Methodology for Template Induced Crystallization

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Abstract

In-Situ Product Removal (ISPR) technologies are being developed at the Dutch National Organization for Applied Sciences (TNO) & Delft University of Technology (TUD) to increase the yield and productivity in fermentation processes. ISPR can in principle be achieved using Template Induced Crystallization (TIC). With TIC, templates are added to the solution as a specific surface upon which the solute preferably crystallizes. The governing principles of TIC are poorly understood and template selection criteria are lacking. Therefore a method to screen for these templates is developed.
The methodology comprises metastable zone width determination, template classification and different types of induction time measurements. The screening method is implemented in robotized equipment for parallel experimentation.
The results show that the induction time of cinnamic acid crystallization is decreased by the addition of TiO_2 templates. Templates enabling crystallization can be distinguished from ineffective templates with relatively simple experiments. The methodology therefore is applicable to evaluate templates upon their potential to promote crystallization. Future work will be focused on the integration of the TIC-principle and fermentation processes.

Keywords: Sustainable processing, *In-Situ* Product Removal (ISPR), Template Induced Crystallization (TIC), screenings methodology, high throughput equipment

1. Introduction

Nowadays a large amount of research is conducted in the field of sustainable processes [1]. The need for such processes originates from the growing human population and the accompanied required growth in availability of (edible) materials and energy. A significant part of the developments is focussed on bio-based processes, which make use of renewable feedstocks, to decrease the use of non-renewable fossil resources.
The higher specificity and efficiency of these processes, the availability of new biochemical routes and the reduction of environmental unfriendly wastes are clearly

advantageous. However, many products formed in fermentation processes have toxic or inhibitory effects on the productivity and growth of the biomass. *In-Situ* Product Removal (ISPR) has the potential to instantaneously separate the formed product from the fermentation broth to overcome these constraints. Due to ISPR, low concentrations of fermentation products are maintained during the bioprocess, thus minimizing product inhibition and optimizing productivity and yield of the bio-based process [2].

The objectives of TNO, as part of B-BASIC (a Dutch research consortium of several universities, institutes and industries working on the development of bio-based processes [3]), are the development of a bulk-chemical producing micro-organism and a set of separation tools for ISPR. *In-situ* crystallization, using the principle of Template Induced Crystallization (TIC), is one of the separation techniques in the toolbox and highlights the potential of ISPR. With this technique fermentation products can be selectively separated from the broth by crystallization on templates. According to [4], templates are defined as particles or surfaces that induce crystallization and simplify the separation of the crystals. Selection criteria of templates, that are specific for a certain compound, are lacking. Therefore a method to screen for these templates is developed upon newly defined selection criteria.

The methodology is represented as a decision tree. It comprises metastable zone width determination (to define the operating area of the TIC process), template classification experiments and different types of induction time measurements. Experiments with templates are compared to blank experiments (where no templates are added to the crystallizing solution), to determine the ability of a certain template to promote crystallization. This paper focuses on pH-track experiments, which is one of the different types of induction time measurements of the screening methodology.

2. Theory

Crystallization consists of the subsequent processes of nucleation and growth. The nucleation work largely defines the metastable zone width [5]. At supersaturations higher than the metastable zone width, both nucleation and growth take place, whereas with lower supersaturations, between the solubility and the metastable zone width, only growth of earlier formed nuclei occurs, because of a too high barrier for homogeneous nucleation.

Heterogeneous nucleation, the type of nucleation usually occurring in solution crystallization, refers to the phenomenon where foreign bodies with heterogeneous surfaces can induce nucleation at supersaturations lower than those required for homogeneous nucleation. This is due to the fact that the introduced surface lowers the nucleation work, resulting in nucleation and subsequent growth [6].

Templates promising for TIC are intended to be such heterogeneous surfaces. The use of such templates thus lowers the nucleation work and as a result the metastable zone width is decreased and nucleation is induced at lower supersaturations [7].

Upon creation of a constant supersaturation a certain instance of time may evolve before a detectable amount of crystals are formed. This period is referred to as the induction time [8]. The addition of templates, lowering the nucleation work, results in decreased induction times, because crystallization is induced at lower

supersaturations. The induction time therefore is considered to be a proper measure for the ability of templates to promote crystallization.

