CENTRO DE INVESTIGACION Y DE ESTUDIOS AVANZADOS DEL INSTITUTO POLITECNICO NACIONAL

UNIDAD ZACATENCO

DEPARTAMENTO DE BIOTECNOLOGIA Y BIOINGENIERIA

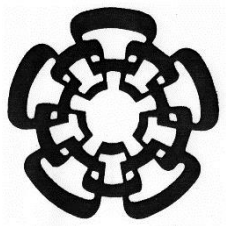
Clonación, expresión y localización de polihidroxialcanoato sintasa tipo II de *Pseudomonas putida* CA-3 en vesículas secretoras de *Saccharomyces cerevisiae* ATCC 9763

Tesis que presenta
GURUSAMY MUNIASAMY

Para obtener el grado de
DOCTORADO EN CIENCIAS
EN LA ESPECIALIDAD DE BIOTECNOLOGIA

Director de la tesis
Dr. FERMIN PEREZ GUEVARA

MEXICO, DISTRITO FEDERAL SEPTIEMBRE, 2014



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Cloning, expression and localization of polyhydroxyalkanoate synthase type II of *Pseudomonas putida* CA-3 onto secretory vesicles of *Saccharomyces cerevisiae* ATCC 9763

Thesis submitted by
GURUSAMY MUNIASAMY

For the degree of
DOCTOR OF SCIENCE
SPECIALIZATION IN BIOTECHNOLOGY

Thesis Director
Dr. FERMIN PEREZ GUEVARA

MEXICO, DISTRITO FEDERAL SEPTEMBER, 2014

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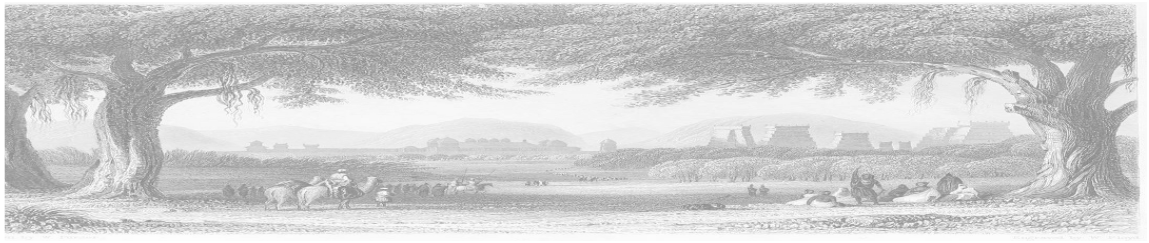

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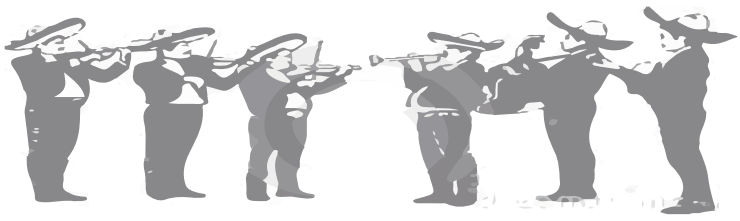

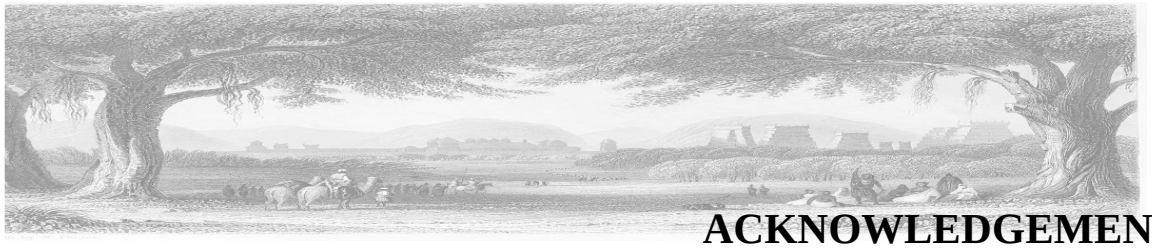

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"Beautiful Memories Last Your Whole Life Long"

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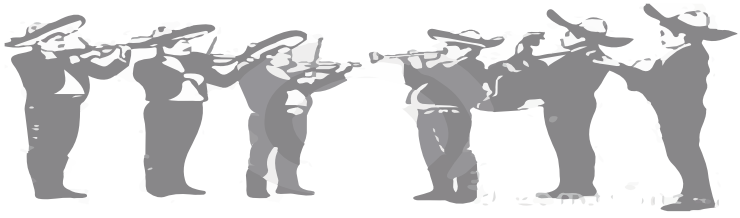

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Resumen

El objetivo del presente trabajo es demostrar que una proteína heteróloga citoplasmática puede ser inmovilizada funcionalmente en las vesículas de *S. cerevisiae* utilizando su proteína de transporte vesicular "v-SNARE Snc2". Para ello, se construyó un casete de fusión conteniendo el gene que codifica para la poly-3-hidroxialcanoato sintetasa (PhaC) de *Pseudomonas putida* CA-3, flanqueada por los dos dominios del gene Snc2p. El gene que codifica para la proteína verde fluorescente mejorada (EGFP) se incorporó en la región N-terminal del casete para permitir la localización de la proteína heteróloga cuando se expresa en *S. cerevisiae*. La localización de la proteína de fusión en las cellulas se confirmó por la marca de florescencia en la membrana citoplasmática empleando microscopía confocal. El propósito de inmovilizar la PHA sintetasa fue para producir y secretar el polímero producido mediante las vesículas secretorias de *S. cerevisiae*. Las transformantes que expresaron la PHB sintetasa quimérica fueron crecidas en medio con glucosa para estudiar la producción y secreción del polímero. Como fue planeado, la inmovilización llevó a la secreción de un polímero no-proteico en forma de nanopartículas de 4-8 nm (FESEM, TEM). La identificación química del polímero secretado está aún en proceso, sin embargo la composición preliminar de la estructura elemental del polímero obtenida a partir de EDX (analizador elemental) muestra la presencia de carbono, hidrógeno, oxígeno y nitrógeno.

Por lo tanto, este es el primer trabajo que muestra la inmovilización "*in vivo*" de una enzima bacteriana activa sobre la membrana de las vesículas secretorias de levaduras, resultando en la secreción de material polimérico no-proteico a través de su sistema vesicular.

Abstract

This work aims to show that a heterologous cytoplasmic protein can be embedded to vesicles using the naturally occurring vesicle-SNARE Snc2p, a transport protein of *S. cerevisiae*. A fusion cassette was constructed containing the gene encoding the bacterial poly-3-hydroxyalkanoate synthase (PhaC) of *Pseudomonas putida* CA-3, flanked by the two domains of Snc2p gene. The gene coding for the Enhanced Green Fluorescent protein (EGFP) was incorporated in the N-terminal of the former cassette to permit the localization of the heterologous protein when expressed in *S. cerevisiae*. The localization of the fusion protein in yeast cells was confirmed by the fluorescence labelling the plasma membrane using confocal microscopy. The purpose of immobilizing PHA synthase was to produce and secrete a polymer through the hydrophobic system across the cell membrane. Then, the transformant expressing PHA synthase fusion protein was grown in glucose containing medium to study the production and secretion of the non-proteinaceous polymer. As predicted, the immobilization resulted in the secretion of a non-proteinaceous polymer in the form of nanoparticles in the range of 4-8 nm (FESEM, TEM). Even if the chemical identification of the secreted polymer is still in process, the preliminary composition of polymer backbone from the EDX (element analyzer) identified carbon, hydrogen, oxygen and nitrogen.

Therefore, this work is the first to show the *in vivo* immobilization of an active bacterial enzyme onto the lipid membrane of secretory vesicles of yeast to secrete non-proteinaceous polymer materials through its vesicular system.

