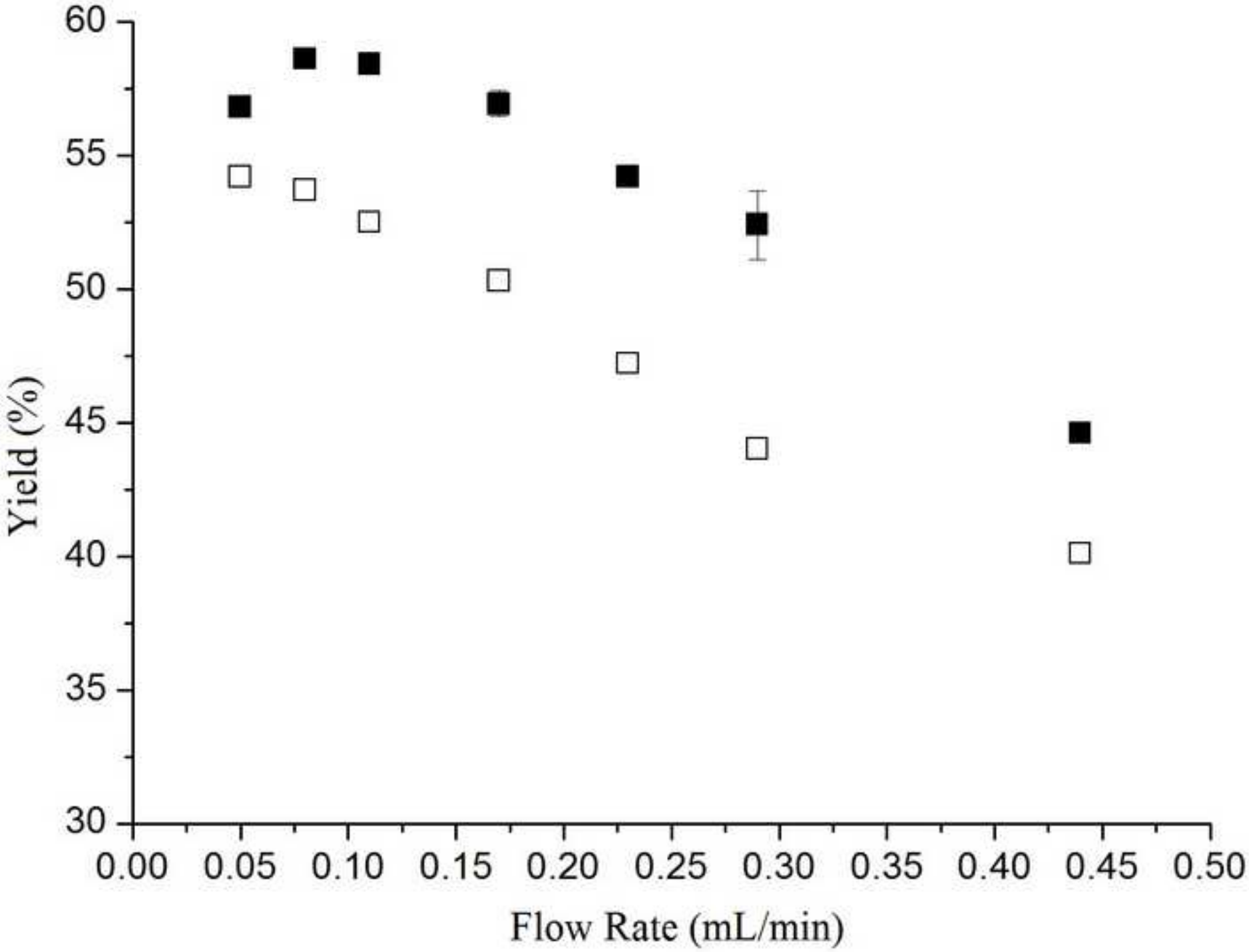
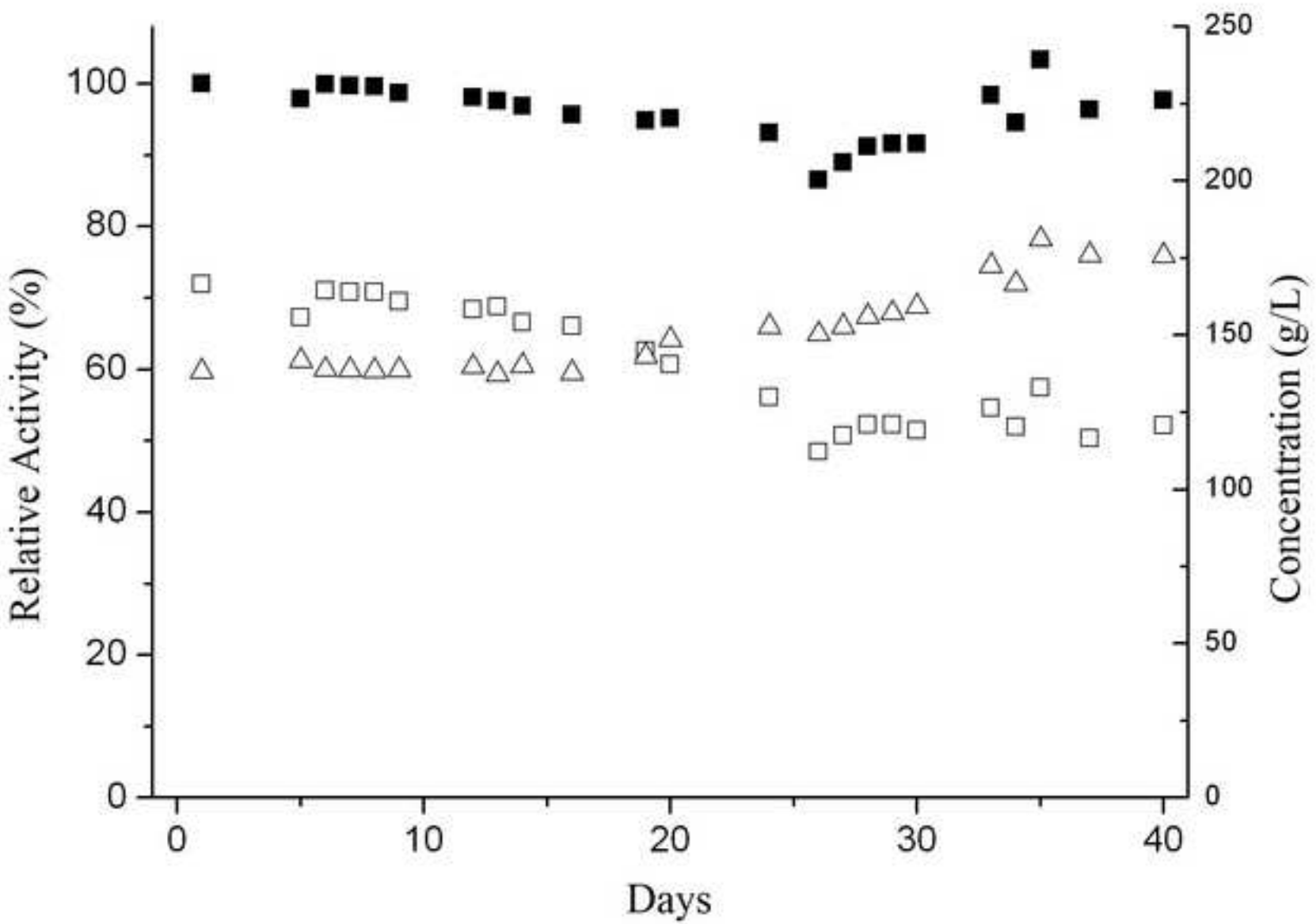

60
55
50
45
40
35
30
Yield (%)
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
0.50
Flow Rate (mL/min)

Figure 4


Relative Activity (%)
Concentration (g/L)
Days
0
20
40
60
80
100
0
50
100
150
200
250
0
10
20
30
40