The supersaturation, which is the driving force for crystallization processes, can be established in several ways. Decreasing the bulk temperature lowers in most cases the solubility of a certain compound. Another method to lower the solubility of a compound is known as anti-solvent crystallization. pH-shift crystallization, a special form of reactive crystallization, is suited for precipitation of compounds which can be (de)protonated, or in other words only for acidic/alkaline substances. The charged species of these compounds are in most cases highly soluble. By shifting the pH towards the less soluble form of the compound, a driving force for crystallization might be created and crystallization could take place.
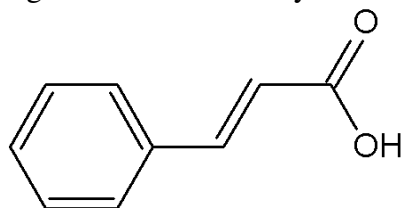

Figure 1: Structural formula of cinnamic acid

Cinnamic acid is chosen as the crystalline model compound to illustrate the screening methodology for templates. Cinnamic acid, of which the structural formula is depicted in figure 1, is an interesting aromatic intermediate for the chemical industry. Furthermore, it is produced by *Pseudomonas putida* S12 [9], so that in future work, TIC can be tested in actual fermentation processes. Cinnamic acid is an organic acid with an equilibrium constant (pK_a) of 4.44. The speciation of cinnamic acid is calculated using equation (1) and the equilibrium constant.

$$CAH_{(aq)} \Leftrightarrow CA^-_{(aq)} + H^+_{(aq)} \tag{1}$$

Concentrations of associated cinnamic acid (CAH) and dissociated cinnamic acid (CA$^-$) are plotted against the pH (figure 2), together with the solubility of CAH. For the calculation of the speciation, a total cinnamic acid concentration (equal to CAH + CA$^-$) of 13.23 mmol·l^{-1} is assumed, which is four times the solubility of CAH (3.3 mmol·l^{-1} at 25 ℃ [10], [11]).
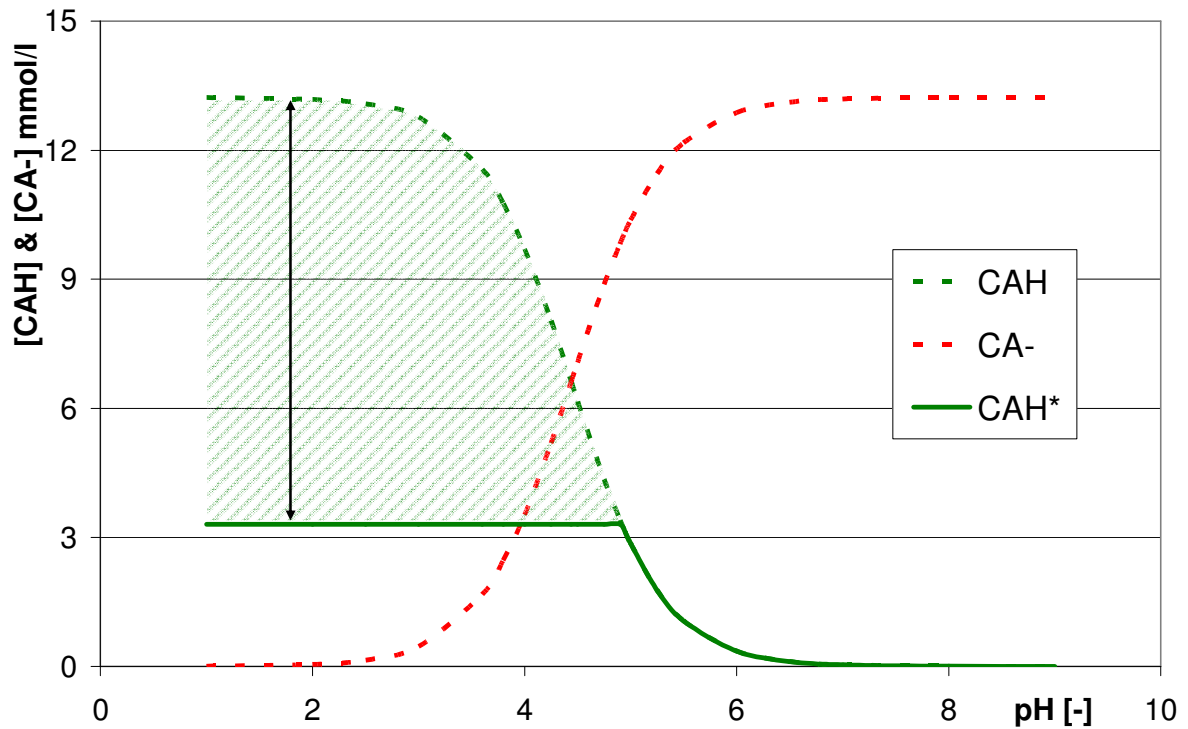
Figure 2: Speciation and solubility of cinnamic acid. [CAH] is the associated cinnamic acid concentration, [CA⁻] the dissociated cinnamic acid concentration and [CAH]* the solubility concentration of associated cinnamic acid.