1. Introduction

1.1 Immobilization and methods

Enzymes are proteins acting as a catalyst in almost every metabolic and biochemical reactions of all living organisms. Enzymes have proven themselves to evolve into a key factor in facilitating chemical reactions even under unfavorable physiological conditions (Norouzian, 2003). The quality of the enzymes (high substrate activity, increased rate of reaction, and selectivity of substrates) have been improved, with enhanced properties, by the recombinant DNA technology. Such newly improved enzyme variants has to be studied in detail. The researchers have found immobilization useful to study enzymatic properties at *in vitro* conditions. This is a process of placing an enzyme onto a physical support in which it is separated into a distinct phase from the substrate containing phase (Norouzian, 2003). The basic principles and substrates of immobilization are summarized in the Fig 1.

In the first place, these techniques were developed to maintain the long term stability and storage of the enzymes. Other benefits that had made enzyme immobilization methods more reliable and feasible are as follows (Spahn & Minteer, 2008; Nisha et al., 2012; DiCosimo et al., 2013): (1) enzymes can be easily separated from the available support because the chosen support usually solubilizes (Betancor et al., 2014) in solvent like water and (2) enzymes released from the support can be reused multiple times without affecting its longer half-lives and less degradation. Lengthening the half-life period of enzymes increases the opportunity to work with the different range of environments by changing conditions of temperature and pH in finding optimal enzymatic activity. Immobilization of the enzymes from product stream in analyzing its characteristics often does not seemly require any purification process (Spahn & Minteer, 2008). The substrates are in the form of either solid or liquid which includes many polymers, sol-gel complexes, polymer sol-gel composites and inorganic materials (Xie et al., 2009). The common immobilization methods are based on principles of adsorption, entrapment, cross-linking and covalent binding (Norouzian, 2003).

Immobilized proteins have been extensively used in various beneficial research areas in the industries from food to environmental. The other known applications involve the immobilization of antibodies, antigens, ELISA receptors and ligands used in the diagnosis and treatment of many diseases (Liang et al., 2000).

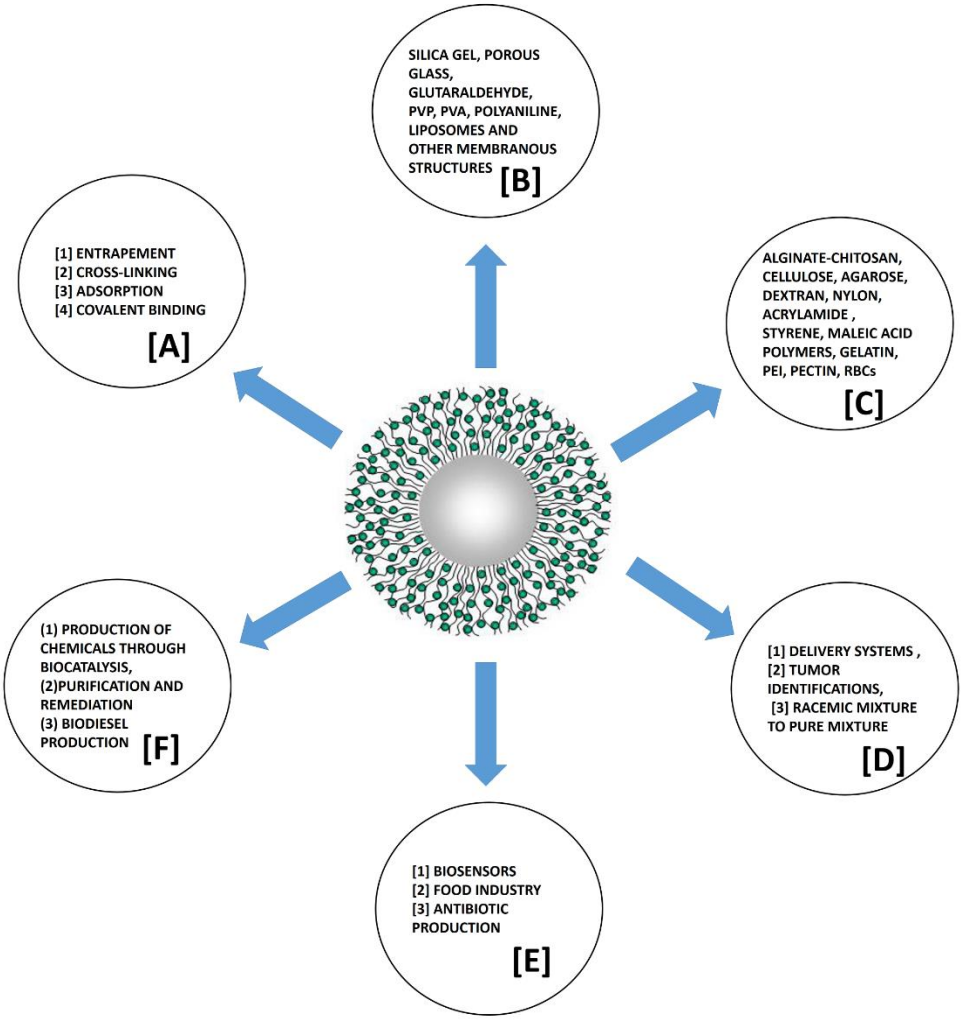


Figure 1 Schematic representation of immobilization process. (A). Methods of immobilization; (B, C). Organic and inorganic materials used in the enzyme immobilization; (D, E, F). Application of immobilized enzymes in various industries and research fields.

1.2 Bacterial structures and synthetic biology in immobilization

The advanced and effective way of immobilization is by functionalization of the support (either the polymer or the membranous particles). Immobilization is allowed by the

interaction of the enzymes directly with the modified (functionalized) support (DiCosimo et al., 2013). The surface of polymer material has been modified by introducing a specific anchorage ligand through which the interesting enzyme can bind enough to immobilize (Liang et al., 2000). The functionalization immobilization had laid a significant background for using some natural polymers, such as polyhydroxyalkanoate (organic) and magnetosomes (inorganic) produced by bacterial organisms, for immobilizing proteins. The identified surface proteins, enlisted in the Fig 2, act as anchors to immobilize target enzymes, onto lipid core of these natural polymers. The enzymes/ proteins targeted to the surface of particles are easily accomplished by recombinant DNA technologies of synthetic biology (Norouzian, 2003). To immobilize enzymes, a single step fusion of the target enzyme gene with the surface protein gene followed by the expression in the corresponding bacterial organisms is needed. The fusion is done to provide a contact with bacterial particles (acting as solid support) permanently. These fusions are designed in a way to immobilize the enzymes in right position with anchor peptides without affecting its structure and activity (Norouzian, 2003).

The formation of these particles is well-known which makes immobilization process more attractive. Another key point is that these polymers are nanoparticles which provide larger surface area and can be modified with respect to their chemical composition. Some of the important works came through the immobilization studies carried out particularly in polyhydroxyalkanoate (PHA) granules, magnetosomes (magnetic nanoparticles), fibrillar structures of proteins and inclusion bodies. The applications of this immobilization to protein production, isolation and purification, water purification, antibody presenting etc. are presented in the Table 1.

