FINAL SCIENTIFIC/TECHNICAL REPORT

Project Title:	Commodity Scale Thermostable Enzymatic Transformations
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Executive Summary: The conversion of corn starch to high fructose corn-syrup sweetener is a commodity process, producing over 3 billion kg/y. In the last step of the process, an enzyme catalyst is used to convert glucose to the much sweeter sugar fructose. Due to incomplete conversion in the last step, the syrup must be purified using a chromatographic separation technique, which results in equal quantities of water being added to the syrup, and finally the water must be evaporated (up to 1 lb of water/lb of syrup). We have estimated the energy requirement in the evaporation step to be on the order of 13 billion BTU's/y. This process inefficiency could be eliminated if a thermostable form of glucose isomerase (GI), the enzyme catalyst used in the final step, was developed.

Our chosen strategy was to develop an immobilized form of the enzyme in which the protein is first crystallized and then chemically cross-linked to form an insoluble particle. This so-called cross-linked enzyme crystal (CLEC®) technology had been shown to be a powerful method for enzyme stabilization for several other protein catalysts. In this work we have developed more than 30 CLEC preparations of glucose isomerase and tested them for activity and stability. We found these preparations to be highly active, with a 10-50 fold rate per gram of catalyst increase over existing commercial catalysts. The initial rates were also higher at higher temperatures as expected, however the efficiency of the CLEC GI preparations unexpectedly rapidly decreased to a low constant value with use at the higher temperatures. At this point, the source of this activity loss is unclear, however during this loss, the catalyst is found to form a solid mass indicating either breakage of the chemical cross-links or simple aggregation of the particles. It is likely that the increased mass transfer resistance due to this agglomeration is a major component of the activity loss. This research suggests that one potentially beneficial outcome could be the reconfiguration of catalyst columns using these highly active catalyst preparations with inerts to prevent agglomeration.

As a result of this work, methods for the preparation of highly active immobilized glucose isomerase preparations were developed along with test methods that are predictive for the stability of these preparations.

This research has been conducted as a team effort. The enzyme is produced using Genencor's glucose isomerase protein and the stabilized form has been prepared using Altus' CLEC technology. ORNL has provided bioprocess engineering and testing expertise, and Cargill, Inc. and Genencor have supplied critical technical consultation and economic assessment.

Project Objective: To enable a more energy efficient high fructose corn syrup process by development of a stabilized cross-linked enzyme crystal form (CLEC) of the enzyme glucose isomerase (GI). Thermostable GI CLEC would allow direct conversion of glucose syrup to 55 percent fructose syrup and eliminate chromatography and energy wasteful water evaporation steps from the current process.

Project Outline – GANNT and Milestone Charts : Achievements and Assessment of Tasks

A summary of the project outline is shown below in GANNT chart form. Tasks 1-22 have been completed as have Tasks 28-30.
An efficient method for crystallization of glucose isomerase (Tasks 2-7) was optimized as a part of this work. It was found that crystallization of the protein using magnesium sulfate, if carefully controlled, could be used to develop polygonal crystals with excellent properties. Moreover, control of crystal size could be effected by controlled seeding. Execution of these tasks was impeded by problems with crystal solubilization. It was found that protein solubility did not decrease continuously with precipitant concentration, but was found to reach a minimum at < 1M magnesium sulfate.

Extensive cross-linking studies were performed in which the effect of cross-linker composition, exposure time and concentration were examined (Tasks 9-12). Conditions which gave high activity yet relatively insoluble CLEC particles were found. Due to the unexpected loss of activity experienced in the stability and column studies, far more effort on optimization was expended than originally planned for. More than 30 unique formulations were developed, many of which with high volumetric activity, however none with acceptable maintenance of activity.

Activity assays based on those of Genencor and Cargill were transferred to ORNL and Altus. ORNL validated and adapted these assays to our studies (Tasks 14-16). In development of stability tests, ORNL found that the originally targeted temperature, 95˚C was too high giving rapid inactivation of all preparations due to acidification and carmelization of syrups. Through a series of studies it was found that 70-75˚C was sufficient to distinguish between stable and unstable preparations in a reasonable time (1 month).

Stability studies of CLEC GI samples indicated that the loss of activity versus time was inferior to that of commercial immobilized GI (Gensweet IGI from Genencor) so as a result, the column studies were by-passed in favor of cross-linking optimization. Several rounds of cross-linking studies followed by stability testing resulted in the finding that such preparations were highly active (10-50X that of Gensweet IGI) consistent with our expectations, however none of these maintained activity at a rate equal to that of IGI. This activity loss was unexpected. The best formulations were scaled to the 25 g scale and tested in column format (Tasks 28, 29 and 30). It was found that the columns very rapidly plugged due to conversion of the discrete catalyst particles to a single mass.

Initially, this loss of activity was attributed to protein denaturation, however observation of aggregation of intact crystals in many of the samples is also consistent with activation loss through aggregation with concomitant increase in diffusion into the catalyst particle. In addition the activity decreased to a low but constant and stable value indicating that some enzyme in the GI-CLEC remained active for extended high temperature periods. Future work will be centered on prevention of particle aggregation by the use of a solid diluent such as silica gel and reversal of aggregation by mechanical or sonochemical means. The results of this will allow us to distinguish enzyme inactivation from diffusional activity loss.

Patents: None.

Publications/Presentations:

None, however it is likely that this work will be presented and published once the source of the catalyst activity loss is identified.

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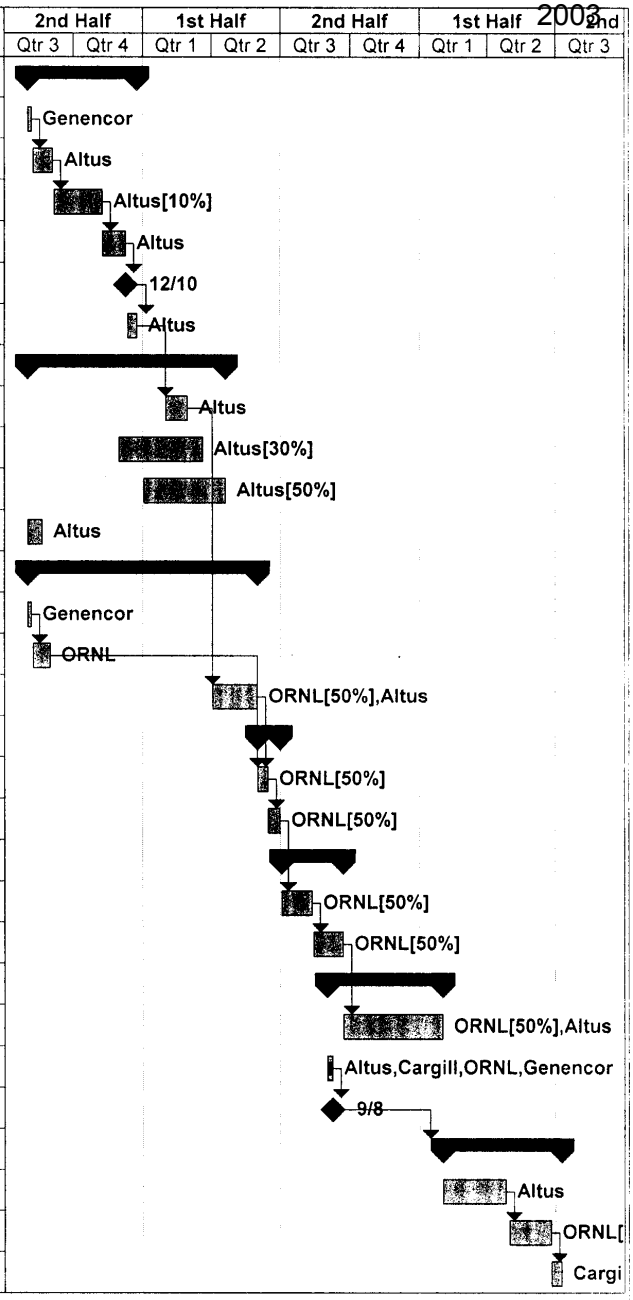
ID	Task Name	Duration
1	**Screen Crystallization Conditions**	**105 days**
2	Supply 100g of GI protein	5 days
3	Analyze, Purify protein	20 days
4	Perform hanging drop screen	46 days
5	Scale up 4 crystallization conditic	23 days
6	Choose 1 for scale up	1 day
7	Prepare 1g of crystals	10 days
8	**Crosslinking Studies**	**188 days**
9	Screen glutaraldehyde levels	21 days
10	Test stability, leaching	79 days
11	Test other crosslinking agents	78 days
12	Optimize temp., pH of crosslinkin	15 days
13	**Assay Development**	**218 days**
14	Supply current assays	5 days
15	Test current product @ 95C	17 days
16	Test prototypes	43 days
17	**Column Testing**	**22 days**
18	Transfer protocols from partner	10 days
19	Test current product @ 95 C	12 days
20	**Prototype Testing**	**59 days**
21	Stability/activity test of prototypes	30 days
22	Column testing	29 days
23	**Non-standard feedstocks**	**109 days**
24	Feed quality effect	94 days
25	Team meeting -project direction	5 days
26	Choice of best system	1 day
27	**Optimization of CLEC**	**113 days**
28	Scale to 100g	60 days
29	Test in column format	40 days
30	Meeting, analyze economics	10 days