Figure 2 shows that cinnamic acid is almost entirely dissociated in neutral environments, while the concentration of associated cinnamic acid [CAH] dominates in acidic environments, below the pK_a value. Below a certain pH, defined as the intersection between the [CAH] and the solubility of CAH, [CAH] will exceed [CAH]*. Shifting the pH towards the associated speciation of cinnamic acid thus leads to an increased driving force for crystallization. The marked area in figure 2 displays the pH-range (within the context of the calculation of the speciation) for which cinnamic acid is supersaturated. According to [5], the supersaturation ratio *S* is defined as the actual activity of cinnamic acid (a_{CAH}) divided by the equilibrium activity of the compound (a^*_{CAH}):

$$S = \frac{a_{CAH}}{a^*_{CAH}} \qquad (2)$$

With this definition for the driving force for pH-shift crystallization, the speciation of cinnamic acid and the pH of the solution are taken into consideration via the equilibrium reaction (equation 1). The supersaturation thus can be established via the CAH concentration and the pH.

3. pH-track methodology

Upon creation of a supersaturation (S>1), nucleation and growth of associated cinnamic acid are induced. The crystallization process consumes CAH, therefore the [CAH] decreases. Due to the equilibrium reaction (see equation 1) dissociated cinnamic acid combines with a proton to form a CAH molecule. Crystallization of

CAH therefore leads to a decreased $[H^+]$ and with that an increased pH. This increasing pH can be monitored as illustrated in figure 3.
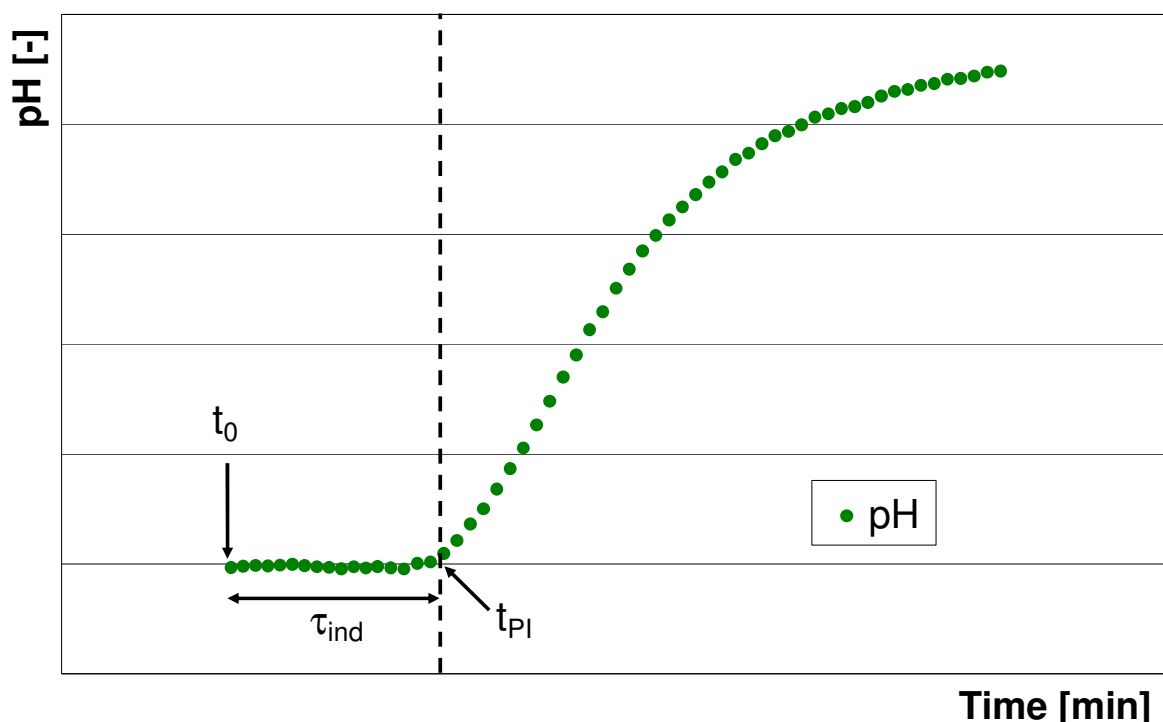

Figure 3: Principle of pH-track. t_0: Start of experiment, t_{PI}: Inflection point, $\tau_{ind} = t_{PI} - t_0$

The start of the pH-track experiment (t_0) is defined as the moment upon which supersaturation is imposed. The point of inflection (t_{PI}) is mathematically determined using a macro (Excel). This program creates a polynomial fit (>0,99) and calculates the inflection point from the third derivative of the fit. The point of inflection is a measure for the start of crystallization of cinnamic acid. The induction time (τ_{ind}) for pH-track experiments therefore is defined as the difference in time between the start of crystallization and the mathematically determined point of inflection.

To be able to qualify a certain template as promoting (CAH) crystallization, the following rules of evaluation are applied.

