July 1, 1993
LCMR Final Status Report -- Summary --Research
LCMR WORK PROGRAM 1991

I. **Synthesis of Biodegradable Plastics in Microbial and Crop Plant Systems -**

Program Manager: Friedrich Srienc
BioProcess Technology Institute and Dept.Chem.Eng. & Matl.Sci.
University of Minnesota
240 Gortner Laboratory
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St. Paul, MN 55108
(612) 624-9776

A. M.L. 91 Ch 254 Art.1 Sec. 14 Subd: 13c Appropriation: $150,000
Balance: $ 0

Biodegradable Plastics-Microbial and Crop Plant Systems: This appropriation is to the commissioner of administration for a grant to the University of Minnesota, Department of Agronomy and Plant Genetics, to genetically engineer yeast and crop plants to produce low-cost polyhydroxybutyric acid, a biodegradable plastic, to substitute for petroleum-based plastics.

B. Compatible Data: NA

C. Match Requirement: None

II. Narrative:

Polyhydroxybutyric acid (PHB) is a commercially valuable, biodegradable plastic-like material that could substitute for petroleum-based plastics. PHB is produced by certain bacteria from which the genes for the PHB synthesis enzymes have been isolated. This proposal is for transferring PHB synthesis genes by genetic engineering into yeast and crop plants to determine if PHB can be produced more cheaply than fermentation of PHB-producing bacteria. Low cost production of PHB will facilitate its substitution for petroleum-based plastics thereby reducing both dependence on petroleum as a substrate for plastics manufacture and solid waste disposal problems associated with plastics. Production of PHB in yeast will provide alternate utilization of agricultural products such as molasses. Engineering PHB production into crops, such as oilseed rape, would provide an alternative cropping system and commodity for Minnesota producers.

III. Objectives

A. **Modify genes for PHB synthesis enzymes for expression and transfer to yeast**

A.1 Narrative: PHB is synthesized in three enzymatic steps from acetyl-coenzyme A. Genes for the enzymes, ß-ketothiolase, acetoacetyl-CoA reductase and PHB polymerase have been isolated from *Alcaligenes eutrophus* and are available to us. The *Alcaligenes* genes for PHB synthesis have been expressed in *E. coli* and confer PHB production to that bacteria. However, the genes have not been expressed in a eukaryotic system. Yeast (*Saccharomyces cerevisiae*), a eukaryote, will be used initially to determine if eukaryotes are capable of PHB synthesis because it is easily transformed, grows rapidly and is genetically well characterized.

In yeast, acetyl CoA is a substrate for fatty acid biosynthesis which takes place in the cytosol. Furthermore, an isozyme of the yeast enzyme acetoacetyl-CoA thiolase, which catalyzes the same reaction as ß-ketothiolase, also is localized in the cytosol. Therefore, only the genes for the last two steps of the PHB synthesis pathway must be transferred to yeast to engineer PHB production. To accomplish this these genes will be combined with regulatory sequences for appropriate expression in the yeast cytoplasm and incorporated into the yeast genome.

A.2 Procedures: The yeast GAL 10 promoter will be fused to the structural genes for acetoacetyl-CoA reductase and PHB polymerase. The GAL 10 promoter is induced by feeding galactose to yeast strains lacking glucose catabolite repression allowing control of expression levels of the PHB synthesis enzymes which may be required if PHB production is deleterious or lethal to yeast.

The PHB synthesis genes will be separately incorporated into homologous recombination plasmids developed for yeast transformation. Homologous recombination will place the introduced genes into known chromosomal locations conferring gene stability and control of gene copy number. The transformation constructs will be introduced separately into two different haploid yeast strains of opposite mating type using the lithium acetate transformation method. Following confirmation of stable gene transfer, the transgenic yeast strains will be crossed to combine genes for the entire PHB synthesis pathway comprised of yeast acetoacetyl-CoA thiolase, and *Alcaligenes* acetoacetyl-CoA reductase and PHB polymerase.

A.3 Budget:

		LCMR Funds
a.	Amount Budgeted	37,500
b.	Balance	0

2. Major Cooperator:

David A. Somers
Associate Professor
Department of Agronomy and Plant Genetics
University of Minnesota

Ph.D. Agronomy

D. Somers's research emphasizes development of cell and tissue cultures and gene transfer technologies for genetic manipulation of crop plants such as corn, oats and soybeans. He has support from the Midwest Plant Biotechnology Consortium and in the past from the Minnesota Soybean Research and Promotion Council to conduct transformation research in these crops.

Selected publications related to proposal:

Parker W.B., Marshall L.C., Burton J.D., Somers D.A., Wyse D.L., Gronwald J.W., Gengenbach B.G.; "Dominant mutations causing alterations in acetyl coenzyme A carboxylase confer tolerance to cyclohexanedione and aryloxyphenoxypropionate herbicides in maize." Proc.Natl.Acad.Sci. USA 87:xx-xx (1990) in press

Somers D.A., Gengenbach B.G.; "Potential for transformation of barley." Proc. Symp. on Biotic Stress of Barley. Montana State Univ. and Intl. Center for Agric. Res.; July 31-Aug.2 1990; Big Sky, Montana.