Bacterial Structures	PHA Granules	Magnetosomes
Identified Protein Anchors	PHA Synthase (phaC) Phasins (phaP) PHA depolymerase (phaZ) PHA Repressor (phaR) Acyl CoA Synthetase Heat Shock Proteins	Mms5, Mms6, Mms7, Mms13 Mms13, Mms16, MagA, MamC, MamF, MamG

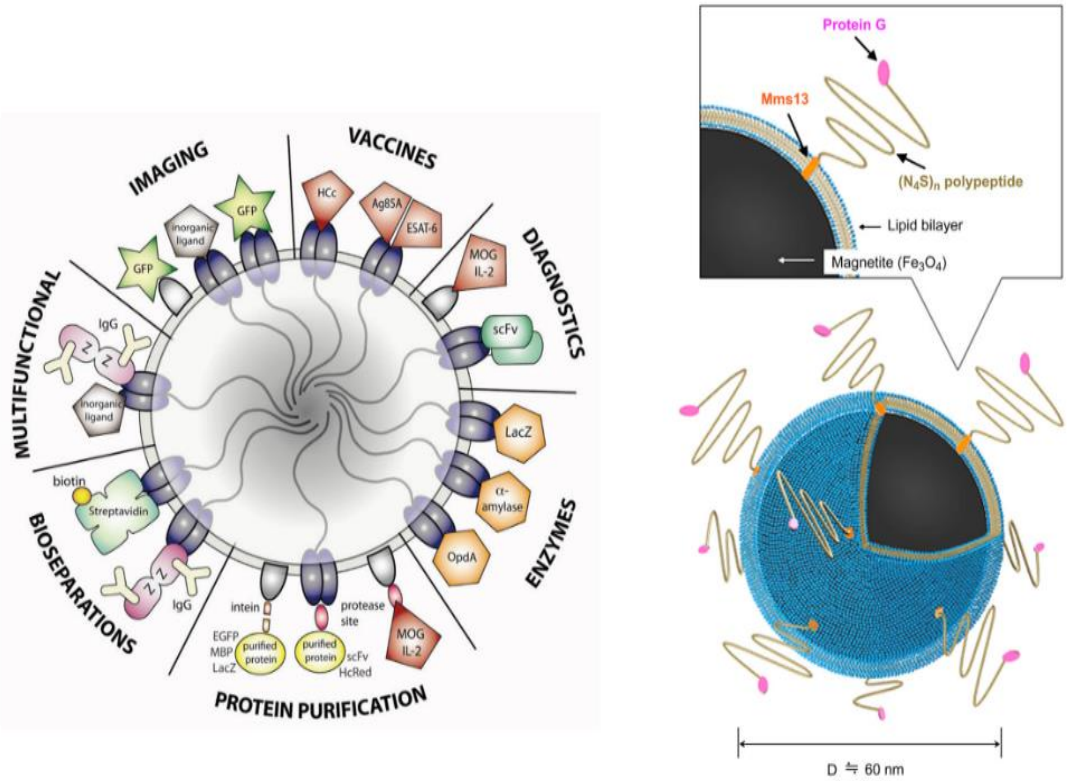


Figure 2 showing protein anchors that have been immobilized to bacterial polymeric nanoparticles; to the right – on magnetosomes; to the left – on polyhydroxyalkanoate granules.

Table 1 Single step immobilization process, using bacterial polymeric and inorganic particles, and its applications.

Bacterial particles	Host protein anchor	Immobilized protein	Applications	Ref.
Polyhydroxyal kanoates granules	Pha depolymerase	Severe acute respiratory syndrome coronavirus envelope protein	Immunoassays	Park et al., 2006
	Pha synthase (PhaC)	Highly thermosTable - amylase (BLA)	Starch liquefaction process	Rasiah and Rehm, 2009
	PhaC	anti-β-galactosidase scFv (single-chain variable 34 fragment)	Antigen presenting and purification	Grage et al., 2011
	N terminus of PhaC	β- glucosidase	Bioprocessing	Peters and Rehm, 2006
	PhaC	Antigen	Antigen delivery system	Parlane et al., 2009, 2011, 2013
	PhaC	Organophosphohydrolase enzyme OpdA	Hydrolyzes neurotoxic pesticides	Blatchford et al., 2012
	PhaR	DNA binding domain	Gene regulation	Wang et al., 2011
	PhaP	Lipopolysaccharide binding protein	Endotoxin removal	Li et al., 2011
	PhaC	Glucose oxidase fused with KVSALKE coil	Bioprocessing	Steinmann et al., 2010
Magnetosomes	Mms13	Immunoglobulin G-binding domain of protein A	Immunoassays	Yoshino and Matsunaga, 2006
	Mms16	G protein-coupled receptors (GPCRs)	Drug targets	Matsunaga et al., 2004
	MagA	Immunoglobulin G-binding domain of protein A	Antigen presenting	Yoshino and Matsunaga, 2006

	Mms13	NS-Polypeptide linked to protein G	Cell-cell interactions	Takahashi et al., 2010
	MamC	EGFP, antibody binding 'ZZ' protein	Antibody binding magnetosomes	Pollithy et al., (2011)

From these studies, it can be seen that organic and inorganic particles obtained from bacterial organisms have proven to be suitable sources for immobilization of proteins. The single step approach, through fusion technology, used in bacterial polymeric particles for immobilizing enzymes is more attractive and less expensive. By this work, we propose secretory vesicles from yeasts as a platform for immobilizing enzymes from yeast organisms. The design of the work involves the selection of membrane protein of vesicles and adapting its transport mechanism to translocate a bacterial protein, PHA synthase. The brief description on the vesicles in the process of secretion is described below.

1.3 The yeasts secretion pathway

In biotechnology, several advantages have been found in using yeasts for synthesizing products: they are larger than bacteria and are, therefore, easier to process; yeast metabolism and nutrition physiology are sufficiently flexible to facilitate the production; wild types are not ecologically harmful. Comparatively with bacillus bacteria, the major advantage in using yeasts, for synthesizing bacterial polymers, is that they do not produce any endotoxins (Glick and Pasternak, 1998). Profoundly, yeasts like *Saccharomyces cerevisiae* and other yeasts have permitted the simple expression and efficient purification of recombinant heterologous proteins from the extracellular medium upon 'secretion'.

In *S. cerevisiae*, a signal peptide at the N-terminal of a protein is required for effective translocation across the endoplasmic reticulum to the extracellular space via the Golgi complex. Mostly, proteins are initially synthesized in the cytoplasm. Later, they are selected and targeted to the plasma membrane for secretion via specific signal peptides (Bonifacino

and Glick, 2004; Pines and Inouye, 1999). At first, the selected protein cargo is transferred from the Endoplasmic Reticulum and packaged as cargo into COPII coated vesicles (early secretory vesicles) bound for the stacks of the Golgi complex. The, protein cargo passes through the cis-Golgi to the *trans*-Golgi network (TGN), where proteins destined for clathrin coated vesicles (late secretory or post-Golgi vesicles (PGVs)) are sorted away from cargo intended for vacuole or for localization at the plasma membrane (Grote et al., 2000).

To secrete the proteins constitutively, vesicles are transported from TGN to plasma membrane which takes place in three stages. First, PGVs are targeted to the vicinity of designated plasma membrane domains via microtubule- and/or actin-based transport systems. Second, after the vesicles arrive at plasma membrane they are tethered to specific plasma membrane domains (Guo et al., 2000; Pfeffer, 1999). Finally, the fusion between PGVs and plasma membrane takes place allowing the secretion of vesicle contents and the incorporation of membrane proteins at specific plasma membrane domains. This specific fusion event is mediated by interaction of proteins present in PGVs membrane (v-SNAREs, snc1p/2p) (SNARE, soluble N-ethylmaleimide-sensitive fusion attachment protein receptors) and plasma-membrane (t-SNAREs; sso1p/2p).

Wild type *S.cerevisiae* strains generate PGVs from 50-70 nm in diameter (Byers, 1981; Matile, 1969). In contrast, some mutant strains deficient in vesicular transport accumulate PGVs within the cell in different size ranges. For example, exo70-35 and exo70-38 mutant cells accumulated PGVs from were 80–100 nm in diameter (He et al., 2007). Forsmark et al., 2011 have determined the protein composition of PGVs obtained from the sec6-4 and sro7 mutant strains for isolation. The protein content identified were mainly involved in vesicle transport, molecules transportation, metabolism of carbohydrates and protein biosynthesis and degradation. The major dominant lipids constituents of membrane are

phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn) and phosphatidylinositol (PtdIns) (Fig.3).