- Measure & calculate (multiple times) induction time of blank experiment
- Calculate probability distribution of blank induction time
- Measure & calculate (multiple times) induction time of template experiments
- Calculate probability distribution of induction time of templates
- Perform (statistical) test: 95 % probability that τ_{ind} (template) < τ_{ind} (blank)
  - If yes: Template promotes (CAH) crystallization
  - If no: Template has no/negative effect on (CAH) crystallization

To ensure that the monitored increasing pH is only due to (CAH) crystallization (and not for example due to ion exchange of the template), the effect of a certain template on the pH is examined as well.

4. Experimental description

The screening methodology is implemented in robotized equipment (HEL Ltd, figure 4) to ensure standardized procedures for cloudpoint and clearpoint determination, template classification and pH-track experiments. The temperature and stirrer speed were set to respectively 30 ºC and 300 rpm (equal to fermentation conditions).
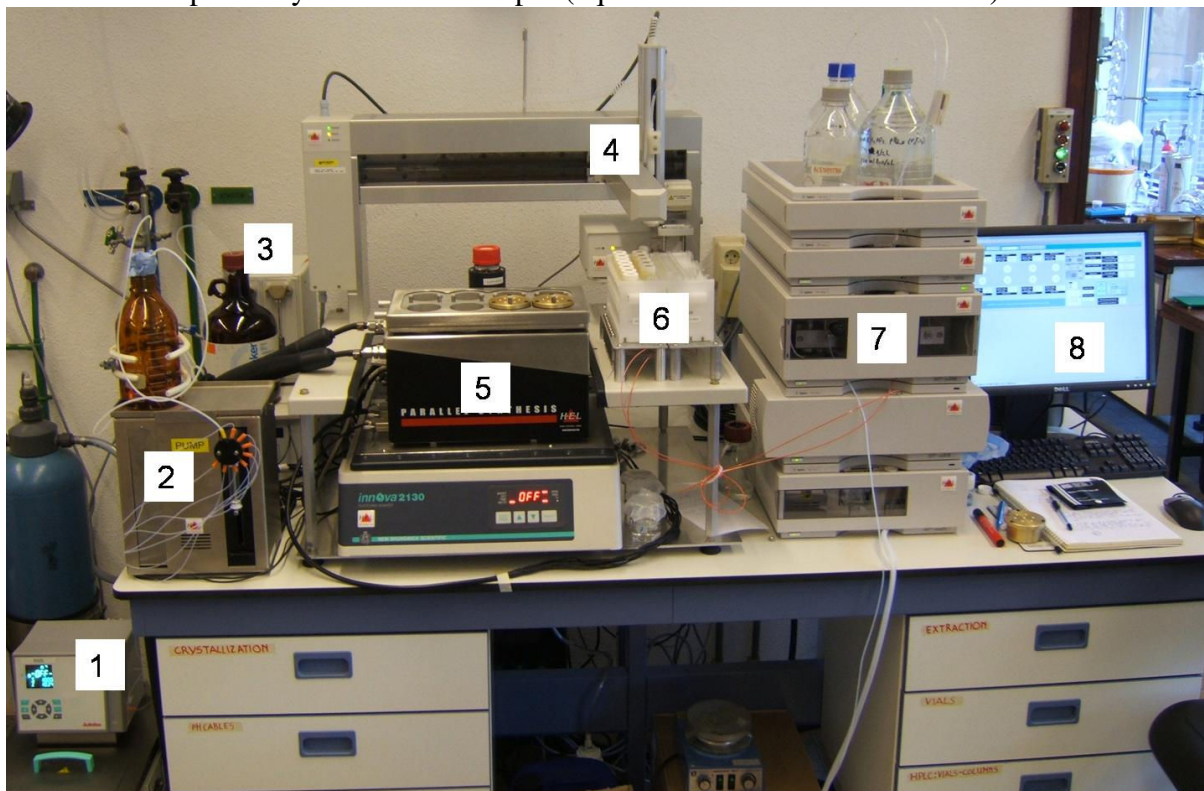

Figure 4: Robotized Equipment – Screenings tool. 1) Temperature control unit 2) acid/base control 3) pump 4) robot 5) polyblock (eight 100 ml reactors with pH/temperature control & *in-situ* sampling) 6) solvent/sample vials 7) HPLC unit 8) computer

In all experiments, except for the determination of the cloud- and clearpoint, the total cinnamic acid concentration ([TCA]) equals 6.75 mmol·l^{-1}. This value is used, because it compares to the [TCA] obtained in fermentation processes executed at TNO [9].

Titanium dioxide (TiO_2) is used as a template to demonstrate the applicability of the screenings methodology.