Kaeppler H.F., Somers D.A., Rines H.W., Cockburn A.F., "A rapid, simple method for introduction of foreign DNA into plant cells." Agronomy Abstracts, in press (1990)

Delzer B.W., Somers D.A., Orf J.H., "*Agrobacterium tumefaciens* susceptibility and plant regeneration of ten soybean genotypes in maturity groups 00 to 11." Crop.Sci. 30:320-322 (1990)

Phillips R.L., Somers D.A., Hibberd K.A., "Cell/tissue culture and in vitro manipulation." In: Corn and Corn Improvement.(G.F. Sprague ed.), Amer. Soc. Agronom., pp 345-387, (1988)

Presentations acknowledging support by the state appropriation recommended by LCMR:

F. Srienc, "Plastics that really are biodegradable", presentation given to the members of the local section of the American Institute of Chemical Engineers (AIChE), Minneapolis, January 16, 1992

Peterson, M.S. and F. Srienc, "Introduction of a bacterial biosynthetic pathway in *Saccharomyces cerevisiae*", Annual Meeting of the American Institute of Chemical Engineers, Miami Beach, FL, Nov. 1-6, 1992

A.C. Eschenlauer, F Srienc, and D.A. Somers, "Genetic Engineering of Polyhydroxybutyrate Production in Maize Tissue Cultures", American Society of Agronomy Annual Meeting, Minneapolis, MN, November, 1992.

D.E.Jackson, M.Peterson, T.Leaf, and F.Srienc, "Physiology of PHA synthesis in prokaryotic and eukaryotic cells", Engineering Foundation Conference: Biochemical Engineering VIII, Princeton, NJ, July 11-16, 1993.

F.Srienc and D. Somers, "Biodegradable Plastics from Yeast and Plant Cells" Energy from Biomass Workshop, Chicago O'Hare Airport, July 28, 1993

M.Narrol, J.J.Hahn, A.C.Eschenlauer, D.Somers and F.Srienc, "Controlled Suspension Cultures of Wild Type and Recombinant Black Mexican Sweet Corn Cells". Annual Meeting of the American Institute of Chemical Engineers, St. Louis, MO, Nov. 7-12, 1993. (to be presented)

VII. Reporting requirements

Semiannual status reports will be submitted not later than Jan. 1, 1992, July 1, 1992, Jan. 1, 1993 and a final status report by June 30, 1993.

1991 RESEARCH PROJECT ABSTRACT
FOR THE PERIOD ENDING JUNE 30, 1993
Funding for this project approved by the MInnesota Legislature (M.L. 91 Ch 254 Art.1 Sec. 14 Subd. 13c) as recommended by the Legislative Commission on Minnesota Resources from the Oil Overcharge Money.

TITLE:	Synthesis of Biodegradable Plastics in Microbial and Crop Plant Systems
PROGRAM MANAGER:	Dr. Friedrich Srienc
ORGANIZATION:	University of Minnesota
LEGAL CITATION:	M.L. 1991, Ch 254, Art.1, Sec. 14, Subd. 13c
APPROP.AMOUNT:	$ 150,000.-

STATEMENT OF OBJECTIVES

To be able to produce the biodegradable plastic with yeast and plant cells the research focussed on (i) modification of bacterial genes for PHB synthesis and introduction into yeast cells, (ii) development of analytical methods for investigating PHB metabolism and expression studies in yeast, (iii) modification and transfer of bacterial PHB synthesis genes into corn cells, (iv) study of conditions of PHB synthesis in corn cells.

RESULTS

A set of yeast plasmids has been constructed containing the bacterial gene cluster or combinations of the three bacterial genes responsible for PHB synthesis in bacteria. The genes have been placed under the control of yeast promoters so that they can be expressed in yeast. Individual plasmids containing the PHB synthesis genes and combinations of these vectors have been introduced into the yeast *Saccharomyces cerevisiae*. Similar gene constructs have been introduced by particle bombardment into Black Mexican Sweet Corn (BMS) tissue culture cells. Analytical methods including gas chromatography, mass spectrometry, HPLC, flow cytometry, enzyme assays and western blotting procedures have been developed to probe for functional expression of the PHB synthesis enzymes in yeast and corn cells. The expression studies with yeast cells have shown that transformed cells are able to functionally synthesise high levels of the bacterial acetoacetyl- reductase, one of the key enzymes in PHB synthesis. It was found that untransformed yeast cells are able to synthesise small amounts of PHB. The PHB level was not increased, however, after introduction of the particular gene constructs. Out of fifty transformed corn cell lines seven were found to be able to synthesise small but significantly elevated levels of PHB.

PROJECT RESULTS USE AND DISSEMINATION

Dr. F. Srienc gave presentations on the project topic to the local section of the American Institute of Chemical Engineers (AIChE) in Minneapolis, January 16, 1992 and at the workshop "Energy from Biomass" held in Chicago in July 1993. Dr. Art Eschenlauer and Dr.Somers gave a presentation on PHB production in maize tissue cultures at the Annual Meeting of the American Agronomy Society in November 1992. Dr. M. Peterson presented the results of the yeast work at the Annual AIChE Meeting in Miami Beach, FL, in November 1992. Furthermore the work has been presented at the recent Engineering Foundation Conference in Princeton, NJ. An abstract has been accepted for presentation at the Annual AIChE Meeting in November, 1993. Matt Narrol is in the process of completing a Masters thesis entitled "A Comparative Growth Study of Controlled Suspension Cultures of Wild Type and Recombinant Black Mexican Sweet Corn Cells".
The results and matching funds of this project have substantially contributed to the attraction of a two year grant ($140,000) funded by the Midwest Plant Biotechnology Consortium.