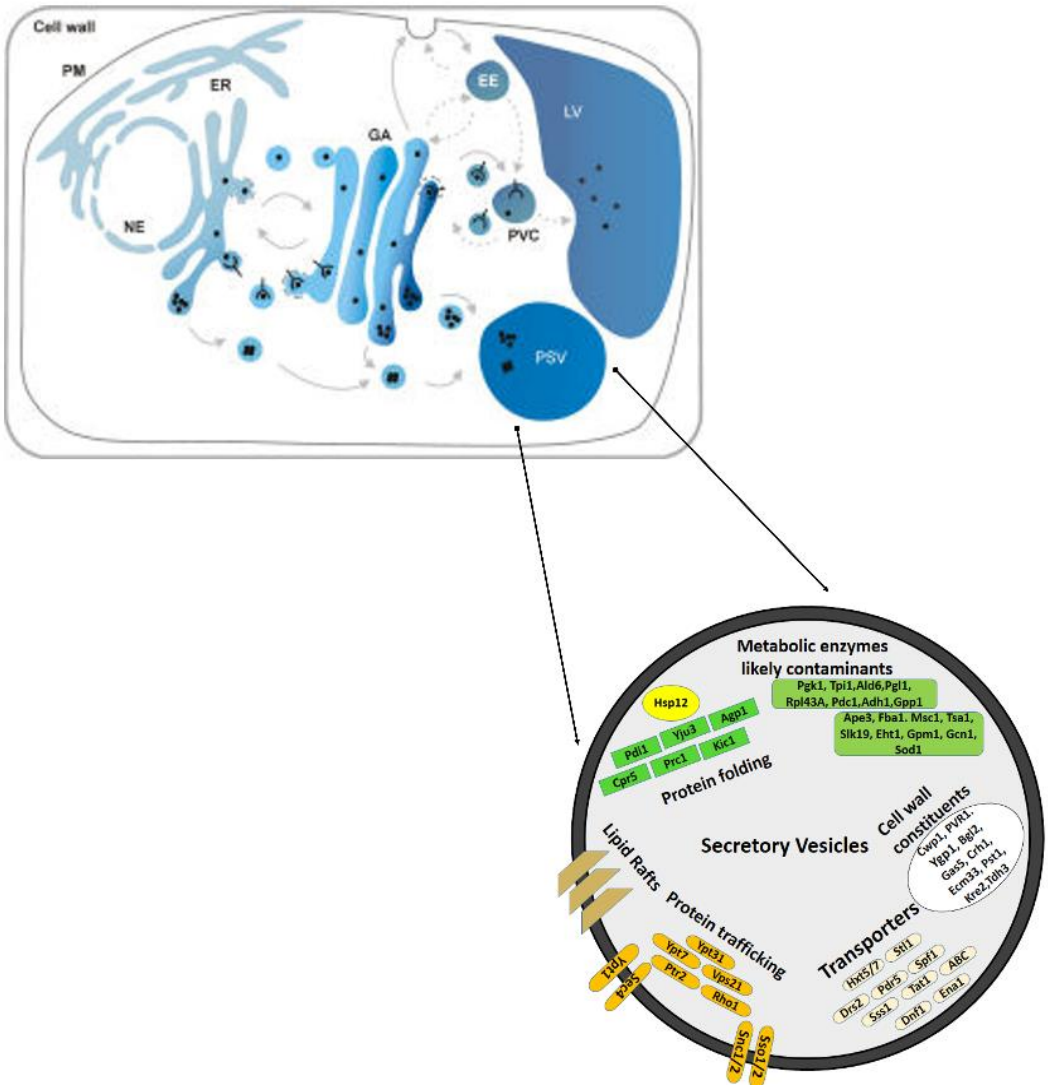


Figure 3 Structure and components of secretory vesicles of *S. cerevisiae*.

1.4 SNAREs – membrane protein of secretory vesicles

The most prominent proteins present on the surface of the yeast secretory vesicles are SNARE proteins which are well conserved in all eukaryotic cells. Synaptobrevin/vesicle-associated membrane protein is one of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. It is proposed to provide specificity for the targeting (t-SNARE) and fusion (v-SNARE) of vesicles with the plasma membrane (Burri and Lithgow, 2004). It belongs to a class of membrane proteins lacking a signal sequence and contain a single hydrophobic segment close to their C-terminus, leaving most of the polypeptide chain in the cytoplasm (tail-anchored) (Kutay et al., 1993). v- SNARE protein is attached to the cytoplasmic surface of vesicles by its C-terminal trans-membrane anchor in an alkali-resistant manner, adopting a trans-membrane orientation (Kutay et al., 1995; Burri and Lithgow, 2004). It localizes to secretory vesicles, interact with t-SNAREs from the plasma membrane and are likely to undergo a dynamic cycle of transport to and retrieval from the plasma membrane through endocytosis.

So far, the mechanism of translocation is well understood for Snc1p and Snc2p, the most studied SNAREs in *S. cerevisiae*. The Snc1p/Snc2p proteins share two conserved amphipathic alpha helices (central coiled-coil); - Helix 1(H1), from 39 to 53 amino acids, is unusually hydrophobic and Helix 2(H2), from 60 to 88 amino acids, is predicted to interact with another hydrophobic segments of membrane proteins (Syntaxin). A variable domain in the N-terminal and a carboxy trans-membrane region, from 96-110 amino acids, is rich in hydrophobic amino acids (alpha-helical) (Protopov et al., 1993). Deletion and mutational studies had been performed to study the regions playing an important role in targeting v-SNAREs to secretory vesicles (Protopov et al., 1993; Banfield et al., 1994; Ungar et al., 2003). Table 2 shows the summary of targeting studies performed by Grote et al., 1995 and Gerst, 1997. These authors concluded that in the absence of helical loops, it is not possible

to target the v-SNAREs. Thus, deletion or gross substitutions in either of the predicted H1or H2 segments result either in the loss of targeting or in a complete loss of its function. This shows that the conserved amphipathic alpha helical regions (32-85 amino acids; complete H1 and H2 region) are essential for the confinement of v-SNAREs. These studies also show that both regions of v-SNARE protein hold a major role in the translocation and integration to membrane of secretory vesicles.

Table 2 Deletion mutational studies on SNARE proteins and its effects on vesicular transport and localization.

Regions deleted	Effects	Ref.
VAMP (2-30)	++	Grote et al., 1995
(2-60)	------	,,
(31-38)	------	,,
(41-50)	------	,,
(61-70)	++	,,
(71-80)	++	,,
Snc1 (2-27)	++	Gerst, 1997
(31-50)	------	,,
(51-82)	N.D.	,,
VAMP (1-90) Snc1 (91-116)	------	,,
Snc1 (1-65) VAMP (65-84) Snc1 (85-116)	++	,,

++ Targeting of v-SNAREs to PGVs membrane; ------ Non-targeting of v-SNAREs to PGVs membrane

VAMP- Vesicle Associated Membrane Protein

Thus, both of the regions of v-SNAREs must be used for demonstrating PGVs as a model system of immobilization.

1.5 Poly-hydroxyalkanoate synthase

PHA synthase is the critical enzyme involved in the cytoplasmic formation and accumulation of polymeric granules in a number of bacteria. The enzyme utilizes ß-hydroxyacyl-coenzyme A (CoA) as the substrate for PHAs synthesis. The PHA synthase is localized in the cytoplasm of exponentially grown cells, which have not yet accumulated PHAs (Haywood et al., 1989). More than 59 PHA synthase genes have been cloned from 45 species of bacteria and PHA synthase enzyme have been categorized into four different classes (Table 3), based on their *in vivo* substrate specificities, primary amino acid sequences, and subunit composition.

Table 3 Different classes of polyhydroxyalkanoate synthase

Class	Subunits	Species	Substrate
I	PhaC ~60-73 kDa	*Ralstonia eutropha*	$3HA_{SCL}$-CoA (~C3-C5) $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA, $3MA_{SCL}$-CoA
II	PhaC ~60-65 kDa	*Pseudomonas aeruginosa*	$3HA_{MCL}$-CoA (~≥C5)
III	PhaC ~40 kDa PhaE ~40 kDa	*Allochromatium vinosum*	$3HA_{SCL}$-CoA ($3HA_{MCL}$-CoA [~C6-C8], 4HA-CoA, 5HA-CoA)
IV	PhaC ~40 kDa PhaR ~22 kDa	*Bacillus megaterium*	$3HA_{SCL}$-CoA

The process of PHA biosynthesis in bacteria (three proposed models of PHA synthase and biosynthesis - Appendix I) involves the polymerization of (*R*)-3-hydroxyacyl-CoA to PHA

which leads to the formation of spherical inclusions of PHA in the cytoplasm (Rehm, 2006). This polymer assembly is mediated by conversion of soluble substrate monomers into insoluble high molecular weight polymer through the activity of PHA synthase. During the polymerization process, the synthase remains covalently attached to the growing polyester chain and continues to incorporate more substrate until certain constraints such as, depletion of substrate or all available cytoplasmic space in the cell has been occupied by the PHA granules, terminate the polymerization procedure. (Rehm, 2006).