4.1. Materials

Cinnamic acid (99% natural, FCC), sodium hydroxide (98% purified), hydrochloric acid (32% puriss) and dispersed (10 wt %) nano-sized (50 nm) titanium dioxide are purchased from Sigma-Aldrich. All solutions were prepared with deionised water and filtered (0,45 μm) before use.

4.2. Cloudpoint & clearpoint determination

The solubility (clearpoint) and metastability (cloudpoint) are experimentally determined with pH-shift crystallization techniques. The procedure for these experiments is based on turbidity and on-line pH measurements. In one experiment, both cloud- and clearpoint are measured. Solutions of cinnamic acid (initial pH = 10) are slowly titrated with HCl until the cloudpoint is observed. The created suspension is the starting point for the titration with NaOH to obtain the clearpoint.

4.3. Template classification

The effect of a certain template on the pH is easily measured. A specific amount of template (equal to the amount that is added in pH-track experiments) is added to a cinnamic acid solution (initial pH = 10, 30ºC). The constantly monitored pH of the obtained suspension is tuned to approximately 4. The total cinnamic acid concentration is smaller than the solubility concentration of cinnamic acid to ensure that no crystallization of CAH occurs. As a result, an increasing pH is due to the effect of the template on the pH.

4.4. pH-track experiment

Induction times of suspensions with templates were determined using the pH-track principle. For this purpose, the pH of a cinnamic acid-template suspension (initial pH = 10) is instantaneously decreased (with a calculated fixed amount of HCl), in order to reach a supersaturation within the metastable zone of the reference. The pH of the (crystallizing) solution is monitored.

5. Results

5.1. Cloudpoint & clearpoint

Figure 5 depicts the experimentally determined cloud and clearpoint curves for pH-shift crystallization of cinnamic acid. The total cinnamic acid concentration (mmol·l^{-1}) is plotted against the pH (-).
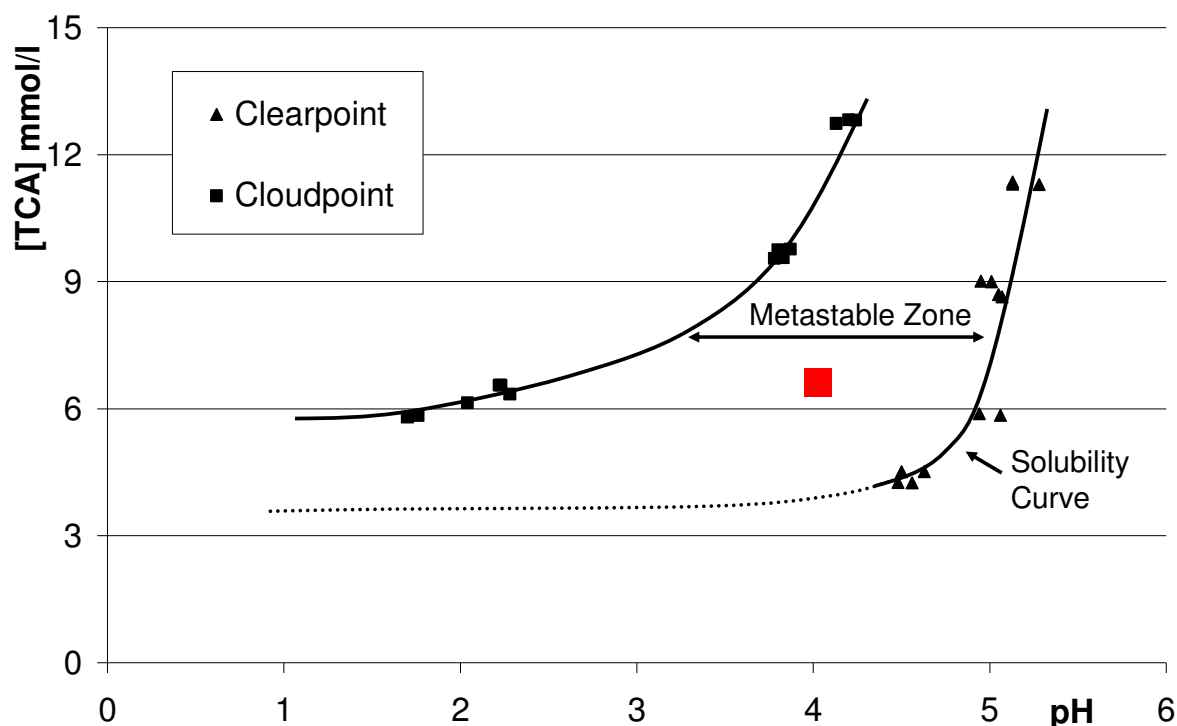
Figure 5: Cloudpoints (squares) & clearpoints (triangles) for pH-shift crystallization of cinnamic acid. Red area indicates [TCA] & pH at which template classification & pH-track experiments are conducted.