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A.3 Budget:

		LCMR Funds
a.	Amount Budgeted	37,500
b.	Balance	0

A.4 Timeline for Products:

	July 91	Jan 92	June 92	Jan 93	Oct 93
Complete gene construction					
Gene transfer					

A.5 Status: A set of yeast plasmids has been constructed carrying the bacterial PHB synthesis genes under different configurations. The work has focussed primarily on the polymerase and the reductase genes as *S. cerevisiae* has a native β- ketothiolase. The bacterial genes have been placed under the control of the yeast GAL1 and/or GAL10/CYC promoters. The gene constructs are located on (i) integrating yeast vectors, (ii) vectors with a centromere element, and (iii) on vectors containing the 2μ origin of replication in order to be able to control gene copy number. The individual vectors and vector combinations have been transformed into yeast cells for expression studies.
It has been demonstrated that (i) the bacterial reductase can be functionally expressed in yeast cells, (ii) that wild type yeast cells are able to synthesise small amounts of PHB. The functional expression of the polymerase has not yet been proven. Current work focusses on overexpressing the combination of reductase and polymerase genes with the anticipation to observe significantly increased PHB levels in yeast.

A list of constructed vectors is given in the table below. The plasmids have been constructed on the basis of yeast vectors obtained from Phil Hieter and L.Guarente. The bacterial PHB gene cluster has been obtained from A. Sinskey. The constucts can be cassified into several goups. Some plasmids can be maintained only in E.coli. These plasmids represent either intermediate constructs or constructs that have been made to isolate fusion proteins for antibody production. The yeast plasmids are either integrating, single copy, or multi copy vectors, depending on the type of ARS and CEN element present. In addition, these plasmids carry the bacterial PHB genes either as the entire gene cluster or as a combination of individual genes under the control of the yeast GAL1 or the GAL10-CYC1 promoter region. In addition the plasmids contain different selection markers that are useful for isolating desired transformants. For expression studies the PHB genes have been transformed into *S. cerevisiae* strains with or without catabolite repression by glucose. The latter strain permits activation of the galactose inducible promoter in the presence of glucose.

PHB Project - Plasmids Available 7/93

Following is a summary of plasmids that are available for study of PHB in in E. coli or yeast. Some plasmids are vectors while others contain various combinations of the three PHB genes.
The listing is arranged according to which PHB genes are carried on the plasmid.
Labelling of the PHB genes follows the convention: C = polymerase, A = ketothiolase, B = reductase.

PHB genes	plasmid name	plasmid type	yeast promoter	yeast selection marker	comments
-	pRS167	YCp			yeast shuttle vector
-	pRS169	YCp	GAL1	URA3	yeast shuttle vector
-	pRS303	Yip	LACZ	HIS3	yeast shuttle vector
-	pRS304	Yip			yeast shuttle vector
-	pRS305	Yip			yeast shuttle vector
-	pRS306	Yip	LACZ	URA3	yeast shuttle vector
-	pMP33-5	Yip	GAL1	URA3	yeast shuttle vector; pRS306/PvuI + pRS169/PvuI
-	pMP40-7	Yip	GAL1	HIS3	yeast shuttle vector; pRS303/PvuI + pRS169/PvuI
-	pMP61-8	Yip	GAL10	LEU2, URA3	shuttle; pRS305/HindIII/BamHI + pLGSD5/HindIII/BamHI
-	pMP62-2	Yip	GAL10	URA3, URA3	shuttle; pRS306/HindIII/BamHI + pLGSD5/HindIII/BamHI
-	pMP71-3	Yip	GAL1	LEU2	yeast shuttle vector; pRS305/PvuI + pRS169/PvuI
-	pLGSD5	2 micron	GAL10	URA3	2 micron plasmid, lacZ gene
CAB	pAeT41	E. coli	-	-	pUC18 containing PHB gene cluster
CAB	pMP32-1	E. coli	-	-	pAeT41: remove BamHI-BstBI, blunt-end, ligate
CAB	pMP63-1	E. coli	-	-	pMP32-1/BamHI, blunt end, ligate (to destroy BamHI site)
CAB	pMP73	E. coli	GAL10	URA3	pLGSD5/BamHI/HindIII + pMP32-1/BamHI/HindIII
CAB	pMP50-1	E. coli	-	-	GST fusion pGEX3X/SmaI/EcoRI + pAeT41/EcoRV/EcoRI
CAB	pMP13-4	YCp	GAL1	URA3	pRS169/HindIII/EcoRI + pAeT41/HindIII/EcoRI
CAB	pMP34-1	YCp	GAL1	URA3	pRS169/HindIII/EcoRI + pMP32-1/HindIII/EcoRI
CAB	pMP37-11	YCP	GAL1	URA3	remove XhoI-XbaI from pMP34-1, to shorten 5' UTR
CAB	pMP57-5	Yip	GAL1	HIS3	pMP40-7/EcoRI/XhoI + pMP34-1/EcoRI/XhoI
CAB	pMP64-6	Yip	GAL1	HIS3	pMP57-5/XhoI/XbaI, to shorten 5' untranslated region
PHB genes	plasmid name	plasmid type	yeast promoter	yeast selection marker	comments
AB	pMP52-2	E. coli	-	-	pAeT41/PstI/AflII + pAeT41/BamHI/AflII
AB	pMP48-6	E. coli	-	-	GST fusion pGEX3X/SmaI/EcoRI + pAeT41/PmlI/EcoRI
AB	pMP28-5	YCp	GAL1	URA3	pMP13-4: remove polymerase gene StuI-XhoI
CB	pMP55-1	E. coli	-	-	GST fusion, remove StuI-StuI from pMP50-1
C	pMP69-1	E. coli	-	-	pMP32-1: remove thiolase and reductase AflII-StuI
C	pMP76	E. coli	GAL10	URA3	pLGSD5/BamHI/HindIII + pMP69-1/BamHI/HindIII
C	pTL77-1	E. coli	GAL10	URA3	pMP76: remove BamHI-NotI forming in-frame fusion
C	pMP65-1	Yip	GAL1	HIS3	pMP57-5: remove thiolase and reductase AflII-StuI
C	pMP70-3	2 micron	GAL1	HIS3	pLGSD5/EcoRI + pMP65-1/EcoRI (2 micron from pLGSD5)
A	pTL81-6	E.coli	-	-	pTL75-2/AflII/partial XcmI to remove reductase gene
A	pTL83-1	YCp	GAL1	URA3	pTL81-6/HindIII/EcoRI + pRS169/HindIII/EcoRI
B	pMP38-7	E. coli	-	-	pAeT41: remove polymerase and thiolase BamHI-XcmI
B	pMP53-6	E. coli	-	-	pAeT41/DraII, only a portion of reductase remaining
B	pMP58-3	E. coli	-	-	pGEX3X/SmaI/EcoRI + pMP53-6/DraII/EcoRI
B	pMP39-1	YCp	GAL1	URA3	pRS169/HindIII/EcoRI + pMP38-7/HindIII/EcoRI
B	pMP41-1	YCp	GAL1	URA3	pMP39-1: remove XhoI-XbaI from 5' untranslated region
B	pMP43-6	Yip	GAL1	URA3	pMP33-5/HindIII/EcoRI + pMP38-7/HindIII/EcoRI
B	pMP51-3	Yip	GAL1	URA3	pMP43-6: remove XhoI-XbaI from 5' untranslated region
B	pMP45-4	2 micron	GAL1	URA3	pLGSD5/EcoRI + pMP43-6/EcoRI (2 micron from pLGSD5)