GI CLEC TIMELINE
Date: Tue 12/4/01

Task		Rolled Up Milestone	
Progress		Rolled Up Progress	
Milestone		Split	
Summary		External Tasks	
Rolled Up Task		Project Summary	

Milestone Status Table:

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U.S. Department of Energy

Milestone Log

OMB Control No.
1910-0400

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DE-SC07-99CH11006, 01/01/02 06/30/03

Identification Number	Description	Planned Completion Date	Actual Completion Date	Comments
Milestones and Activities. See project GANNT chart for Task Identification Numbers and original timeline.				
11	Test Other Crosslinkers	Q2, Yr 2	Q3, Yr 2	Unexpected reversal of crosslinking occurred at 70° C
16	Test prototypes	Q2, Y2	Q3, Y2	Complete
20	Prototype Testing Iteration 2	Q4, Yr2	Q4, Y2	Complete
27	Optimization of CLEC	Q4, Yr2	Ongoing	Task moved forward from original plan
28	Scaled to 100g	Q4, Y2	Q4, Y2	Done, Scaled to 50g
29	Column Testing	Q4,Y2	Q2,Y4	Column plugged due to agglomerate or gel formation
30	Meeting, Analyze Economics	Q4, Y2	Q2, Y3	Complete, recommendation to produce additional materials
New Task	Iteration 3 , Crosslinking optimization	Q1, Y3	Done	Additional funds approved to prepare and test final set of materials
"	Column Testing, Iteration 3 and 4	Q3, Y3	Q3 and Q4,Y3	Two additional sets of 5 samples each produced on 10+ g scale

Budget Data (as of 6/30/03): The approved spending should not change from quarter to quarter. The actual spending should reflect the money actually spent on the project in the corresponding periods.

			Approved Spending Plan			Actual Spent to Date		
Phase / Budget Period			DOE Amount	Cost Share	Total	DOE Amount	Cost Share	Total
	From	To						
Year 1	8/99	08/00	160,000	160,449	320,448	98464	111464	207928
Year 2	09/00	08/01	200,000	200,449	440,448	150581	121581	272162
Year 3	09/01	12/01	0	0	0	65340	74340	139680
Year 3	01/02	10/02	25,000*	25,000*	50,000*	53,258	85,248	138506
Year 4	10/02	5/03	0	0		17,357	75,181	92,538
		Totals	385,000	385,898	770,000	385,000	467,814	852,814

*Additional funds approved in ‘02

Background:

The production of high fructose corn syrup is a commodity operation with over 3 B kg/y. The current glucose isomerases operate at 60°C, which yields an equilibrium concentration of 42% fructose HFCS stream. The glucose/fructose mixture is partially separated by ion exchange and the glucose rich portion recycled. A higher fructose content (55%) is necessary to give a product of sufficient sweetness. Any increase in temperature shifts the equilibrium to more fructose and less glucose. This will improve the process efficiency, increase the rate of isomerization and substantially decrease the need for the ion exchange separation. Current disadvantages of the HFCS process are temperature equilibria-based limitations on the fructose composition, a pH optima different from preceding process steps, and poor stability of glucose isomerase requiring more frequent replacement [Cheetham, **Applied Biocatalysis**, 1994]. The basis of the enzymatic high fructose corn syrup process is the isomerization of glucose, produced from starch hydrolysis, to fructose using glucose isomerase. The basis of our research strategy was the stabilization of glucose isomerase (GI) in the form of a cross-linked enzyme crystal (CLEC) to allow the direct conversion of glucose syrup to 55% fructose syrup, thus eliminating the need for subsequent chromatographic enrichment of fructose and resulting in substantial energy savings. Altus Biologics, Inc. has basic patents on a powerful new approach to producing a highly stable dense enzyme catalyst using cross-linked enzyme crystal (CLECs®). These CLECs can be made of any purified enzyme and have been demonstrated with other hydrolases to have better stability to temperature and solvents. Oak Ridge National Laboratory has expertise in continuous bioprocess design, biocatalyst immobilization, and in the modification of enzymes for use in nonaqueous environments. Genencor International has a great deal of expertise in the commercial application of GI in HFCS production and produces one of the most efficient commercial forms of immobilized GI. Cargill is one of the world's largest producers of HFCS and has extensive knowledge and expertise in the technical and economic considerations in commercial HFCS production. Thus, a four-way collaboration between ORNL, Altus, Cargill and Genencor was created with the goal of developing a highly improved process for HFCS production.

The stabilization of GI in CLEC form would afford a catalyst with maximal volumetric activity, allowing rapid attainment of equilibrium with short residence times. Moreover, the high volumetric activity would allow the process to be run outside the optimal pH range, thus minimizing the sugar decomposition that occurs at higher temperatures. Finally, a glucose isomerase form with enhanced organic resistance would also allow a lower quality dextrose feed to be used.

A higher fructose content (55%) is necessary to give a product of sweetness equal to that of sucrose. Any increase in temperature shifts the equilibrium to more fructose and less glucose.

Table 1. Effect of Temperature on Equilibrium Concentration of Fructose[1]

Temp. (°C)	30	40	45	55	65	75	85	90
% Fructose	46.5	47.9	48.2	50.0	51.5	53.1	54.7	55.6

A rapid contact time is also needed to prevent degradation of the sugars at high temperature and the formation of unacceptable color. Higher temperature will cause the current GI to deactivate more rapidly increasing the contact time required in a GI column to reach the final concentration. Cost considerations based on enzyme rate, enzyme life, and by product formation have caused 42% HFCS to become the final intermediate product concentration. The 42% refers to the fraction of fructose sugars in the product stream, with 55% remaining as glucose and the remainder as other sugars.

Comparison of Current HFCS Process with CLEC HFCS Process:

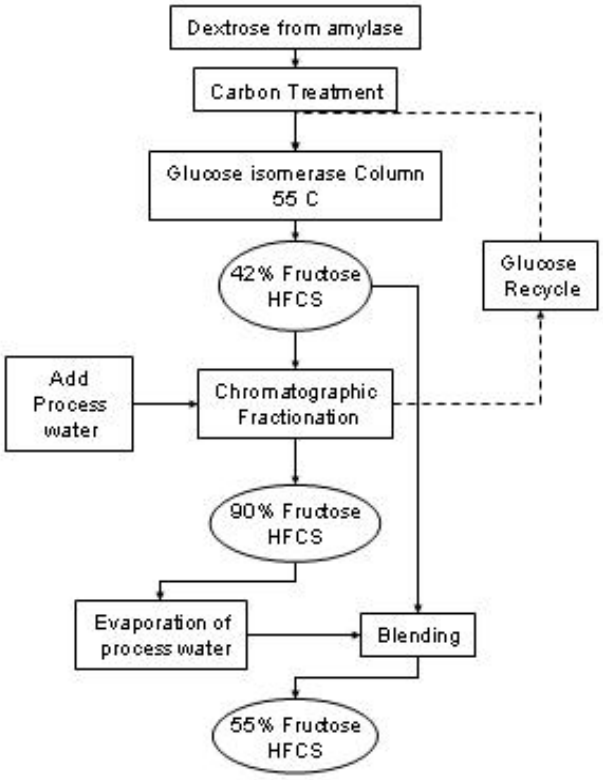
Figure 1 shows a schematic of the overall process. [for reviews see Coker et al. 1985, Kirk-Othmer, 1977; Bhosale et al, 1996] The starch is solubilized and hydrolyzed into glucose. It is also prepared for the isomerization step by removal of color and other impurities and pH adjustment. The control of pH is important and requires balancing the optimal pH for activity (pH 8) with the optimal for enzyme stability (pH 7-7.5). Calcium is removed and Magnesium added to activate the enzyme. The core process is the GI isomerization of the 96% glucose stream (with 40 wt % sugar solids) into a 42% fructose stream. At these conditions (temperature near 60 C) the enzyme has a half-life of 70-120 d. Therefore the flowrate is constantly decreased during operation to allow near constant conversion. Unfortunately, this results in increasing the contact time from 0.5 to 4 h resulting in additional color formation. Typically, multiple enzyme columns are operated in parallel to allow a consistent average product stream.