Table 4 Signal peptides used for translocation of PHA synthase

Organism	Signal peptide	Target organelle	Ref.
Arabidopsis thaliana	Chloroplast transit peptide	plastids	Nawrath et al., 1994
A. thaliana	Rubisco	plastids	Slater et al., 1999
S. cerevisiae	Isocitrate lyase	peroxisome	Poirier et al., 2001
S. cerevisiae	----------	cytosol	Leaf et al., 1996
Pichia pastoris	Isocitrate lyase	peroxisome	Poirier et al., 2002
A. thaliana	PTS1	peroxisome	Matsumoto et al., 2006
Tobacco	Cloned between rbsL and accD	chloroplast	Wang et al., 2005
Tobacco	psbA	plastids	Bohmert-Tatarev et al., 2011
Sugarcane	peroxisomal type 1 targeting sequence, RAVARL,	peroxisome	Anderson et al., 2011
Monocot and Dicot species	PTS1 sequence of spinach glycolate oxidase	peroxisome	Gnasambandam et al., 2012

The PHA synthase enzyme has been expressed heterologously in several organisms including bacteria (*E. coli*, *R. eutropha* PHB-4), yeasts (*S. cerevisiae*, *P. pastoris*) and plants

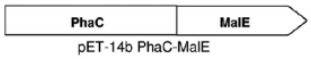
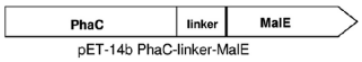
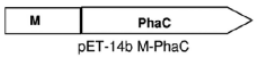
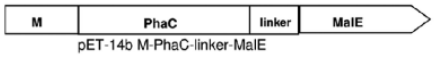
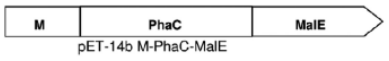
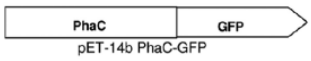
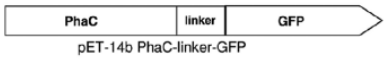
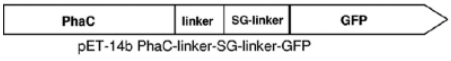
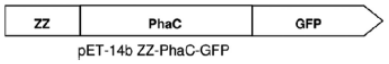
for the production of polyhydroxyalkanoates. Specifically in yeasts and plants, distant from the cytoplasmic expression, enzyme was designed and expressed in a way to translocate to certain organelles such as peroxisomes, plastids, chloroplasts with the organelle specific signal peptides. The table 4 shows some of the signal peptides used for targeting PHA synthase to different organelles.

1.6 Chimeric PHA synthases – role of regions in PHA synthesis

Numerous fusion studies had concluded that the PHA granule formation was not affected, when proteins of different functionality are fused to PHA synthase enzyme. The carboxy terminal end of PHA synthase plays an important role for its functionality. In the same time, the hydrophobicity of the fusion protein also affected the function of PHA synthase. Fusion protein analysis indicated that fusion points located at the N terminus of the conserved α/ß hydrolase fold region, and at a variable surface-exposed loop based on the current protein model, are tolerated by PHA synthases (Rehm and Steinbuchel, 2002; Rehm, 2003).

The N terminus, i.e. the first about 100 amino acid residues relative to the *Cupriavidus necator* Pha synthase, represents the most variable region of the protein and has been found to be dispensable for activity (Rehm, 2003; Schubert et al., 1991). The class I PHA synthase (PhaC) from *C. necator* was investigated regarding the functionality of its conserved C-terminal region and its ability to tolerate translational fusions to its C terminus. MalE (maltose binding protein) and green fluorescent protein (GFP) were considered reporter proteins and translationally fused to the C terminus.

Table 5 Chimeric PHA synthases and their functional activity in PHA production.

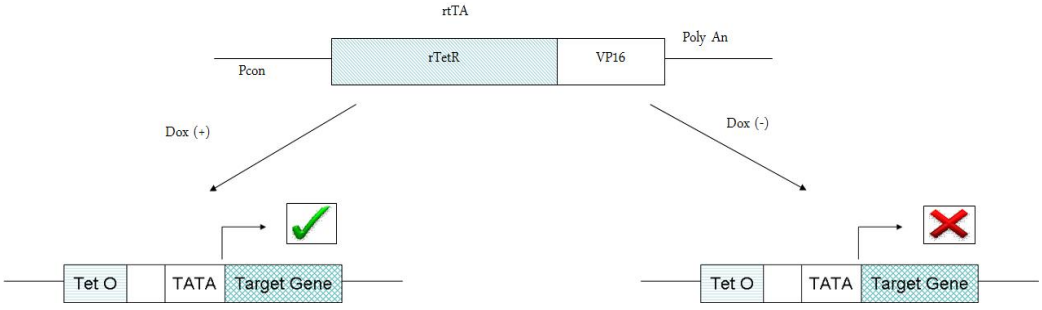
Hybrid Gene	PHB production	Activity of fusion partner	Surface display of fusion partner
 pET-14b PhaC-MalE	-	-	-
 pET-14b PhaC-linker-MalE	+	-	+
 pET-14b PhaC-linker-SG-linker-MalE	+	-	+
 pET-14b M-PhaC	+	-	N/A
 pET-14b M-PhaC-linker-MalE	+	+	+
 pET-14b M-PhaC-MalE	+	-	+
 pET-14b PhaC-GFP	+	+	+
 pET-14b PhaC-linker-GFP	-	+	+
 pET-14b PhaC-linker-SG-linker-GFP	+	+	+
 pET-14b ZZ-PhaC-GFP	+	+*	+*

In contrast to N-terminal fusions, some C-terminal fusions inactivated the PHA synthase. For example, MalE directly fused to the synthase C- terminus led to inactivation, while a direct fusion of GFP to the C terminus of PhaC allowed PHB granule formation and a functional fusion protein. A hydrophobicity analysis of the N-terminal regions of MalE and GFP showed that GFP comprises an extended hydrophobic region similar to the designed linker region, whereas the N-terminal region of MalE was found to be hydrophilic. This suggested that the hydrophobic N terminus of GFP, when directly fused to PhaC, does not interfere with the proposed anchor function of the hydrophobic C terminus of the PHA synthase. (Jahns and Rehm, 2009). Maintaining an hydrophobic environment around the C terminus of the PHA synthase was found to be required for activity (Jahns and Rehm, 2009). The PHA synthase activity of various constructed fusion cassettes is shown in the Table 5.

1.7 Tetracycline mediated expression system

The protein expression system in heterologous hosts often provides a time of regulation and varies in the level of expression. Various promoters have been developed for use in *S. cerevisiae* and have been applied in other yeasts with varying levels of success. Among them, the Tet system is the most widely used inducible gene expression technology in eukaryotes, for both, *in vivo* and *in vitro* applications. Tet-controlled systems are based on the *Esecherichia coli* Tn10 Tet resistance operon, which consists of the gene coding for Tet repressor protein (TetR) and the Tet operator DNA sequence (TetO DNA). In the absence of Tet or its derivate doxycycline (Dox), the TetR protein gets attached to the TetO DNA sequence which turns off gene expression.