A.6 Benefits: Construction of the bacterial PHB synthesis genes for proper expression and localization in yeast will enable us to investigate PHB synthesis in a eukaryotic cell system.

B. **Investigate expression of PHB synthesis enzymes in yeast.**

B.1 Narrative: Methods to detect expression of PHB synthesis enzymes and production of PHB is essential for optimization of PHB synthesis in eukaryotic systems. Antisera will be produced to specifically detect each PHB synthesis enzyme and PHB itself.

B.2 Procedures: Isolated genes of the three synthesis enzymes will be inserted into a transcription/translocation system (pGEX plasmid available from Pharmacia) creating a fusion protein with glutathione-S-transferase and each enzyme. The fusion plasmid will be transferred into E. coli, and the fusion protein will be purified according to the manufacturers recommendation using a glutathione affinity column. Following cleavage of the glutathione-S-transferase molecule from the PHB synthesis enzymes, the purified proteins will be used to immunize chickens for antiserum production. Oligomers of chemically cleaved PHB will be covalently attached to keyhole limpet hemocyanin and used to immunize chickens.
Resulting antisera will be used in western blots and immunofluorescence to detect the expression of the PHB enzyme in transgenic yeast cells. PHB production will be detected using ELISA and flow cytometry.

B.3 Budget:

		LCMR Funds
a.	Amount Budgeted	37,500
b.	Balance	0

B.4 Timeline for Products

	July 91	Jan 92	June 92	Jan 93	Oct 93
Clone synthesis enzyme genes into pGEX		———	———		
Develop and characterize specific antibodies		———	———		
Develop PHB antibodies and develop PHB quantitative immunoassays		———	———		
Investigate enzyme expression and PHB synthesis in transgenic yeast cells			———	———	———

B.5 Status: In order to obtain antibodies against enzymes involved in PHB synthesis we have constructed glutathione reductase fusion proteins with carboxy terminal portions of the bacterial PHB synthesis enzymes β-ketothiolase, reductase and polymerase. The proteins have been expressed in *E.coli* and affinity purification has been applied to obtain pure protein fragments. These have been injected into chickens and antibodies have been prepared from eggs. The expression and isolation of the fusion protein with the reductase protein fragment was not possible as the protein apparently was degraded in *E.coli*. We have isolated the β-ketothiolase and polymerase proteins and obtained polyclonal antibodies of high affinity