The clearpoint curve (triangles) represents the solubility of cinnamic acid as a function of pH. The area between the cloudpoint curve (squares) and the clearpoint curve corresponds (for the applied conditions) to the metastable zone.

The conditions ([TCA] & pH) for TIC processes should be such that the crystallizing solution is supersaturated with respect to cinnamic acid. Supersaturations applied should be low enough to be able to distinguish between induction times of blank experiments and templated experiments. The metastable zone therefore determines the operating area for TIC.

The red area indicates the conditions at which template classification and pH-track experiments were conducted. The combination of this [TCA] and pH results in a supersaturation in between the clearpoint and cloudpoint curves. TIC experiments thus are conducted within the metastable zone.

Based on the conditions indicated in figure 5, a theoretical maximum of crystalline mass can be calculated using equation 1&2, the equilibrium constant and the solubility of cinnamic acid. From this calculated crystal mass (~ 0,1 gram solid CAH/liter), the amount of templates that should be added in TIC experiments can be deduced. Schubert and Mersmann [7] state that enough free surface area should be provided for heterogeneous nucleation on foreign particles. To ensure this, an excess of 0,1 gram/l TiO_2 templates are added to TIC-experiments.

5.2. pH-track

Figure 6 (pH versus time) displays typical results of a blank (green) and a TiO_2 template (red) pH-track experiment. Between t_0 (the starting point) and t_{PI} (the

inflection point), the pH remains constant. After t_{PI}, both graphs clearly show an increasing pH, indicating crystallization of cinnamic acid. Both graphs have the same starting point, but different inflection points ($t_{PI, template} < t_{PI, blank}$). This is an indication that the induction time of cinnamic acid crystallization is decreased by the addition of TiO_2 templates.
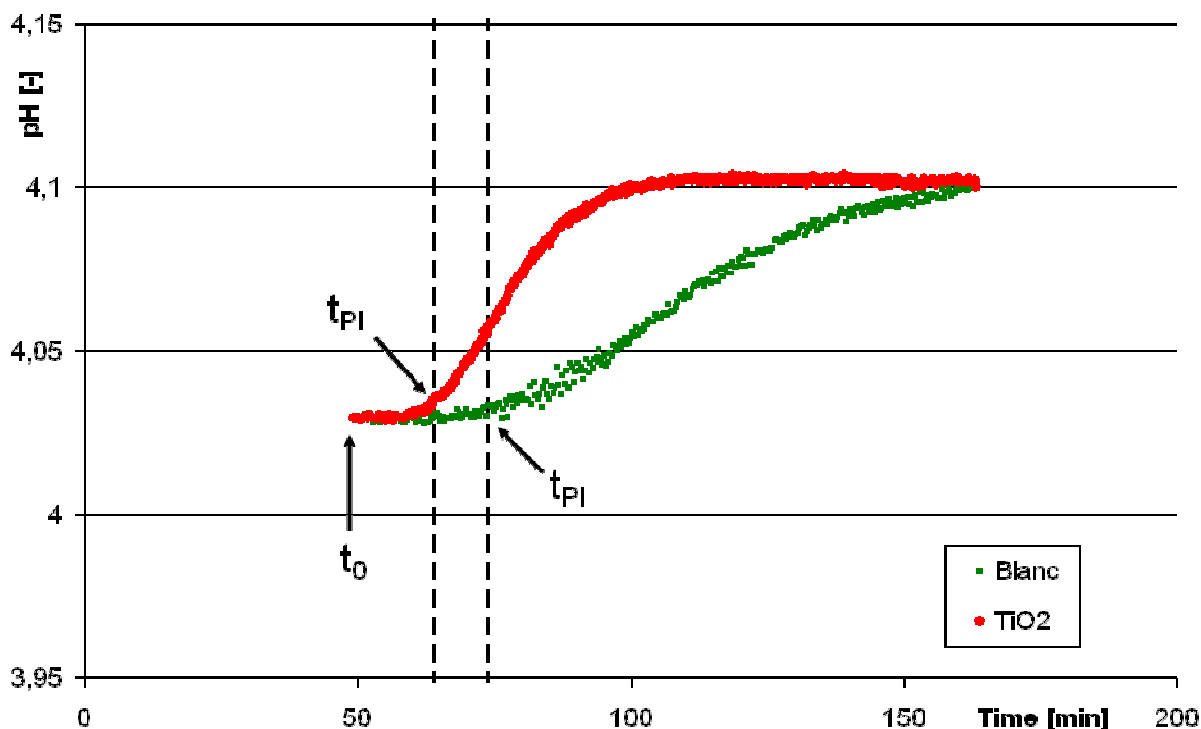

Figure 6: pH-track results of blank experiments (green squares) and TiO_2 template experiments (red dots)