This stream is polished again and concentrated by evaporation. The fructose concentration is then enriched in a chromatographic fractionation stage. This results in the 90% fructose ratio stream that has been diluted during the chromatographic separation. It is concentrated by evaporation and blended with the 42% ratio stream to give the final standard 55% HFCS syrup where 55% of the sugars are fructose and it has been concentrated to 70% solids.[2]

[1] Bhosale et al, *Microbiol. Rev.* **60**:280-300, 1996

[2] Cheetham, **Applied Biocatalysis**, 1994. Coker, L.E., K. Venkatasibramaniam, "Starch Conversion Processes," Chap 39 in Comprehensive Biotechnology vol. 3 ed., M. Moo-Young, Pergamon Press, 1985. Verhoff, F.H., G. Bogulawski, O. J. Lantero, S. T. Schlager and Y. C. Jao, "Glucose Isomerase," Chap 42 in Comprehensive Biotechnology vol. 4, ed. M. Moo-Young, Pergamon Press, 1985. Kirk-Othmer Encylopedia of Chemical Technology, J. Wiley & Sons, New York NY, 4th ed., 1997, vol 23:595 (1997)]

Current Glucose Isomerase Process



Proposed Glucose Isomerase Process

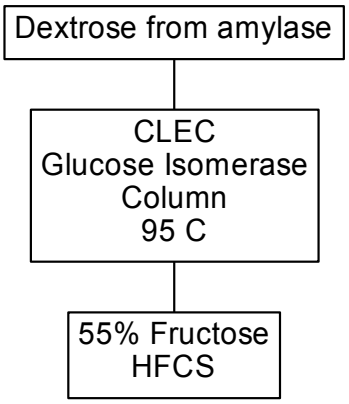


Figure 1. Comparison of Current HFCS Process and GI-CLEC Process

Energy Savings/Waste Reduction:

The potential to eliminate a chromatography step used to enrich fructose in the HFCS process would result in dramatic decrease in energy consumption by eliminating the need to remove the water added for chromatography. The energy cost associated with water removal is substantial;

water (2kg/kg of HFCS) must be added before chromatography and then removed by evaporation. For >3 billion kg of HFCS/y, this amounts to 6 billion kg of water with an energy cost estimated at 13 trillion BTU's (@ 2150 BTU/kg to evaporate water). There is also the possibility to eliminate a pH shift requirement by the current pH range of glucose isomerase and the preceding process steps with a biocatalyst of improved stability. Due to the large volume (>3 B kg fructose/y), the elimination of acid/base neutralization will decrease raw materials required and salt wastes produced by more than several hundred thousand kg/y. An improved conversion will also decrease the glucose recycle stream and decrease pumping and heating requirements. The large volumes of this process will amplify any energy impact.

Detailed Experimental Results and Discussion

Production of Glucose Isomerase Cross-Linked Enzyme Crystals

Development and scale-up of cross-linked enzyme crystal preparations of glucose isomerase were performed in this study. The initial samples were made following the general method of CLEC preparation disclosed by Navia and St. Clair in U.S. Patent #5,618,710. In this technique, the enzyme is first crystallized and then the slurry of enzyme crystals is treated with a chemically reactive bi-functional agent such as glutaraldehyde. The cross-linking must be carried out in such a manner that the crystallinity is maintained, the crystal maintained as discrete particles and that the activity of the enzyme is maintained as much as possible. In this work, long term stability under exposure to dextrose and fructose syrups at high temperature was an additional requirement. Thus our strategy was to develop efficient methods of crystallization which gave control of crystal size. As it became evident that structural integrity of the GI-CLECs was an issue, numerous additives were tested in an evident to increase the strength of covalent linkages within the crystals.

The protein, soluble glucose isomerase was supplied throughout the study by Genencor International along with proprietary assay procedures and protocols for testing of the enzyme in column format. Genencor also provided extensive technical support for the technical transfer of these assays, test methods and advice on scaling the protocols. Genencor supplied samples of their current enzyme product, Gensweet IGI which is immobilized on an ion exchange resin. The current immobilized product and process were used as a benchmark for the project. Analyses of activity and stability were performed by ORNL and Genencor. Cargill and Genencor provided commercial assessment of the work and information on existing industry practices.

The first major research milestone of the project achieved was the development crystallization conditions in which crystal shape and size can be controlled on a large scale. Crystallization studies conditions were developed at Altus Biologics in which crystals of the morphology shown in the photomicrograph (Figure 2) could be prepared in size ranging from 25 to 100 microns.

Several lots of 50 g of pure protein were prepared for cross-linking studies. The commercial enzyme Gensweet, was provided as a solution in a preservative buffer. The buffer was removed by exhaustive dialyzing against distilled water. Four hundred ml of formulated enzyme was exchange with 4X 1000 ml of distilled water using a 30,000 MW cutoff hollow-fiber membrane. A small aliquot of this protein solution (200 mg/ml final concentration) was treated with $MgSO_4$ to give a final concentration of 0.125 M $MgSO_4$ and 75 mg/ml protein. This solution was stored overnight at 4˚C until crystals were observed to form. These crystals were collected by centrifugation and used as seed crystals for the remaining protein concentrate. The remaining solution was adjusted to 0.125 M $MgSO_4$ and 50 mg/ml protein, and then cooled to 4˚C. The seed crystals were added and the mixture stirred gently using an overhead stirrer overnight. The resulting crystal slurry was used within 1 day for all subsequent cross-linking studies.

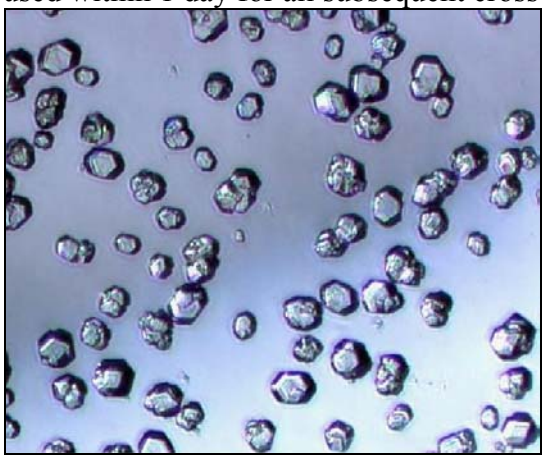


Figure 2. Photomicrograph of Glucose Isomerase Crystals from $MgSO_4$

A key finding in this work was that the solubility of glucose isomerase exhibits a minimum that is dependent on both concentration of magnesium sulfate and on temperature (Figure 3) and therefore minimal protein is lost in the mother liquor through crystallization under these minimum solubility conditions. The solubility of the protein increases dramatically at higher and lower concentrations of precipitant.

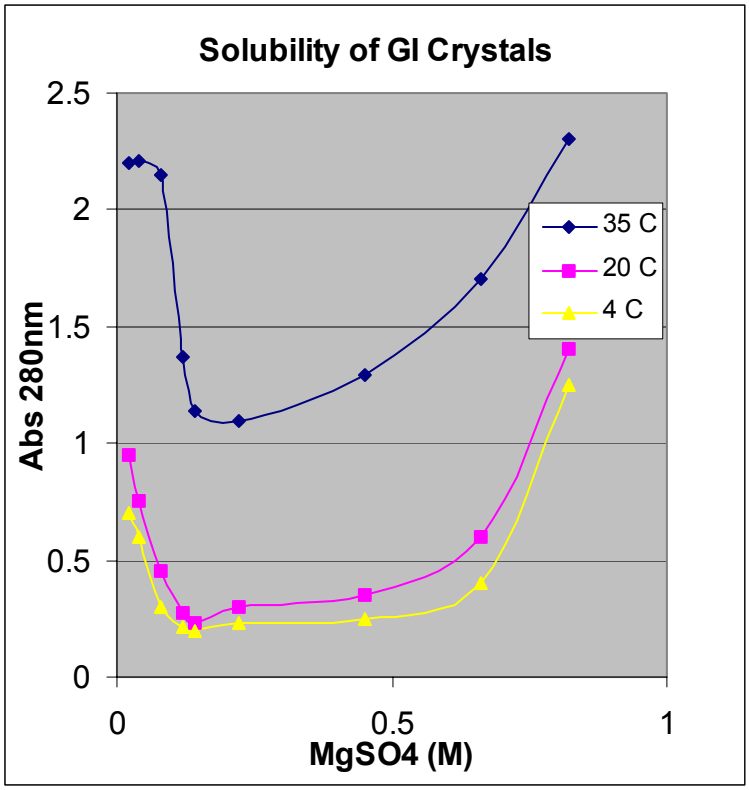


Figure 3. Solubility of Glucose Isomerase as a Function of Magnesium Sulfate Concentration and Temperature

Stability and Activity: Development and Validation of Activity and Stability Test Methods

In order to test activity and stability of CLEC prototypes, protocols were developed in which 50-100 mg of sample could be tested. Stability is typically tested in heated columns over several weeks. This test was deemed unsuitable for screening due to the large sample size and prolonged testing period. As a result, several rapid screening tests were evaluated which involved incubating the samples at elevated temperature with and without glucose syrup.

Activity was measured by adapting Genencor International's proprietary assay method for the interconversion of glucose to fructose to the appropriate scale. Degree of conversion was measured using HPLC chromatography using a method supplied by Genencor. A plot of the ratio of fructose to glucose over a 30 min assay for several levels of soluble enzyme and at four different temperatures is shown below in Figure 5. As seen from the plot, the activity determination is in the linear range under these conditions. It is also clear that the enzyme rate increases with increasing temperature up to 80 °C in these initial rate tests.