against β-ketothiolase and of lower specificity against the polymerase. In addition, enzyme assays have been developed to probe the PHB synthesis enzymes. GC and NMR procedures have been adapted to probe for PHB in cellular material. Furthermore a staining procedure has been developed to analyze PHB in single cells using flow cytometry. The developed procedures are crucial for studying the physiology of PHB formation in transgenic organisms. To date significantly elevated PHB levels in yeast have not yet been detected. Work is underway to overexpress the polymerase and also the β-ketothiolase. The established basis of gene constructs and analytical methodology promises a fast progress of the work towards the initial goal.
A significant amount of effort has been invested into developing the analytical tools for studying PHB synthesis in organisms. A brief description of the different types and the rational for developing the analytical procedures is given below.
Gas Chromatography (GC) The quantity of PHB in a sample can be determined gravimetrically after separation of the polymer from the biomass. However, for small amounts of polymer the most accurate method of quantitation is gas chromatography. The polymer can be extracted from microbial or plant tissue with organic solvents or other chemical treatments. The polymer is hydrolyzed to the monomers which are then reacted to form the propyl esters. The esters are volatile, thus they can be separated from other components on a gas chromatography system. Benzoic acid is used as an internal standard. Currently, a Hewlett-Packard 5890A GC is used with an automatic injector. The column is a fused-silica capillary column containing DB-WAX 30W (J & W Scientific). Very small quantities of PHB can be detected with this method (see section C).
Gas Chromatography - Mass Spectroscopy (GC-MS) GC analysis of tissue extracts shows many different peaks. When only trace amounts of PHB are present, GC-MS is used to confirm that the peak is due to the propyl ester of b-hydroxybutyrate. This technique gives the molecular weight of fragments of the compound. Since the structure is known, the size of possible fragments can be determined and compared to those indicated by the GC-MS.
Nuclear Magnetic Resonance (NMR) Spectroscopy Bacterial systems have been shown to synthesize PHAs containing two or more types of monomers, one of them usually b-hydroxybutyrate. These types of polymers are known as copolymers. ^{1}H- and ^{13}C-NMR are used to determine the structures of the monomers and to calculate the ratio of the different monomers. This gives us information on how the carbon sources are metabolized and channeled into PHA by the organisms. ^{13}C-NMR can also be used to determine the sequence distribution of a copolymer. This is necessary to determine if the different types of monomers are randomly mixed or are divided into distinct regions within a polymer chain.
Flow Cytometry The analytical methods discussed above are used to confirm PHB production, to quantitate the PHB in a mass of tissue, and to determine the structures of other monomer types. All of these techniques can give only population average values to characterize the polymer produced by a culture of organisms. A distribution of properties in a population can be determined with flow cytometry. This instrument measures several different parameters of a large number of cells one cell at a time. We have developed a staining procedure based on Nile red that permits to fluorescently label PHB in individual cells. Furthermore, the enzymes involved in PHB synthesis can be quantified using immunofluorescent labelling techniques based on the developed antibodies. We have shown

that this methods can be readily applied to *Alcaligenes eutrophus* as well as to *E.coli*. We anticipate that it will be also useful for evaluating PHB synthesis in the transgenic eukaryotic cells.

High Performance Liquid Chromatography (HPLC) To determine at what rate the various carbon sources are utilized by the microorganisms and at what fraction of these carbon sources are used for polymer production, the carbon source concentrations are measured with HPLC. Organic acids and some sugars are separable on an Aminex A7 column. This column is used in a Hewlett-Packard 1082B Liquid Chromatograph equipped with refractive index and ultraviolet light detectors.

B.6 Benefits: Using immunological approaches, we will be able to determine if the PHB synthesis enzymes are properly expressed in transgenic yeast cells and to determine the rate of PHB synthesis. This will allow us to define conditions for optimal PHB synthesis in yeast.

C. **Modify PHB synthesis genes for expression in and tranfer to plant cells.**

C.1 Narrative: Acetyl-CoA is a substrate for plant fatty acid synthesis which takes place in chloroplasts in leaves and in plastids in nongreen tissues such as developing oilseed embyros. Our goal is to engineer maize plants to produce PHB in the seed by utilizing acetyl-CoA derived from sucrose that normally would be incorporated into starch during seed biosynthesis. However, before this can be accomplished, it must be demonstrated that PHB can be produced by maize cells.

To determine if PHB can be produced by maize cells, the three *Alcaligenes* PHB synthesis genes must be combined with promoter sequences that will provide inducible regulation.

C.2. Procedures: To determine if PHB will be synthesized in genetically engineered plant cells, the three synthesis genes will be fused with the cauliflower mosaic virus 35S promoter. The promoter is constitutive in all plant species whereas transit peptides, if included, will direct PHB synthesis gene products into the chloroplasts. The PHB gene constructs will be fused with 3' nopaline synthase sequences to confer stability to the transgene mRNAs.

In initial experiments to test the gene constructs and the function of the transgenes, it will be most efficient to transfer the genes into a model plant tissue culture system that is readily transformed such as tobacco maize tissue culture. The PHB synthesis genes will be integrated into transformation plasmids such as pBI221 designed for transformation which in turn will be transferred into maize tissue culture by means of particle acceleration. Transformed maize callus tissue and eventually shoots will be isolated by selection on medium containing kanamycin.

The first experimental configuration will be to introduce each synthesis gene individually into a maize transformant to determine that the PHB enzymes are expressed and correctly compartmentalized in the transgenic cells. Antiserum developed against the enzymes (section B) will be used in Western blots to detect transgene products. We will evaluate transgenic callus tissue growth rate to determine the effects of each transgene on tissue growth, if PHB is produced, and the effect of PHB synthesis on tissue growth.

To combine the three *Alcaligenes* PHB synthesis genes into a single genome a number of strategies may be employed. We plan to construct an additional transformation plasmid that carries both the B-ketothiolase and acetoacetyl-CoA reductase genes into the plant genome in one step. Plants regenerated from transformed callus carrying both genes will be crossed with regenerated plants transformed with the PHB polymerase gene. This hybrid seed will then carry all three PHB synthesis genes. investigated 1) if PHB is produced and 2) the effects of PHB synthesis on plant growth and development.