[a]



[b]

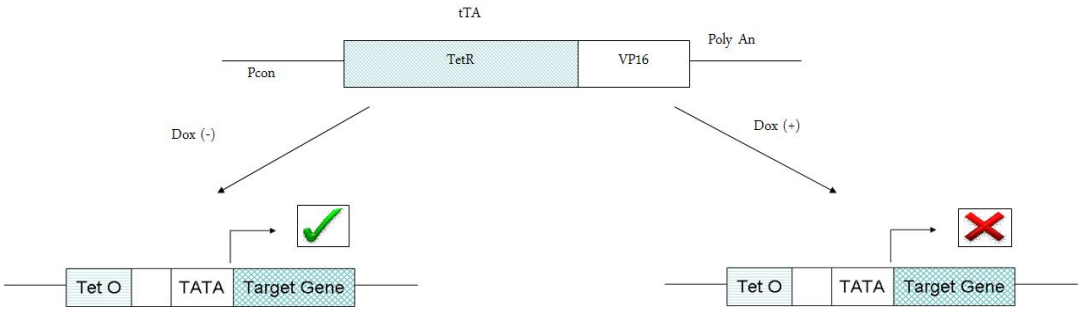


Figure 4 depicts working principles of (a). Direct tet system and (b). Reverse tet system.

While in the presence of the tetracycline and its analogs, TetR changes its conformation to detaching from the DNA and so turns on gene expression (Barens and Hillen, 2003; Aleksandrov et al., 2009). Based on the working principles of TetR, two different regulators tTA and rtTA were developed. The elements of the tetracycline controlled transcriptional activators and working system are outlined in Fig 4. Two or seven repeats of the TetO DNA sequence, referred as TRE (for Tet-responsive elements), are fused upstream of a minimal CMV promoter (PminCMV), forming the promoter PCMV-1 (Barens and Hillen, 2003). In the absence of Dox, tTA binds to the TREs thus activating PCMV-1, which results in the expression of the adjacent gene (Fig 4a). If Dox is present, tTA changes its conformation, detaches from the TREs and gene expression is initiated. The Tet-Off system is useful only if gene expression must be maintained switch-on for a long time. To turn the system off, continued administration of Dox would be required and the activation of transgene expression depends mainly on the kinetics of Dox clearance (Stieger et al., 2009).

Four mutations in the TetR domain of the trans-activator tTA were shown to reverse the behavior of the protein to interact with the TRE with the presence or absence of Dox (Belli et al., 1998). Rather than attaching to the TRE in the absence of Dox, this mutant reverse Tet repressor protein (rTetR) binds to the TREs in the presence of Dox. When rTetR is fused to the VP16 domain, the resulting trans-activator protein is called rtTA and the system changes to a Tet-On system: only the presence of the inducer drug allows expression of a transgene (Fig 4b). Activation of transgene expression was found to be more rapid compared to the Tet-Off system. However, to induce transgene expression using the Tet-On system, high concentrations of Dox were required, raising the question of whether these concentrations were possible to achieve higher expression (Stieger et al., 2009; Belli et al., 1998).

The most advantageous parts of Tet-controlled expression system are as follows: (1) No interference in the metabolism of host organism, (2) Antibiotics used for the gene regulation

does not shown any deleterious effects on the growth of *S. cerevisiae* and (3) The gene expression is easily tuned and tight regulation is feasible with these systems (Wishart et al., 2005; Vogt et al., 2005).

In view of the successful use of the tetracycline expression system in yeasts, we have utilized the same for the regulating the expression of the heterologous PHA synthase gene of our interest.

1.8 Overcoming downstream issues

The high production costs of PHA have its limited widespread use in industrial packaging, tissue engineering and protein purification (Lee 1996; Choi and Lee, 1997; van Wegen, 1998; Castilho, 2009). Specifically, downstream processing can account for as much as 50% of the total production expense (Jung et al., 2005). The solvents usually employed include chloroform, methylene chloride, propylene carbonate, dichloroethane and sodium hypochlorite. A microorganism with ability to secrete the produced PHA to the culture medium may ease its recovery and potentially would be a lower costs alternative, by eliminating the need for chemical or mechanical cellular membrane disruption, which could also lead to a continuous production system (Jung et al., 2005).

Cetin et al., (2006) and Sabirova et al., (2006) had reported the deposition of cytoplasmic PHA granules extracellularly *i.e.* outside of the cells. These observations has shown that the extracellular deposition of PHAs reduces the downstream process with increase in the yield of PHAs. However, the mechanism by which PHAs are transported is unknown. This presence of PHA particles in the extracellular medium let the doubt whether they were being released from the cells by either lysis or without lysis of cell wall. Recently, Rahman et al., 2014 had shown the deposition of phasin bound PHB outside of the cells and into the culture medium. The authors used for the PHB deposition by utilizing the type I secretion system with its HlyA signal in *E. coli*. Likewise, the present work is focused primarily to use the

eukaryotic secretion system (*S. cerevisiae*) and its components (constitutive vesicles) to secrete a non-proteinaceous polymer (PHB) to the extracellular medium.

1.9 Justification

The single step immobilization of the enzymes in biological sources had been advantageous and attractive so far. Until now, the vesicular transport system of *S. cerevisiae* had been engineered to secrete variety of proteins (i.e., amylase, glucanases, and invertase) to the extracellular medium. The present work has taken similar advantage to engineer pathways of eukaryotes to immobilize the heterologous proteins onto secretory vesicles. As well as determining secretory vesicles as support for immobilization, these nanostructures are engineered for secreting non-proteinaceous polymeric substances into the extracellular medium using naturally occurring vesicular transport system. The non-proteinaceous polymer is expected to belong to the polymer family of polyhydroxyalkanoates. The v-SNARE (Snc1p/Snc2p) domains would be a suiTable to immobilize the proteins onto the membrane of vesicles. The type II PHA synthase of *Pseudomonas putida* CA-3 was chosen as a model to show that the v-SNARE domains can be used as anchors of heterologous proteins to the membrane of *S. cerevisiae* secretory vesicles. Generally, this PHA synthase is unexpressed in the natural conditions of *P. putida* CA-3 cultivation (Ward et al., 2005; Ward and Connor, 2005) but Lopez-Cuellar et al., (2014) have reported that this enzyme is active with wide substrate specificity and capable to polymerize monomers from short chain length (scl) to medium chain length (mcl). The report stated that the monomers present in the synthesized polymer were 3-hydroxybutyrate, 3-hydroxyhexanoate, 3- hydroxyl-valerate and 3-hydroxyoctanoate.

The synthetic biological engineering of *S. cerevisiae* secretion system by the introduction of PHA synthase fusion protein with SNARE domains will leads to major investigation on the research of polyhydroxyalkanoates. If the immobilization gets successful, PGVs would

become a broad representative as a substrate for the integration of other heterologous proteins. Apart from the secretion studies, the immobilization of the proteins to membrane maintains the stability and the activity of enzymes for longer time in a wide range of environments with lesser degradation. Furthermore, the vesicular system could demonstrate itself to be an alternative method to the protein/enzyme immobilization with unique advantages.

2. Hypothesis

1. Cloning and expression of poly-3-hydroxyalkanoate polymerase enzyme of *P. putida* CA-3 together with the domains of v-SNAREs will ensure the immobilization of the enzyme on vesicles of recombinant *S. cerevisiae* ATCC 9763.

2. The active PHA synthase fusion protein immobilization will result in the secretion of the non-proteinaceous polymer recoverable in the extracellular medium.

3. Objectives

3.1 General objective

The expression of a fusion cassette comprising the poly-3-hydroxyallkanoate synthase gene flanked with two domains of Snc2, v-SNARE transport protein in *S. cerevisiae* for the immobilization of the functional enzyme protein onto secretory vesicles.

3.2 Particular objectives

- To design the fusion cassette to localize an heterologous protein on the secretory vesicles of *S. cerevisiae*.
- To construct the expression plasmid pMKFMJ01 (plasmid harboring $TetO_7$ systems with 2µ origin).
- Construct the plasmids pMKFMJ26, pMKFMJ86, pMKFMJ87 for the localization studies.
- Genetic transformation of *S. cerevisiae* ATCC 9763 with the expression plasmids carrying fusion cassette.
- Expression and localization of the fusion protein in recombinant *S. cerevisiae* strains.
- Microscopical observations of non-proteinaceous nanoparticles secreted in the extracellular medium of recombinant *S. cerevisiae*.