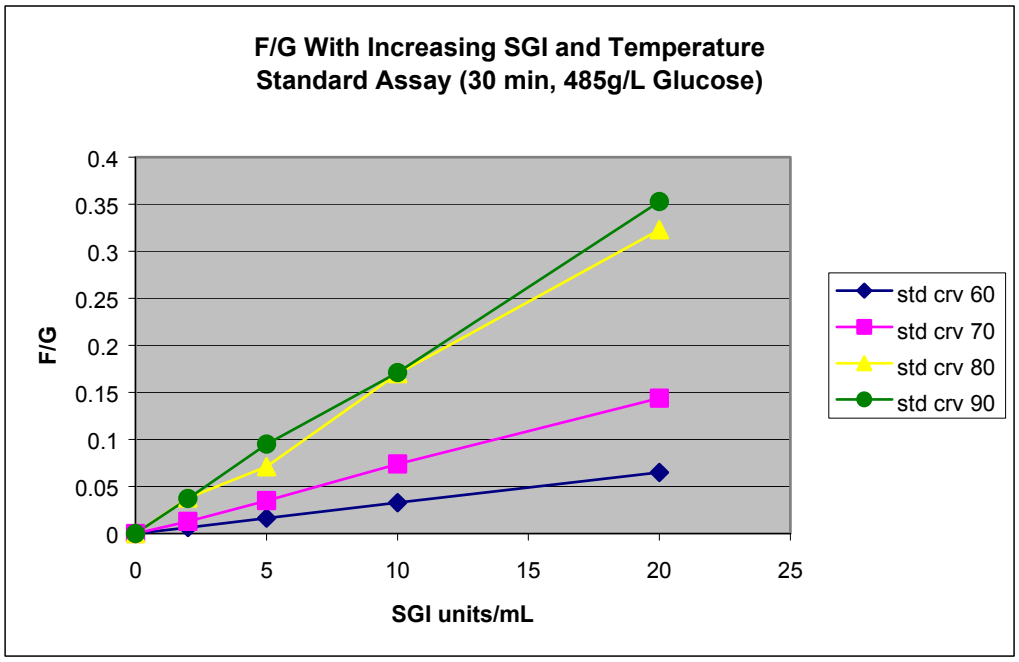


Figure 4. Conversion of Glucose to Fructose vs. Assay Temperature

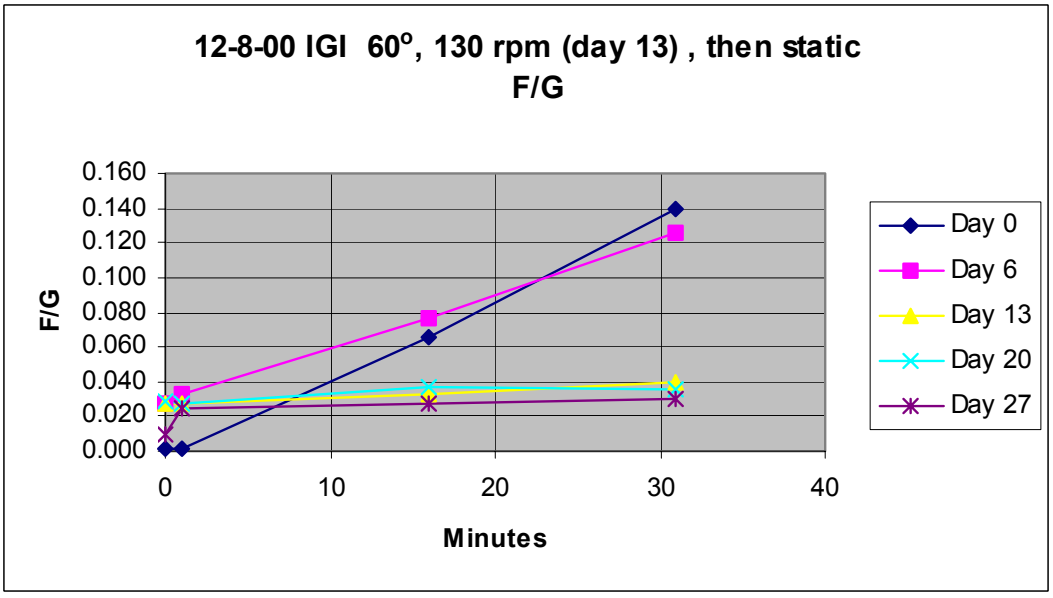


Figure 5. Stability Testing of Genencor IGI at 60˚C

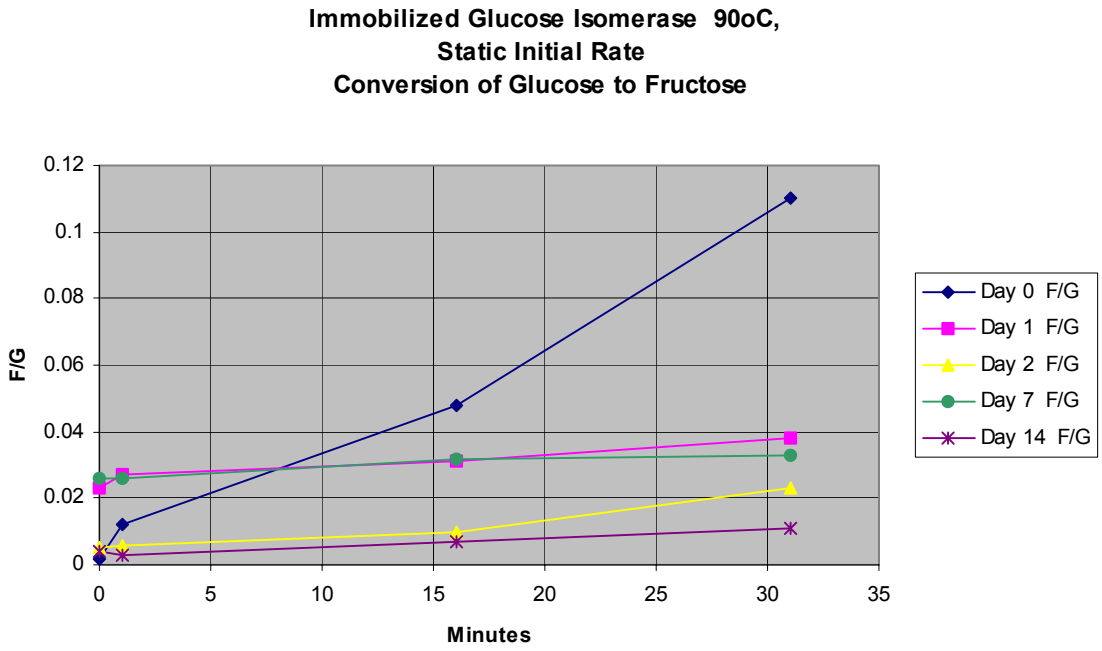


Figure 6. Stability Testing of Genencor IGI at 90˚C

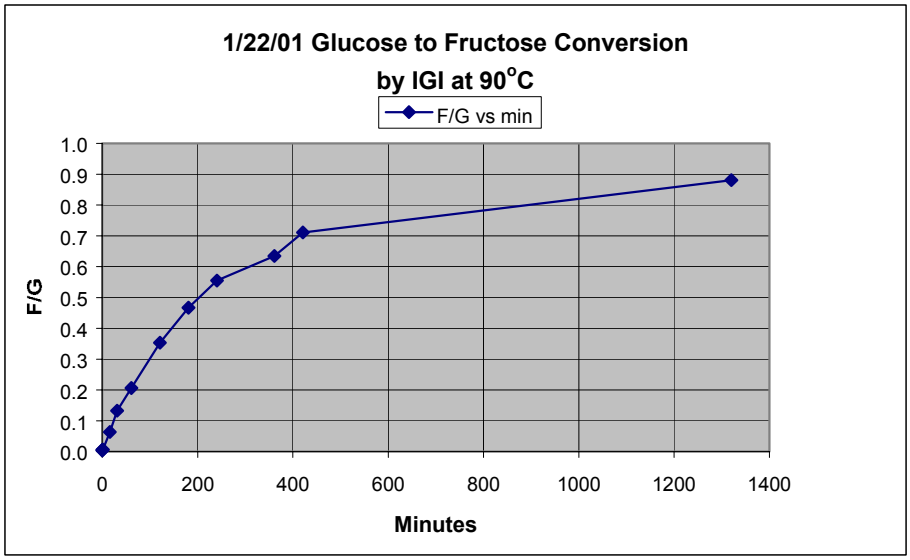


Figure 7. Conversion of glucose to Fructose by IGI at 90˚C

The GI activity protocols were modified by ORNL to accommodate the immobilized enzymes and the higher temperatures. Thermal stability was determined using an incubation assay developed by ORNL as a part of this work. In brief the sacrificial samples of the immobilized enzymes (GI-CLECs and IGI) were incubated in buffer at the desired temperature for the stated time. The sample was then withdrawn and the activity determined under the standard assay conditions of sugar and temperature. This allowed the temperature effect on stability to be separated from the increased rate at higher temperatures. The samples were incubated in buffer, extended incubation of the sugar solution at all temperature caused carmelization and acidification of the sugars. The pH would decrease to under pH 3 within hours; these low pH were determined to be denaturing to the GI. In real operation, the sugar solution is only heated for brief periods (minutes) as it flows through the IGI column; therefore the incubation in buffer is a more realistic test of thermal stability. Due to the extremely high specific activity in the GI-CLECS, on occasion the amount of biocatalyst or the duration of the activity test was decreased in order to keep the F/G conversion ratio near 0.1 for a 'linear' rate.