C.3. BUDGET

a.	Amount budgeted	37,500
b.	Balance	0

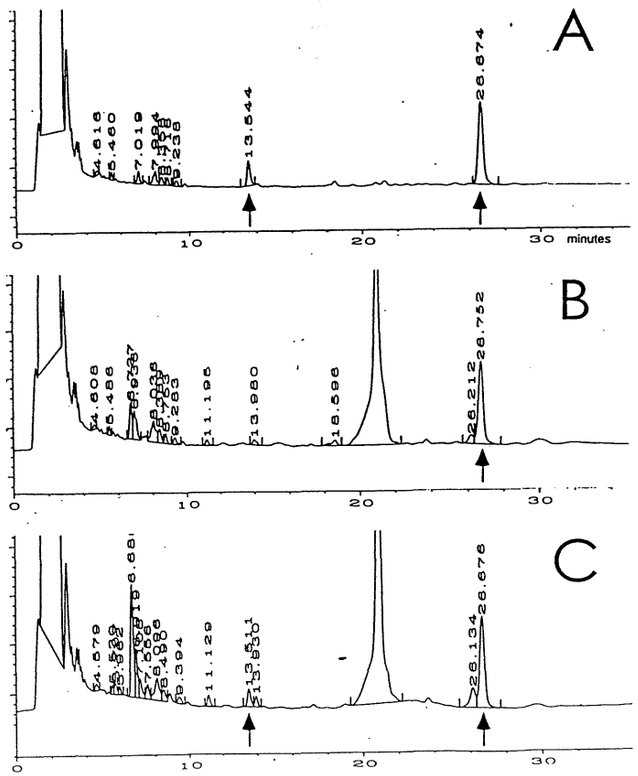
C.4. TIMELINE

	July91	Jan92	July92	Jan93Oct 93
Gene construction and transfer to tobacco		———		
Investigations of gene expression			———	
Complementation of PHB pathway by crossing and investigation of PHB synthesis				———

C.5. Status: Chris Somerville has kindly provided the gene constructs he used to demonstrate PHB synthesis in *Arabidopsis*. In our work, Black Mexican Sweet maize tissue culture cells (BMS) have been transformed with the β-ketothiolase, acetoacetyl-CoA reductase, and PHB polymerase genes via microprojectile bombardment and several transformed cell culture lines were established.
Reductase activity was measured in genetically engineered callus cultures and elevated reductase levels were observed in some cell lines. Elevated ketothiolase levels have been found in callus of ketothiolase transformed maize

Maize cells transformed with all three PHB synthesis genes, were analyzed to confirm the presence of the genes; about half of the selected calli have stably integrated the genes. Fifty genetically engineered tissue cultures were analyzed using gas chromatography (GC) to determine whether they produce PHB. To date, seven of these genetically engineered tissue cultures have been shown to be producing small but significant amounts of PHB. Investigations of these engineered tissue cultures will permit more precise conclusions to be drawn about the interdependence at the cellular level of metabolism and PHB production than would be possible with intact plants. Since the inputs to the PHB pathway are central to the energetics (acetyl-CoA) and biosynthetic activities (reduced hydrogen) of the cell, we will evaluate the optimal balance between utilization of the inputs in PHB synthesis and utilization in other activities in the plant cell. We can then use this information to design more effective whole-plant transformation experiments.Additional gene construction work for generating maize specific expression vectors with the PHB genes is underway. Culture conditions for controlled cultivation of maize cells in suspension have been established.

The key result obtained in this research part is the demonstration that corn cells indeed are able to synthesise PHB when equipped with the appropriate genes. The included Figure below shows the GC data which reveal PHB synthesis. The nature of the PHB peak has been confirmed with the previously described GC-mass spectrometry method.



HPLC profiles of pure PHB (A) and of BMS maize tissue culture cells without (B) and with (C) the genes for PHB synthesis. Plant cell material was acetone-dried, subjected to acid propanolysis, and passed over a DBWax 0.5 µm, 0.32 mm ID, 30 m column (J & W Scientific). Benzoic acid was added to the propanolysis reaction as an internal standard, and is seen in the profiles as a peak with a retention time of about 26.7 minutes. The peak corresponding to PHB has a retention time of about 13.5 minutes and is visible in profiles A and C but not in B (untransformed BMS).

C.6. Benefits: Determination of requirements for PHB synthesis in plant cells is critical to sucessfully genetically engineer crop plants to produce PHB in seed.

D. **Synthesis of PHB in maize seed.**

D.1. Narrative: PHB will likely be most economically produce in and extracted from crop seed. PHB synthesis will be engineered to compete for starch biosythesis substrates in maize seed in which starch biosynthesis is extremely rapid during seed development. Maize is the crop that is currently most amenable to transformation. The value-added production of PHB in maize in addition to starch, oil and protein meal production will likely improve the returns for this crop for Minnesota producers.

D.2. Procedures: For expression of PHB synthesis genes in maize seed a promoter sequence that is embyro-specific must be combined with the structural gene sequences described in C.2. Promoter sequences of the maize storage protein zein genes confer embryo-specific expression in transformed maize and would be expected to function properly in maize seeds. This promoter will be combined with the PHB synthesis structural genes.