4. Materials and methods

4.1 Strains

The bacterial and *S. cerevisiae* strains used in this study are listed in Table 6.

Table 6 Strains used in this study

Strains	Characteristics	References
S. cerevisiae ATCC 9763	Industrial polyploidy strain	Department of Biotechnology and Bioengineering, CINVESTAV
S. cerevisiae pMKFMJ26	Transformant carrying pMKFMJ26 plasmid and express chimeric PHA synthase protein	This study
S. cerevisiae pMKFMJ86	Transformant carrying pMKFMJ86 plasmid and express EGFP tagged chimeric PHA synthase protein	This study
S. cerevisiae pMKFMJ87	Transformant carrying pMKFMJ87 plasmid and express EGFP protein	This study
E. coli DH5α	dlacZ Delta M15 Delta(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-mK+) supE44 thi-1 gyrA96 relA1	A gift from Dr. Jesus Valdes Flores
GBE 180		A gift from Dr. Jesus Valdes Flores
BL21 DE3	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	A gift from Dr. Juan Pedro Luna Arias

4.2 Plasmids

The plasmids and primers used in this study are shown in the Table 7 and 8 respectively.

Table 7 PCR primers used in this study

Primers Used in this Study		Enzyme
Target Gene- ADH1 Terminator		
ADH1 F	5'- GGGCCCCAATTGGTAATTCGCGCCACTTCTAAAT-3'	MfeI
ADH1 R	5'- CCCCCGGAATTCTGATCTGCCGGTAGAGGTGT- 3'	EcoRI
Target Gene - Polyhydroxyalkanoate synthase PhaC2 P.putida CA-3		
PHA F	5'-GCGCCCCGGGCCACCATGTCTTGGTACAAGGATCTAATGACTG- 3'	SmaI
PHA R	5'-CCAACCCGGGTCATTATCTAGTCAAAACATAAGTACCTG-3'	SmaI

Table 8 Plasmids used in this study.

Plasmids	Characteristics	Reference
pTEF1/Zeo (3556 bp)	Cytoplasmic vector, Zeo^r, Amp^r, pUC ori	A gift from Dra. Aida Rodriguez
pYES2 (5956 bp)	Amp^r, 2 Micron origin, URA3 marker	A gift from Dr. Jesus Valdes Flores
pCM173 (10683 bp)	Centromeric yeast plasmid, marker TRP1, tetracycline repressed expression of lacZ under control of tetO7, tTA system	EUROSCARF
pCM252 (7626bp)	Centromeric yeast plasmid, marker TRP1, tetracycline inducible expression of target gene under control of tetO7, rtTA system	EUROSCARF
pEGFP-C1 (4731bp)	Mammalian EGFP fusion expression vector; CMV promoter; N-terminal cassette, Km^r	A gift from Dr. Nicolas Villegas
pRSETB (2.9kb)	Amp^r, Col E ori, N-Terminal His Tag (T7 promoter)	A gift from Dr. Juan Pedro Luna Arias
pRSETA (2.9kb)	Amp^r, Col E ori, N-Terminal His Tag (T7 promoter)	
pMKFMJG	pTEF1/Zeo lacking PstI site (HindIII,AccI)	This study
pMKFMJS	pMKFMJG Harbouring 2 micron origin of replication(SwaI, ClaI) from pYES2 plasmid	This study
pMKFMJP	pMKFMJS lacking XhoI site (XhoI)	This study
pMKFMJK	pCM173 harbouring ADH1 terminator (EcoRI) a PCR product obtained from pCM173	This study
pMKFMJT	pMKFMJP harbouring Tet Direct System cassette with one more copy of ADH1 terminator upstream of CMV promoter (EcoRI, BamHI)	This study
pMKFMJ01	pMKFMJT harbouring Tet Reverse system cassette (PvuII, XhoI) from pCM252	This study
pMKFMJ26	pMKFMJ01 harbouring fusion cassette (Pha synthase sequence of Pseudomonas putida CA-3 flanked between two domain sequences of Snc2)	This study
pMKFMJ86	pMKFMJ26 harbouring Enhanced Green Fluorescent Protein in the N-terminal of fusion cassette	This study
pMKFMJ87	pMKFMJ01 harbouring Enhanced Green Fluorescent Protein	This study
pRSETB-Snc2-PHA	pRSETB harbouring fusion cassette	This study
pRSETA-EGFP-Snc2-PHA	pRSETA harbouring EGFP fusion cassette	This study

4.3 Microorganisms conservation

The microorganisms used, *S. cerevisiae* and *E. coli*, were cryo-conserved with glycerol as described in Sambrook, Fritsch and Maniatis, 1989. They were distributed in 1ml of aliquots using 2ml Eppendorf tube and stored at -80ºC. Culture mediums used for the conservation of microorganisms are listed in the Table 9 (A and B):

Table 9 (A and B) Composition of conservation media

[A]

Reagents	LB medium (g/L)	SOB medium (g/L)
Tryptone	10	20
Yeast extract	5	5
NaCl	10	0.5

[B]

Reagents	YPD medium (g/L)
Yeast Extract	10
Polypeptone	10
Glucose	10

4.4 Antibiotics used

The antibiotics employed in the selection and propagation of *E. coli* and *S. cerevisiae* cells was prepared according to Sambrook, Fritsch and Maniatis (1989). They were filtration sterilized using filters of 0.22 µm in diameter and maintained at -20ºC. The concentration of antibiotics are indicated in the Table 10.

Table 10 Antibiotics used in the experiments

Antibiotics	Solution stock (mg/mL)	Dissolving agent	Concentration final (µg/mL)	Trademark
Ampicillin	100	Distilled water	100	Geopen, Pfizer
Kanamycin	50	Distilled water	50	Kantrex, Bristol
Chloramphenicol	37	Ethanol	37	Sigma
Zeocin	50	HEPES buffer, pH 7.25,	50	Invitrogen

4.5 Sequences of enzymes

The amino acid sequences of enzymes PhaC2 polymerase type II of *P. putida* CA-3 (AAU44815) (Appendix II) and SNARE proteins (Snc 1p/2p; AAC05002) (Appendix II) of *S. cerevisiae* were obtained from the Pubmed database. The frequency of codon usage of *S. cerevisiae* was obtained from 'Codon usage database'. For the efficient expression, the reported amino acid sequence of $PhaC2_{P.\ p\ CA\text{-}3}$ was edited according to the codon usage of *S. cerevisiae* for the efficient expression. The edited sequence was aligned with the original sequence using Clustal W 2.0.8 to confirm that amino acids were identical in both cases.

4.6 Plasmid transformation – *E. coli* strains - Fiche technique (Heat shock method)

Overnight cultures of *E. coli* DH5α were prepared by inoculating 50 µl in a 5 ml of SOB medium with 10mM $MgSO_4$. 500 µl of the overnight culture of *E. coli* was inoculated into 50 ml of SOB medium complimented with 50 µl of 10mM $MgSO_4$. The optical density was measured at 595 nm at periodic 30 min time interval. When the O.D. reached 0.5, the cells were centrifuged at 3,000 rpm for 15min maintained at 4^{o}C. 16 ml of TFB1 (Appendix III) was added to the pellet and mixed it gently. The suspension was kept in ice for 15 min and centrifuged at 3,000 rpm for 15 min. 4 ml of TFB-2 (Appendix III) was added to pelleted cells and stored in ice for 20 min. Aliquots of 200 µl were distributed and stored at -80 ^{o}C. To the 200 µl of the aliquots, approximately 1-10 µg of plasmids were added and placed in ice for 5 -10 min. Then, the mixture was placed in a water-bath for 90 sec at 43 ^{o}C. After the treatment, it was kept in ice for 5 min. Later, 800 µl of the SOB was added to it and incubated for 1 hour 20 min at 37 ^{o}C. Finally, a 100 µl of the culture was inoculated in the plates containing antibiotics for selection and the remaining was stored for further use.