Preparation of CLECs and Assessment of Activity and Stability

Summary

Early samples that were lightly cross-linked with glutaraldehyde dissolved at 70˚C.
Activity tests were compared to soluble and immobilized GI using a modified industrial protocol from Genencor. Fructose and glucose were analyzed by HPLC. Soluble GI is active up to 90˚C with increasing rates; however it is unstable fro even an hour at the condition. The Figure shows both the effect of increased temperature of initial rates of Soluble GI at four elevated temperatures during the 30 minutes standard test. The initial rates increase with temperature as expected. The GI is still active at 90˚C; however, the GI is already beginning to denature within the 30 min at 90C. This encouraged us that high catalytic acitivty could be achieved due to both high enzyme concentration in CLECs as well as thermally increased rates - if a stable CLEC could be produced.

The GI-CLECs were compared to immobilized GI (IGI). IGI is immobilized by Genencor on diatomaceous earth and lightly crosslinked. The IGI is somewhat more thermostable than the soluble GI. An intermediate set of GI-CLECs were produced; several formulations were physically stable and did not leach protein (as measured by absorbance at 280 nm) but lost nearly all activity in 24-48 h at 70˚C. In the last set the GI-CLECs were again physically stable. Figure 3 shows a greatly improved thermal stability. Deactivation required two weeks. However, an industrial use requires a stability of several months. This could require one or two more steps in formulation improvement.

Several iterations of cross-linking, scale-up and testing were performed over the course of the study. The most stable formulations developed used 0.125M MgSO4, 4°C to maintain crystallinity, the glutaraldehyde was treated with borate, and a series of water-soluble, polyethylglycol diamines were added as co-linkers to alter cross-linker length. The cross linking procedure required a GI crystal suspension (50 mg/ml) which was resuspended in 4°C co-linker solution under gentle agitation for 24 h. They were tested for dissolution at RT and 70°C. The adjacent Figure 4 shows one of the final CLEC formulations.

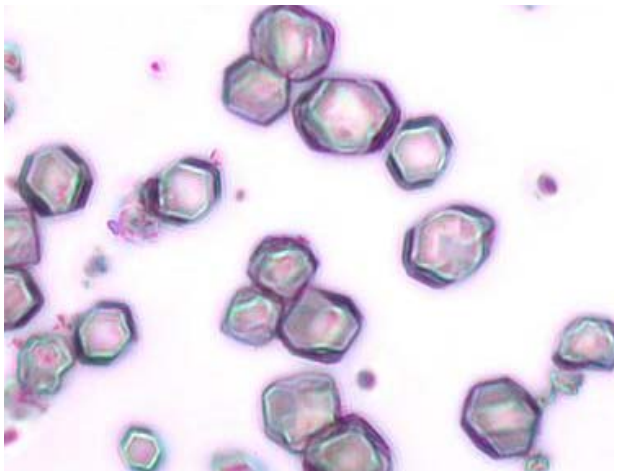


Figure 8. Cross-Linked Glucose Isomerase Crystals

Detailed Description of Cross-linking of GI Crystals and Evaluation of their Activity and Stability

A slurry of crystals in MgSO4 solution (as described above) was used for each batch of cross-linking. The cross-linking conditions are designed such that five parameters are variables: with or without additional MgSO4, two levels of lysine, untreated glutaraldehyde, and glutaraldehyde treated with borate as summarized in Table 2. The resulted materials were examined visually and by optical microscopy and then tested for activity in the conversion of glucose to fructose.

Table 2. Summary of Cross-Linking Conditions*

Sample	$MgSO_4$	Lysine	GA (untreated)	GA (treated)	Physical Appearance
B1M1[1]	16 ml (1.5M)	1.5g	3mL (50% H2O)		Solid Gel
B2M1	11 ml (1.5M)	1.1g	1.6 ml		Solid Gel
B1M2	16 ml (1.5M)	1.5g		30 ml (4%)	Discrete crystals
B2M2	11 ml (1.5M)	1.1g		20 ml	Solid Gel
B1M3		2.5g	3mL (50% H2O)		Solid Gel

B2M3		1.5g	1.6 ml		Solid Gel
B1M4		1.5g		30 ml	Crystals/partial dissolution
B2M4		1.1g		20 ml	Discrete crystals
B1M5	15 ml	1.5g		15 ml	Discrete crystals
B2M5	10ml	1.1g		10 ml	Crystals/partial dissolution
B1B1[2]	16	1.5g	3mL (50% H2O)		Solid Gel
B2B1	11	1.1g	1.6 ml		Solid Gel
B1B2		1.5g		30 ml	Solid Gel
B2B2		1.1g		20 ml	Discrete crystals
B1B3		3 g		30 ml	Solid Gel
B2B3		2 g		20 ml	Crystals/partial dissolution
B1B5	15	1.5g		17 ml	Discrete crystals
B2B5	10	1.1g	2 ml		Solid Gel

10 mL crystal slurry, dry weight ~3.2g for B1, 2.5g for B2

As evident from the high conversion in Figure 9, the materials were too active under that test conditions used. The conversion of glucose to fructose is shown for samples incubated at 60, 75C and without a temperature treatment (ni). In order to distinguish activity differences, the

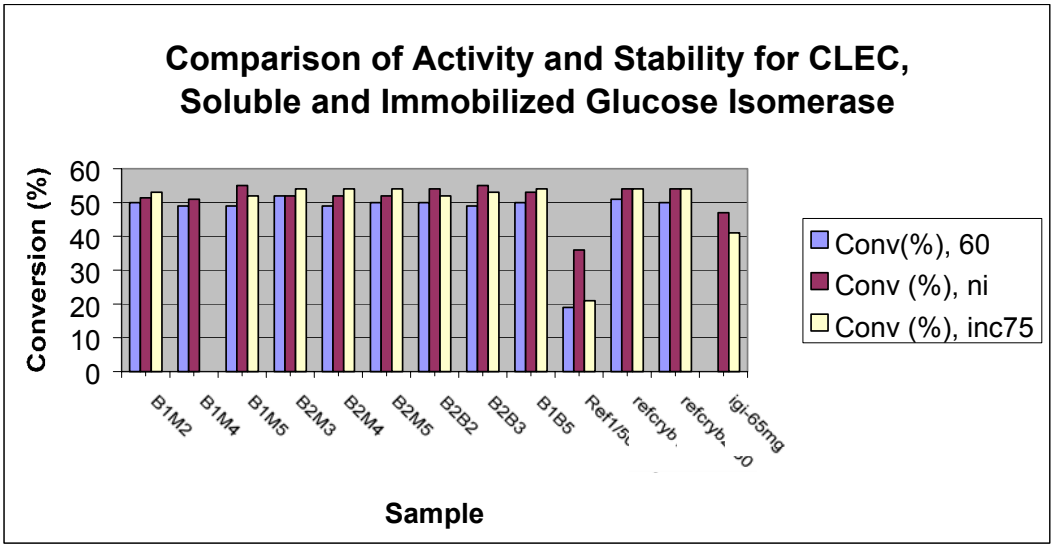


Figure 9. Activity Comparison of CLEC, Soluble and IGI

amount of the enzyme was reduced by 5-fold in the subsequent test. Figure 10 show result of the activity determination using a 10 and 30 minute incubation. From the conversion over 10 minutes it is evident that sample B1M2 gave the highest activity.