The PHB gene constructs will be incorporated into regenerable maize tissue culture cells by particle acceleration. Regenerated transformed plants will be self-pollinated to derive lines that are homozygous for the different transgenes. Southern blots and immunological methods will be used to confirm stable transformation and expression to the transgenes. Seed production will be evaluated to determine the effects of the transgene products on seed and seedling viability. Because the zein promoters are expressed only in developing embryos, the presence of the transgenes should have no effect on plant growth and development.

Plants that are homozygous for the transgene carrying B- ketothiolase and acetoacetyl-CoA reductase will be crossed to plants homozygous for the PHB polymerase gene to transfer genes for the entire pathway into hybrid seed. PHB production and seed development in hybrid oilseed rape will be investigated in detail.

D.3. Budget

a. amount budgeted: 37,500
b. balance: 0

D.4. Timeline

	July91	Jan92	July92	Jan93Oct 93
Develop gene constructs	_____			

Transformation _____

Development of correct genetic materials _____

Investigation of PHB synthesis in hybrid seed _____

D.5. Status: A 10 liter controlled bioreactor has been set up in order to study how PHB production affects the metabolism of maize cells cultivated in suspension. Conditions have been determined for vigorous growth of untransformed BMS. Growth rate and utilization of sucrose and nitrogen have been characterized together with the uptake rates of oxygen and evolution rates of carbon dioxide. The data permit to evaluate the conditions of PHB formation as a function of cellular background and culture conditions. The cell lines that have shown elevated PHB levels are currently evaluated in the biorector and compared to untransformed cell lines. We anticipate to obtain a significant amount of information on the altered cell physiology in transgenic cells from the reactor studies before we will attempt to regenerate plants.
We have modified a microbial bioreactor to be suitable for plant cell cultivation. The modifications included addition of a draft tube so that the reactor can be operated as an airlift reactor with marine type impeller assisted circulation of the growth medium. The reactor is interfaced with a computer for data acquisition and with a mass spectrometer for on-line determinatioin of the rate of oxygen consumption and carbon dioxide production. Several growth sudies have been carried out with wild type BMS cell cultures to establish the optimum operating conditions. The reactor set up has the advantage that large amount of cell material can be harvested for determination of the parameters that are associated with PHB synthesis. These include the PHB levels, the concentrations of nutrients, the levels of PHB synthesis enzymes and the information of gas utilization rates which permit to accurately balance the reaction which forms PHB. Currently the transgenic corn cells that produce PHB are being investigated in the reactor and compared to the wild type cell cultures.

D.6. Benefits: This experiment will demonstrate the feasibility of PHB production in crop plant seed. It is likely that refinements of the promoters and transformation strategies will be required to develop maize varieties that produce optimum levels of PHB. Therefore, plants resulting from this experiment will be used a germplasm for development of PHB producing varieties.

IV. Evaluation

For the FY92-93 biennium the program can be evaluated by its ability to: (1) carry out the appropriate gene constructions for expression in yeast and plant cells; (2) transform [illegible] gene constructs into yeast and plant systems; (3) develop the analytical

methodology for detection of enzyme levels and PHB in yeast and plant cells; (4) obtain functional enzymes that catalyze synthesis of PHB; (4) provide information as to what conditions are optimal for PHB synthesis; (5) develop strategies for constructing crop plants that synthesise PHB and ultimately obtain crop plants that synthesise PHB.

In the long-term, evaluation of this project's success will be the development of yeast and plant systems that will produce inexpensive PHB on the basis of the resources available to Minnesota. PHB might replace many plastics that are currently produced by the petrochemical sources. Increased application of PHB will protect our environment because it is a biodegradable plastic. In addition, the success of this project can be measured by a demonstrated record of attracting additional funds to pursue scientific questions related to and evolving from the project.

V. Context

Poly-β-hydroxybutyric acid (PHB) is a bacterial storage material that is accumulated intracellularly under specific growth conditions. The material has gained significant attention over the past decade because of its commercial value as biodegradable plastic material. Because of its properties it could substitute in many applications the use of conventional plastics that currently are not biodegradable and that significantly contribute to the pollution of our environment. The bacterially produced polymer is however still too expensive to efficiently compete with polymers derived from petrochemical sources. Following reasons contribute to the high production costs of this polymer: (a) the production technology is limited to bacterial systems that utilize expensive sugars as raw material, (b) the extraction and purification technology of the polymer needs further development, (c) the properties of the polymer and their specific modifications are not fully understood yet.

A recent advancement in research relating to PHB was that the three genes that are responsible for PHB synthesis in an organism have been cloned. This opens up the possibility to use genetic engineering technology to modify organisms incapable of PHB synthesis to make this polymer. Prof. Anthony J. Sinskey from M.I.T. is heading one of these groups that have cloned the genes and he indicated that the genes are available to the principal investigators. In fact, discussions are underway to set up a collaboration.
Although, this grant has been placed into effect only October 7, 1991, the start of the research work related to this project has been significantly facilitated by a one year grant (starting February 1, 1991) awarded by the Agricultural Research Utilization Institute (AURI). A multidisciplinary research proposal related to the ongoing project has been submitted to the National Science Foundation in October 1991.
NSF was not able to provide funding, apparently due to lack of funds in the program. A proposal submitted to the Midwest Plant Biotechnology Consortium has been selected to be awarded. The award conditions are currently being finalized.