To guarantee that the observed enhanced crystallization of cinnamic acid by TiO_2 templates is not due to solvent-template interactions, the effect of the addition of TiO_2 nanoparticles on the pH is determined following the template classification procedure. Figure 7 (pH versus time) displays the results of this experiment.
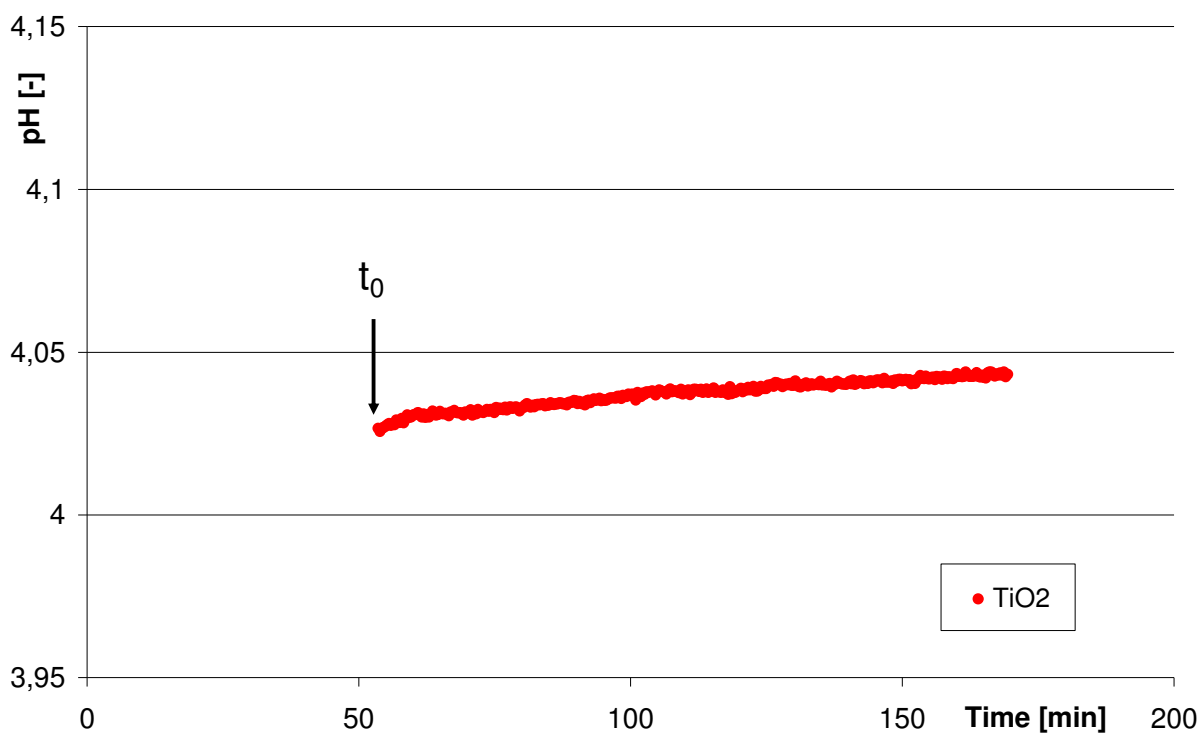
Figure 7: Effect of TiO$_2$ template on pH

HCl is added at t_0 to set the pH to approximately 4. From this point on, a small gradual increase in pH can be observed. This increase however does not explain the distinct decrease of induction time by addition of TiO$_2$ nanoparticles and does not contribute to the observed difference in slope between the blank and template experiments. Besides this, cinnamic acid crystals were visually detected. This clearly indicates that solvent-template interactions do not play a role and therefore that TiO$_2$ templates do enhance crystallization of cinnamic acid.
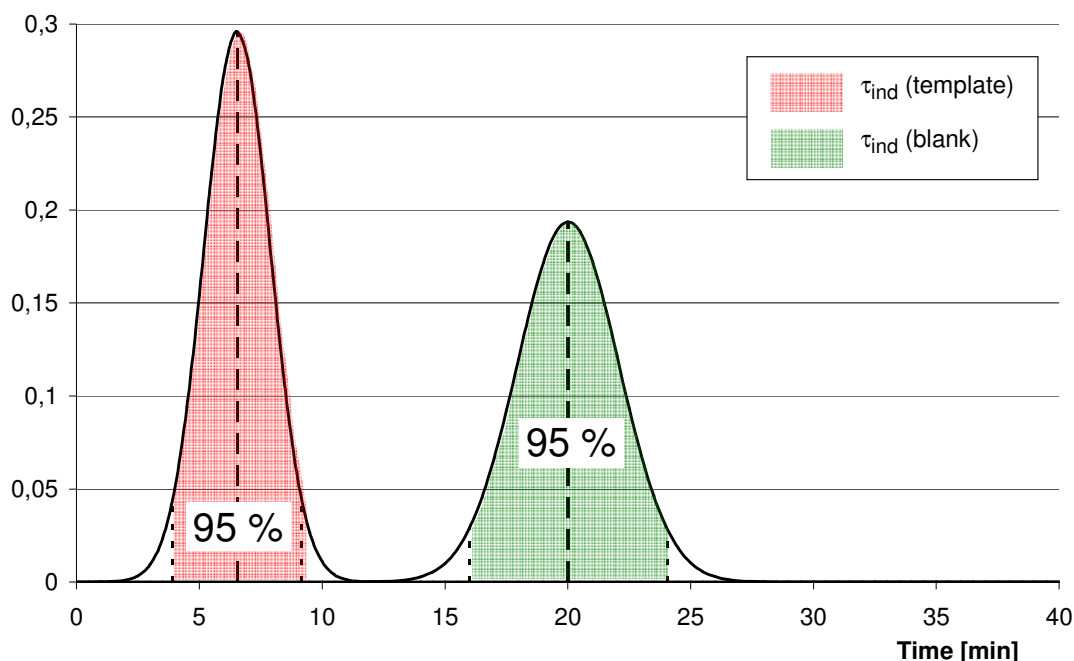
Figure 8: Probability density function of populations of induction times of blank and TiO_2 templated cinnamic acid crystallization ([CAH] = 1 g/l, pH ~ 4, T = 30 ℃).