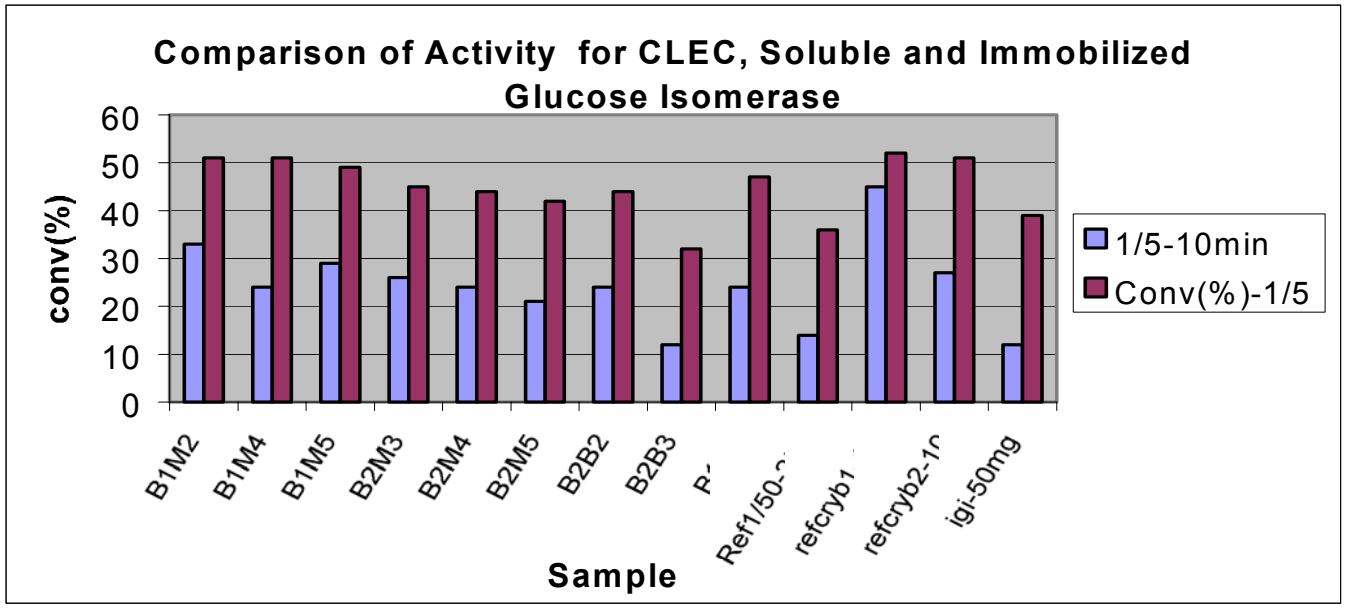


Figure 10. Activity Comparison of CLEC, Soluble and IGI

Cross-linking Using Amine Additiives

Experiments were designed and executed using Jeffamines as co-linkers and cross-linking directly in Tris buffer with or without MgSO4 added. The experiment conditions are listed in Table 3 and activity in Figure 11.

Table 3. Cross-Linking Conditions* : Glutaraldehyde and Additives

Sample	$MgSO_4$	Jeffamine T-403	Jeffamine XTJ-509	GA add treated	Physical Integrity
M-J1a	16 ml (1.5M)	1.5g	-	30 ml (4%)	swelling
M-J1b	16 ml (1.5M)	1.0g	-	30 ml	swelling
M-J2a	16 ml (1.5M)	-	1.5g	30 ml (4%)	
M-J2b	16 ml (1.5M)	-	1.0g	30 ml	
Tris-J1a	-	1.5g	-	30 ml (4%)	swelling
Tris-J1b	-	1.0g	-	30 ml	swelling
Tris-J2a	-	-	1.5g	30 ml	
Tris-J2b	-	-	1.0g	30 ml	

- Start with 10 ml crystal slurry, ~3.2g for B1, 2.5g for B2

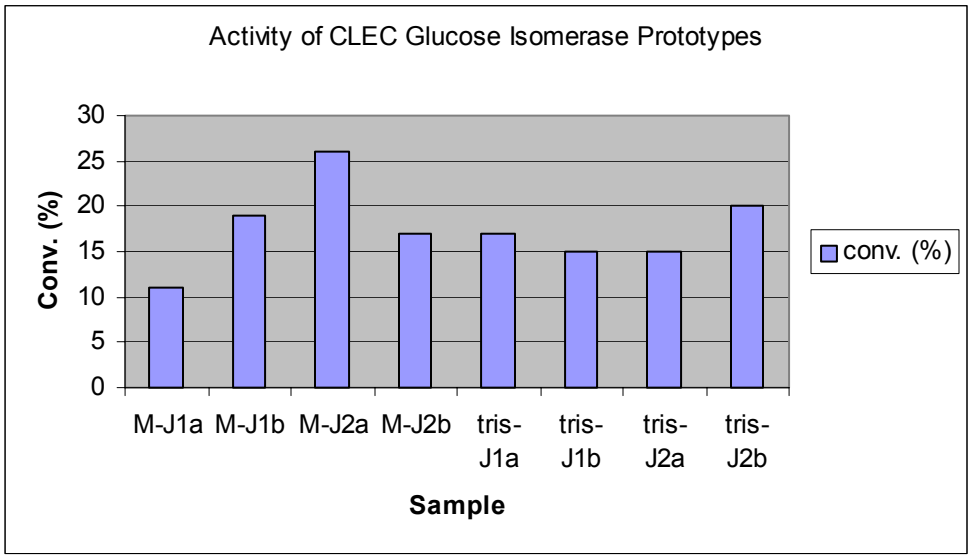


Figure 11. Activity Comparison of CLEC Samples

Activity and Stability Test

As a result of assay development work over the course of the study. An incubation and assay and protocol method was finalized. The GI samples were incubated in individual microcentrifuge tubes and held at the incubation temperature over the period indicated. In most cases, the IGI was use at 10X

weight/volume relative to the CLEC due to the difference in activity density. The GI samples were held in a glucose syrup buffer at the designated temperature.

Outline of Protocol

IGI = 50mg in 1.0mL GSB
CLECs = 5mg in 1.0mL GSB

Experiment to be run in Eppendorf centrifuge tubes floating in a small 70^{o}C water bath with circulating flow.

Prep of 250mL Glucose Substrate Buffer (GSB) for IGI

1. To 175 mL hot ddH_2O, add 117.5g anhydrous dextrose. Cool

2. While stirring, add 0.75g Tris, 0.25g $MgSO_4$-7H_2O and 0.004g Na bisulfite.

3. pH to 8.0 and QS to 250mL with Milli-Q H_2O

4. Measure glucose (g/L) on the YSI and record.

Prep of 250mL Glucose-Free Substrate Buffer (NGB) for IGI

Follow steps 2 and 3 above and QS to 250mL

Procedure:

Rehydration of IGI

1. Weigh 50mg IGI to glass tube and add 3mL "no glucose" substrate solution*
2. Incubate at RT for 30' at RT, swirling every 10 minutes. Let solids settle. Remove supe to 1mL, place IGI and remaining supernatant into an Eppendorf tube and spin on high for 2 min.

2. Carefully remove supernatant and discard. Add 1mL "no glucose" buffer, store at 4˚C.

1. To 0.9mL "no glucose" buffer in 8 (each) color-coded tubes, add appropriate amount of CLEC.
2. Spin at high speed 2min in microfuge. Discard supernatant
3. Add 1.0mL NGB to each of 7 tubes/CLEC and float in covered 70˚C bath.
4. For IGI, weigh 8x50mg, adding each 50mg to a tube. Rehydrate. Follow steps 2 and 3.
5. A tube of GSB placed in water bath for observation and pH determination.

Assay

0-time

1. To remaining 8th tube for each CLEC and IGI, add 1.0mL GSB, mix and place into a second 70^{o} C water bath for 10 min. Remove and spin. Collect supe in a clean tube.
2. Dilute in 5mM H_2SO_4 for HPLC analysis.
3. Add 1mL NGB to pellet and store in refrigerator.

Day 1, Day 7, Day 15…

1. Select a tube from incubating water bath and repeat as for 0-time.

Data Record HPLC determinations of glucose and fructose and plot F/G.

Preparation of Fourth Iteration of CLECs

CLECs were prepared in a manner as described above using the proportions described in Table 4. The samples were tested for activity and stability using the optimized protocols. The activity over 15 days for the best samples is summarized in Figures 12 and 13 The activity of the CLEC samples are much higher than the commercial IGI, but the activity is seen to drop off over time more rapidly for all of the CLEC samples.