VI. Quantifications

1. Program Manager:

Friedrich Srienc
Associate Professor
Department of Chemical Engineering and Material Sciences
Institute for Advanced Studies in Biologigal Process Technology
University of Minnesota

Ph.D. Biotechnology

F. Srienc is very familiar with the PHB system since it was the topic of his Ph.D. thesis. The objective of this research was to genetically modify the bacterium Alcaligenes eutrophus to improve the strain for PHB synthesis. This bacterial strain is the source for the PHB genes that have been cloned. His current research focusses on the physiology and dynamics of growth of eukaryotic cell populations. His work is supported by two grants from the National Science Foundation. He has a joint appointment at the BPTI and the Dept. of Chem. Engineering and Materials Science.

Selected publications related to proposal:

F. Srienc, "Possibilities for genetic improvement of the production of poly-ß-hydroxybutyric acid (PHB) with *Alcaligenes eutrophus* H16." PhD thesis, Technical University Graz, Austria, 1980.

F. Srienc, B. Arnold, J.E. Bailey, "Characterization of intracellular accumulation of poly-ß-hydroxybutyrate (PHB) in individual cells of *Alcaligenes eutrophus* H16 by flow cytometry." Biotechnol. Bioeng. 26:982-987 (1984).

F. Srienc, J.L. Campbell, J.E. Bailey, "Analysis of unstable recombinant *Saccharomyces cerevisiae* population growth in selective medium." Biotechnol. Bioeng. 28:996-1006 (1986).

P.D. Eitzman, J.L. Hendrick, F. Srienc, "Quantitative immunofluorescence in single *Saccharomyces cerevisiae* cells." Cytometry 10:475-483 (1989).

P.D. Eitzman, F. Srienc, "Dynamics of activation of a galactose inducible promoter in *Saccharomyces cerevisiae*." J. Biotechnol. 21:63-82 (1991).

2. Major Cooperator:

David A. Somers
Associate Professor
Department of Agronomy and Plant Genetics
University of Minnesota

Ph.D. Agronomy

D. Somers's research emphasizes development of cell and tissue cultures and gene transfer technologies for genetic manipulation of crop plants such as corn, oats and soybeans. He has support from the Midwest Plant Biotechnology Consortium and in the past from the Minnesota Soybean Research and Promotion Council to conduct transformation research in these crops.

Selected publications related to proposal:

Parker W.B., Marshall L.C., Burton J.D., Somers D.A., Wyse D.L., Gronwald J.W., Gengenbach B.G.; "Dominant mutations causing alterations in acetyl coenzyme A carboxylase confer tolerance to cyclohexanedione and aryloxyphenoxypropionate herbicides in maize." Proc.Natl.Acad.Sci. USA 87:xx-xx (1990) in press

Somers D.A., Gengenbach B.G.; "Potential for transformation of barley." Proc. Symp. on Biotic Stress of Barley. Montana State Univ. and Intl. Center for Agric. Res.; July 31-Aug.2 1990; Big Sky, Montana.

Kaeppler H.F., Somers D.A., Rines H.W., Cockburn A.F., "A rapid, simple method for introduction of foreign DNA into plant cells." Agronomy Abstracts, in press (1990)

Delzer B.W., Somers D.A., Orf J.H., "*Agrobacterium tumefaciens* susceptibility and plant regeneration of ten soybean genotypes in maturity groups 00 to 11." Crop.Sci. 30:320-322 (1990)

Phillips R.L., Somers D.A., Hibberd K.A., "Cell/tissue culture and in vitro manipulation." In: Corn and Corn Improvement.(G.F. Sprague ed.), Amer. Soc. Agronom., pp 345-387, (1988)

Presentations acknowledging support by the state appropriation recommended by LCMR:

F. Srienc, "Plastics that really are biodegradable", presentation given to the members of the local section of the American Institute of Chemical Engineers (AIChE), Minneapolis, January 16, 1992

Peterson, M.S. and F. Srienc, "Introduction of a bacterial biosynthetic pathway in *Saccharomyces cerevisiae*", Annual Meeting of the American Institute of Chemical Engineers, Miami Beach, FL, Nov. 1-6, 1992

A.C. Eschenlauer, F Srienc, and D.A. Somers, "Genetic Engineering of Polyhydroxybutyrate Production in Maize Tissue Cultures", American Society of Agronomy Annual Meeting, Minneapolis, MN, November, 1992.

D.E.Jackson, M.Peterson, T.Leaf, and F.Srienc, "Physiology of PHA synthesis in prokaryotic and eukaryotic cells", Engineering Foundation Conference: Biochemical Engineering VIII, Princeton, NJ, July 11-16, 1993.

F.Srienc and D. Somers, "Biodegradable Plastics from Yeast and Plant Cells" Energy from Biomass Workshop, Chicago O'Hare Airport, July 28, 1993

M.Narrol, J.J.Hahn, A.C.Eschenlauer, D.Somers and F.Srienc, "Controlled Suspension Cultures of Wild Type and Recombinant Black Mexican Sweet Corn Cells". Annual Meeting of the American Institute of Chemical Engineers, St. Louis, MO, Nov. 7-12, 1993. (to be presented)

VII. Reporting requirements

Semiannual status reports will be submitted not later than Jan. 1, 1992, July 1, 1992, Jan. 1, 1993 and a final status report by June 30, 1993.