To be able to perform the statistical test (as described above in "pH-track methodology") to qualify a certain template as promoting CAH crystallization, a total of 26 blank and 10 TiO_2 pH-track experiments were performed under identical conditions. The statistical test assumes that the sample is a good representation of the population.

Figure 8 displays the probability functions of the populations of induction times of blank and TiO_2 experiments. The averaged induction time of blank experiments is 20 ± 2 minutes and the averaged induction time of template experiments is 6,5 ± 1,5 min. The graph clearly shows that the induction time of TiO_2 experiments is outside the 95% reliability interval of blank induction times. The induction time of cinnamic acid crystallization thus is significantly decreased by addition of TiO_2 nanoparticles; therefore it is concluded that TiO_2 does promote crystallization of cinnamic acid.

6. Discussion

The observed variation for induction times of cinnamic acid crystallization is largely determined by the stochastic character of (primary & secondary) nucleation processes. Part of the variation might also arise from the error margin (approximately 5%) of the pumps instantaneously dosing HCl to the crystallizing cinnamic acid solution (to impose the required supersaturation). A 5% error margin results in a variation in initial (t_0) pH between 3,9 and 4,1. Obviously, different initial pH's translate to slightly different (initial) supersaturations and therefore also to different induction times.

Experimental conditions used to acquire induction times of blank cinnamic acid crystallization are within the experimentally determined metastable zone. Induction times for solutions without templates therefore were expected to be large. Results

show that in this way an optimal balance between accuracy and distinguishing capacity of the methodology is obtained.

The pH-track methodology, based on pH-shift crystallization, is developed in the scope of fermentation processes. In the specific case of cinnamic acid being produced by the organism *Pseudomonas putida* S12, fermentations are executed in neutral environments, whereas crystallization of the model compound occurs at about pH = 4. It is noted that the application of pH-shift crystallization as the driving force in a fermentation process with *Pseudomonas putida* S12, therefore is quite a challenge. However, cinnamic acid is very useful as a model compound for developing the screening methodology.

Application of the TIC principle in fermentation reactions can in principle be achieved using pH-shift crystallization for other model systems (where an (in)organic/aromatic acid is produced at low pH's, e.g. fumaric acid produced by *Rhizopus oryzae* [12]), or other crystallization techniques (e.g. local pH-shift crystallization by means of for example electrochemical processes [10], or anti-solvent/reactive crystallization for bulk crystallization) for fermentation processes in general.

7. Conclusions & Future work

The induction time of cinnamic acid crystallization is significantly decreased by the addition of TiO_2 nanoparticles. This template enhances nucleation and growth of cinnamic acid.

The pH-track methodology to evaluate templates, comprising cloud & clearpoint experiments, template classification experiments and induction time measurements, is proved. The methodology is likely to be generic for all (charged) molecules affected by pH.

For different techniques to initiate a driving force for crystallization (e.g. cooling, anti-solvent or reactive crystallization) other experiments are required to evaluate the ability of a template to promote crystallization. However, the principle of comparing induction times of blank and template experiments can still be used.

Based on the developed generic methodology, TIC experiments will be conducted to find suitable templates for the crystallization of cinnamic acid. Future work will focus on the development of an integrated process of crystallization and fermentation. For this purpose a suitable driving force has to be created (bulk or locally) and a template separation and regeneration tool has to be developed.

8. Acknowledgements

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