Table 4. **Cross-Linking Conditions* : Glutaraldehyde and Additives**

Sample	Glutaraldehyde	Co-Linker	Dissolution at RT	Dissolution, 72h at 70°C
283-1	Untreated, 1g	T403	No	Swelling
283-2	Untreated, 1g	D2000	Complete	Complete
283-3	Untreated, 1g	XTJ509	Partial	Swelling
283-4	Untreated, 1g	Lysine	No	**No**
283-5	Untreated, 1g	None	Complete	Complete
283-6	Treated, 1g	T403	No	**No**
283-7	Treated, 1g	D2000	No	Swelling
283-8	Treated, 1g	XTJ509	No	Partial
283-9	Treated, 1g	Lysine	Complete	Complete
283-10	Treated, 1g	None	No	Swelling
283-11	Treated, 1.25g	T403	No	**No**
283-12	Treated, 1.25g	D2000	No	Swelling
283-13	Treated, 1.25g	XTJ509	No	**No**
283-14	Treated, 1.25g	Lysine	Partial	Partial
283-15	Treated, 1.25g	None	No	**No**

1.5g protein, 0.62g co-linker, 4°C, pH maintained at 7

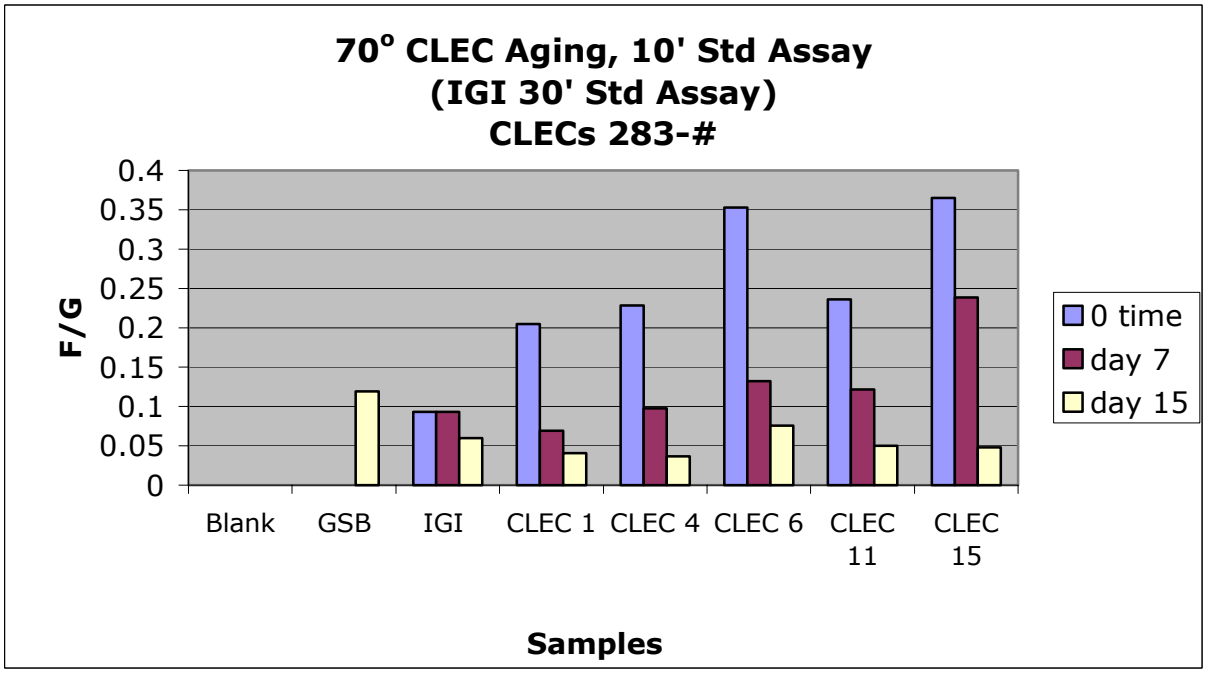


Figure 12. Activity Comparison after 70˚C Incubation

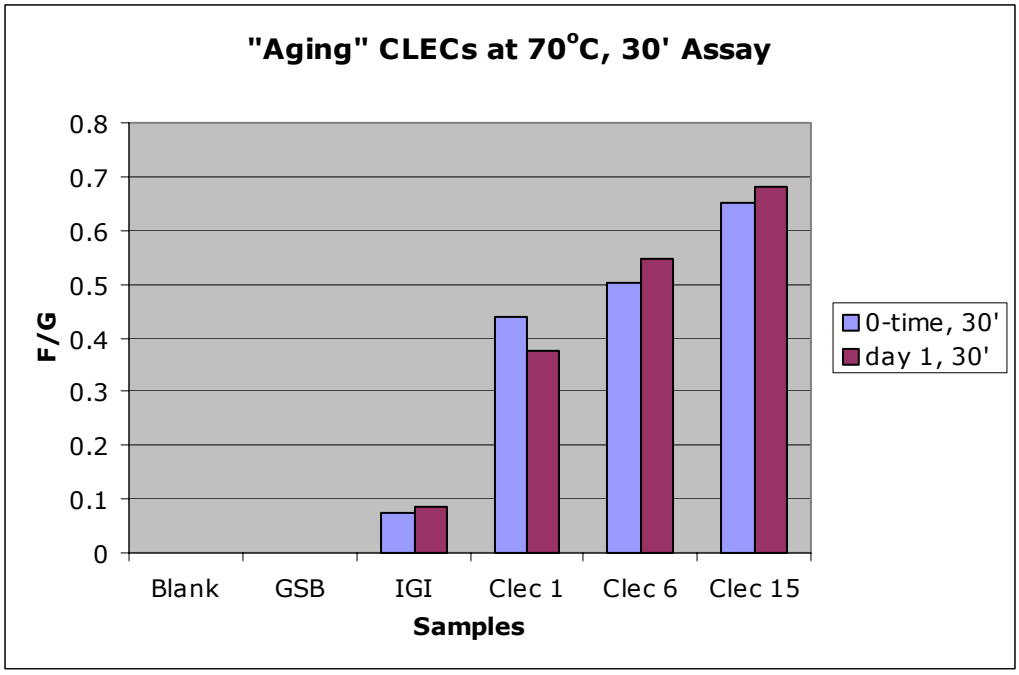


Figure 13. Activity Comparison after 70˚C Incubation

Manganese effects:

The GI enzyme requires Manganese for retained activity in the active site. This metal can leach out of the soluble and IGI forms so low levels of Mn are added to the sugar solutions. We tested the possibility that elevated temperature might increase the loss of Mn from the active site by incubating the GI-CLECs and IGI at 10-50X Mn levels. The effects were within experimental error indicating that Mn loss was not a contributor to thermal deactivation.

Preparation of CLEC Samples

Based on the results of the prior round of CLEC experiments, additional samples were prepared using the optimize MgSO4 crystallization method and increased cross linking times and level of cross linker (Table 5). In addition, sodium alginate was tested as an additive to improve retention of divalent metal ions. As seen in Figures, 14 and 15, the CLEC samples are several fold more active than the IGI, but the activity of these samples decreases much more rapidly.

Table 5. Cross-Linking : Samples: 87-61-X

Sample	Protein (50 mg/ml)	Cross-Linker (borate/glutaraldehyde)	Dry Weight CLEC (mg/ml)
87-69-A	110 ml	165 ml	7
87-69-B	110 ml	440 ml	19.7
87-69-C	110 ml	165 ml + 165 ml 1% alginate	11.1

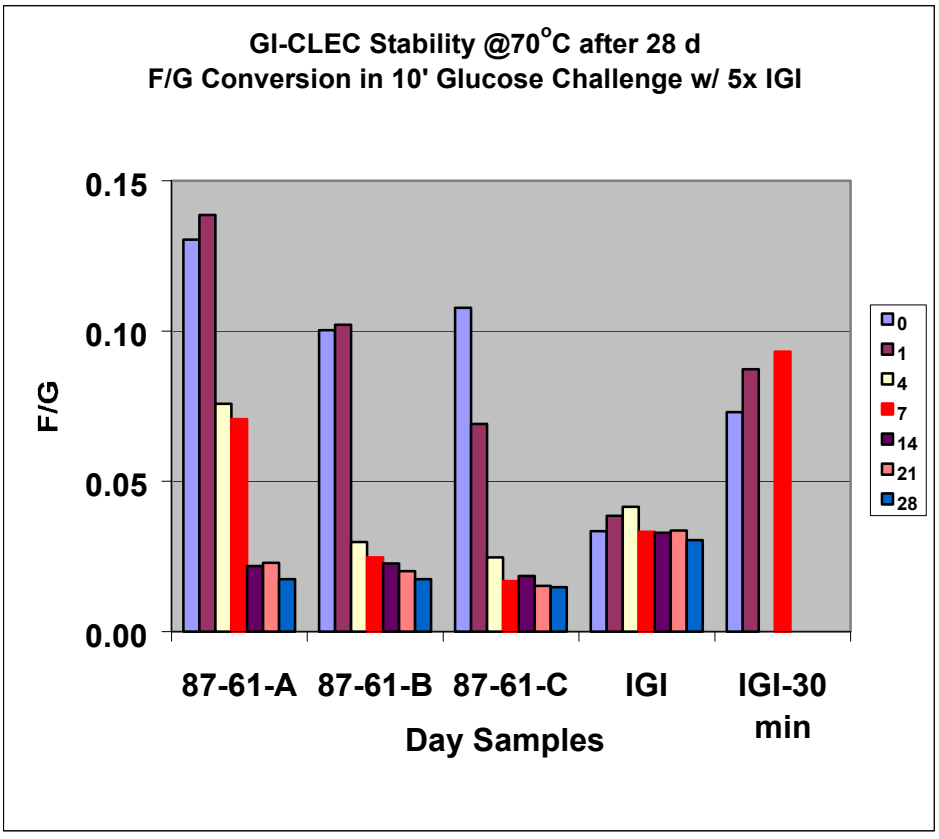


Figure14. Activity of CLEC and IGI After Incubation at 70˚C

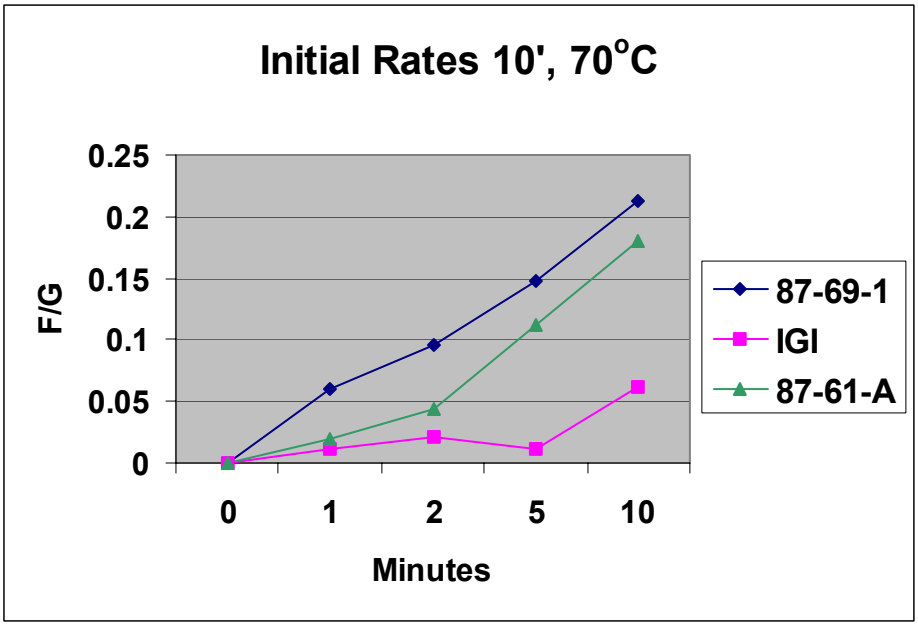
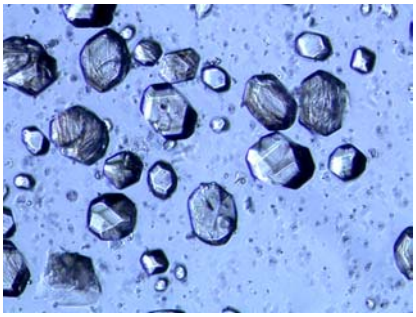


Figure15. Initial Rate Comparison of CLEC and IGI Samples

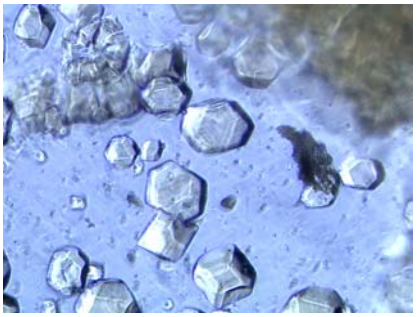
Inactivation via Denaturation or Aggregation

The effect of storage on the GI CLECs in this last round of cross-linking trials was a substantial loss of activity. This was initially attributed to destruction of the CLEC and denaturation of the enzyme, but micrography of some of the samples revealed that the crystalline structure was intact for some of these samples, however they had formed large aggregates of crystals (See series of photomicrographs below). This aggregation may explain some or all of the loss of activity – diffusional losses for large immobilized enzyme particles are common.

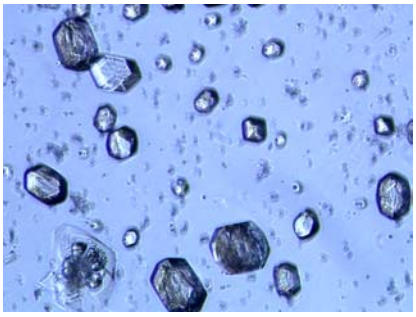
In order to distinguish diffusional loss of activity and enzyme denaturation, we have devised two experiments. The first is simple disruption of the aggregates via mechanical means or by ultrasound. The second is to repeat the model HFCS studies by suspending the GI CLEC in a slurry of silica gel to prevent crystal aggregation.



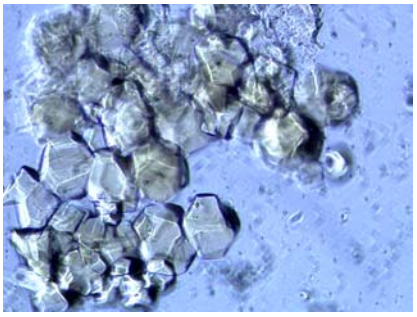
87-69-1 Day 0



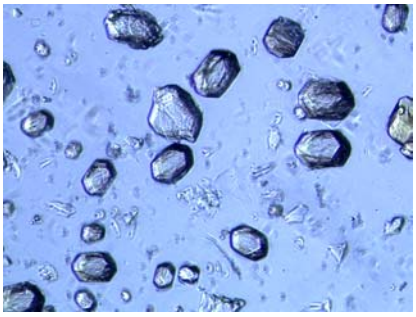
87-69-1 Day 28



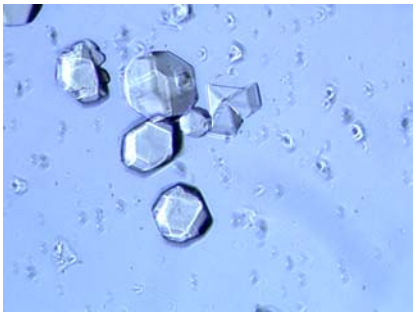
87-69-2 Day 0



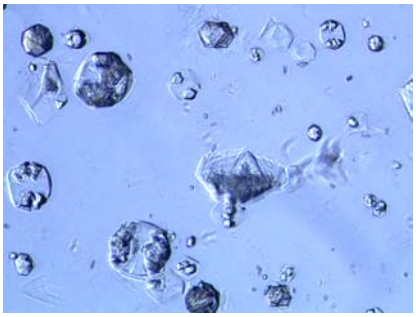
87-69-2 Day 28



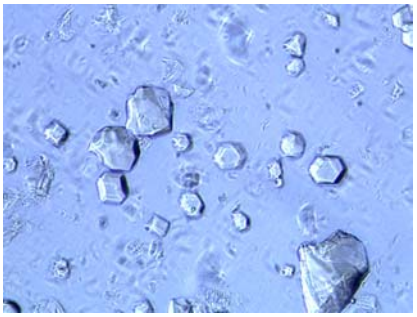
87-69-3 Day 0



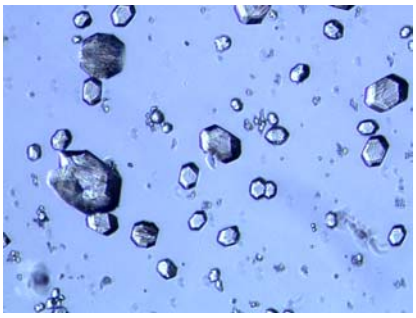
87-69-3 Day 28



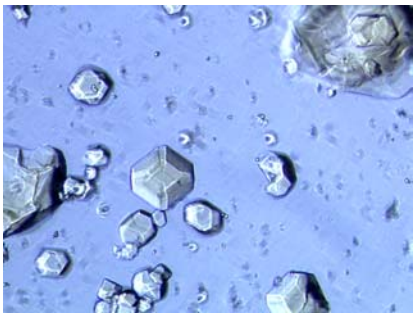
87-61-A Day 0



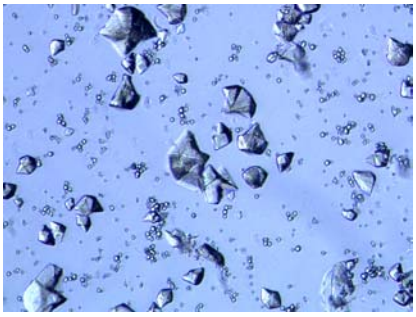
87-61-A Day 28



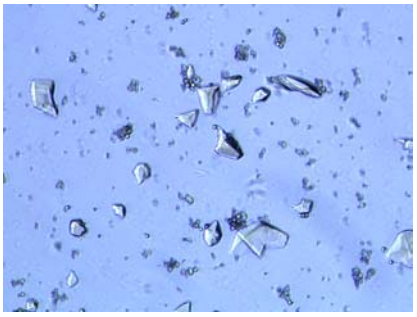
87-61-B Day 0



87-61-B Day 28



87-61-C Day 0



87-61-C Day 28

fold more active than the current commercial form of the immobilized.

Recommendations for Future Work

The most pressing issue is the source of activity loss. To determine the source of this loss, we will perform two experiments; determination of stability in the presence of an inert support and column studies using an inert solid diluent. Analysis of the results of these experiments will tell us whether the loss of activity is due to agglomeration. If the determination is that the agglomeration is the dominant source of activity loss, we will re-evaluate the economics in the diluted GI-CLEC column process. If the activity loss is due to denaturation, the investigation of cross-linkers which form more rugged covalent linkages such as alkylating agents or acylation agents.