4.7 Plasmid DNA isolation from E. coli DH5α

4.6.1 Boiling prep method

The method described is a modification of the boiling prep technique developed by Gianino Del Sal (1988). A single colony (*E. coli* DH5α cells having plasmids) was inoculated in 5 ml of LB-Broth (100 μg/ml ampicillin) and cultured overnight. The cells were spun in an Eppendorf microfuge, suspended in 200 µl of STET buffer (8%w/v sucrose, 0. 1%v/v TritonX-100, 50 mM EDTA, 50 mM Tris-HCl pH 8.0) and incubated at room temperature (RT) for 5 min after addition of 4 µl of lysozyme (50 mg/ml). Samples were boiled for 60 seconds and centrifuged for 15 min at 12000 rpm. The pellet was removed using a toothpick; 10 µl of CTAB (5%w/v) (Sigma H5882) were added and the precipitate was obtained by centrifuging for 15 min at 12000 rpm. The pellet was dissolved in 300 µl of 1.2 M NaCl, by vigorous vortexing, and re-precipitated by addition of 750 µl of ethanol and centrifugation for 10 min. The final pellet was rinsed in 70% ethanol/water dried under vacuum and dissolved in distilled water.

4.6.2 Quick plasmid mini-prep kit

The protocol was designed by Invitrogen technologies to isolate high quality plasmid DNA (up to 30 µg) from *E. coli* cells in 30–45 min. A single colony was inoculated in 5 ml of LB-Broth (100 µg/ml ampicillin) and cultured overnight. Bacterial cells were spun in an Eppendorf microfuge and 250 µl of re-suspension buffer was added to it. Pellet was suspended well and 250 µl of lysis buffer (L7- Appendix III) was added. It was mixed gently by inverting the capped tube until a homogeneous mixture was formed. To this homogenous suspension, 350 µl of precipitation buffer was added and mixed immediately. The suspension was centrifuged for 10 min at 12,000 rpm and the supernatant was loaded onto a spin column in a 2 ml wash tube. The spin column was centrifuged at 12,000 rpm for 2 min

and the flow-through was discarded. 700 µl of wash buffer (W9- Appendix III) with ethanol was added to the column and centrifuged at 12,000 rpm for 1 min. After discarding the flow-through, the column was centrifuged again to remove the remains of wash buffer in it. To elute plasmid DNA, 30-50 µl of water or TE buffer (pH.8) was added to the column and centrifuged at 12,000 rpm for 1 min to collect in a 2 ml Eppendorf tube.

All the genetic manipulations were performed following the standard procedures (Sambrook and Russell 2001). The enzymes such as restriction endonucleases, Klenow fragment and T4 DNA ligase were obtained from New England Biolabs, Invitrogen and Fermentor (USA). They were utilized according to protocol of company with some modifications (Table.11).

The reaction mixtures and its methodology are shown in Table 11.The plasmid isolation and restriction analysis were realized by agarose gel electrophoresis at a concentration of 1% with TAE buffer as a regulator. The voltage utilized was dependent on the time of

Table 11 Modified protocols followed in the experiments of restriction digestion, Klenow treatment, de-phosphorylation and ligation

Restriction digestion

Incubated at 37^{O}C for 2 hours. The enzyme was inactivated by heating it at 65^{O}C for 20 min. 10X BSA is used, if needed.

DNA (~100 ng/µl)	10 µl
10X React buffer	2 µl
Restriction enzyme (10U/µl) (1:10 dilution)	1U/ µl
Distilled water	7 µl
Total volume	20 µl

Klenow treatment

Incubated at 37^{O}C for 1 hour. The enzyme was inactivated by heating it at 75^{O}C for 20 min.

DNA (~100 ng/µl)	10 µl
10X React 2	2 µl
Klenow large fragment (10U/µl) (1:10 dilution)	0.5U/ µl
Distilled water	7.5 µl
Total volume	20 µl

Dephosphorylase treatment

Incubated at 37^{O}C for 1 hour and gel purified.

DNA (~100 ng/µl)	10 µl
10X Buffer	2 µl
Shrimp Alkaline Phosphatase (10U/µl) (1:10 dilution)	1U/ µl
Distilled water	7 µl
Total volume	20 µl

Ligase treatment

Incubated at 16^{O}C overnight and transformed.

Vector DNA (~200 ng/µl)	1 µl
Insert DNA (~50 ng/µl)	4 µl
5x Ligase buffer	1 µl
Ligase enzyme (5U/µl)	1 µl
PEG	1 µl
Distilled water	2 µl
Total volume	10 µl

electrophoresis, size of the gel and electrophoresis apparatus (Cleaver Scientific Ltd, MP-250V). The bromophenol blue stain was used as a running dye and ethidium bromide was used to observe and take the photographs of the DNA bands (Sambrook and Russell 2001). The 1kb plus DNA ladder was obtained from the Invitrogen (10787-018) and Fermentor (SM0312).

4.8 Purification of gene fragments

The restriction digestion of the plasmids results in number of gene fragments depending on the number of the sites accessible to the respective enzyme in plasmid. The interested gene should be purified from the rest of the fragments and enzymes for ligation. Such process of the purifying needs running the restriction mix in the agarose gel for the separation of the fragments and purification is proceeded by either of the following methods.

4.8.1 Method I

This method was followed from the protocol of "Gel extraction kit – QIAEX II of qiagen". The restriction mix was run in the agarose gel and the gel segment containing the gene fragment was cut and separated. The sliced gel segment was weighed and measured. With respect to the measure, thrice amount of solution I was added i.e., To 100 mg of gel, 300 µl of solution I was added. 10 µl of matrix solution II was added and kept in water bath at 55^{o}C for 10 min. The suspension was centrifuged at 13,000 rpm for 30 sec. The pellet was again washed with solution I and centrifuged at 13,000 rpm for 30 sec. The pellet was washed twice with the solution II and it was followed by centrifugation at 13,000 rpm for 30 sec. The pellet was air dried completely, till it turned clear white. To it, 20 µl of water was added and kept it in room temperature for 5 min. It was followed by centrifugation for 30 sec. The supernatant was transferred to fresh tubes and purified gene fragment was confirmed by agarose gel electrophoresis.

4.8.2 Method II

The restriction mix was run in the agarose gel and the gel segment containing the gene fragment was cut and separated. The sliced gel segment was weighed and measured. With respect to the weight of the gel segment, five volumes of QG buffer were added and kept in a water bath at 55ºC for 10 min. One gel volume of isopropanol was added to it and mixed well. The suspension was poured to Qia-quick column and centrifuged at 12,000 rpm for 1 min. The flow through was discarded and 700 µl of PE buffer was added to it. This was centrifuged at 12,000 rpm for 2 min and flow through was discarded. Again, the column was centrifuged to discard the remaining of PE buffer. Later, elution was done with 20 µl of water or TE buffer followed by centrifugation at 12,000 rpm for 2 min. The flow through was collected in a fresh tube and purified gene fragment was confirmed by agarose gel electrophoresis.

4.9 Construction of expression plasmid pMKFMJ01

An illustration of the plasmids construction design is presented in the Fig. 5. Plasmid pMKFMJG was a modified version of pTEF1/Zeo in which PstI recognition site was eliminated by digestion of AccI and HindIII. A 1535 bp fragment including 2 micron origin was removed from pYES2 plasmid by ClaI-SwaI digestion and it was made blunt by klenow fragment DNA polymerase. The blunt ended fragment carrying 2 micron origin was ligated to PvuII digested vector pMKFMJG. This plasmid was named pMKFMJS. A simple modification was made in the plasmid pMKFMJS, in which the XhoI recognition site was eliminated by the digestion and refilling with the klenow fragment to generate pMKFMJP.

A hot start PCR was made to amplify the gene ADHI terminator with the primers listed in the Table 7. EcoRI - MFeI digested PCR amplified fragment was inserted to EcoRI digested pCM173vector to construct pMKFMJK plasmid. The ADHI terminator generated was

inserted upstream to the Tetracycline Trans-activator gene (a fusion of tet repressor TetR and

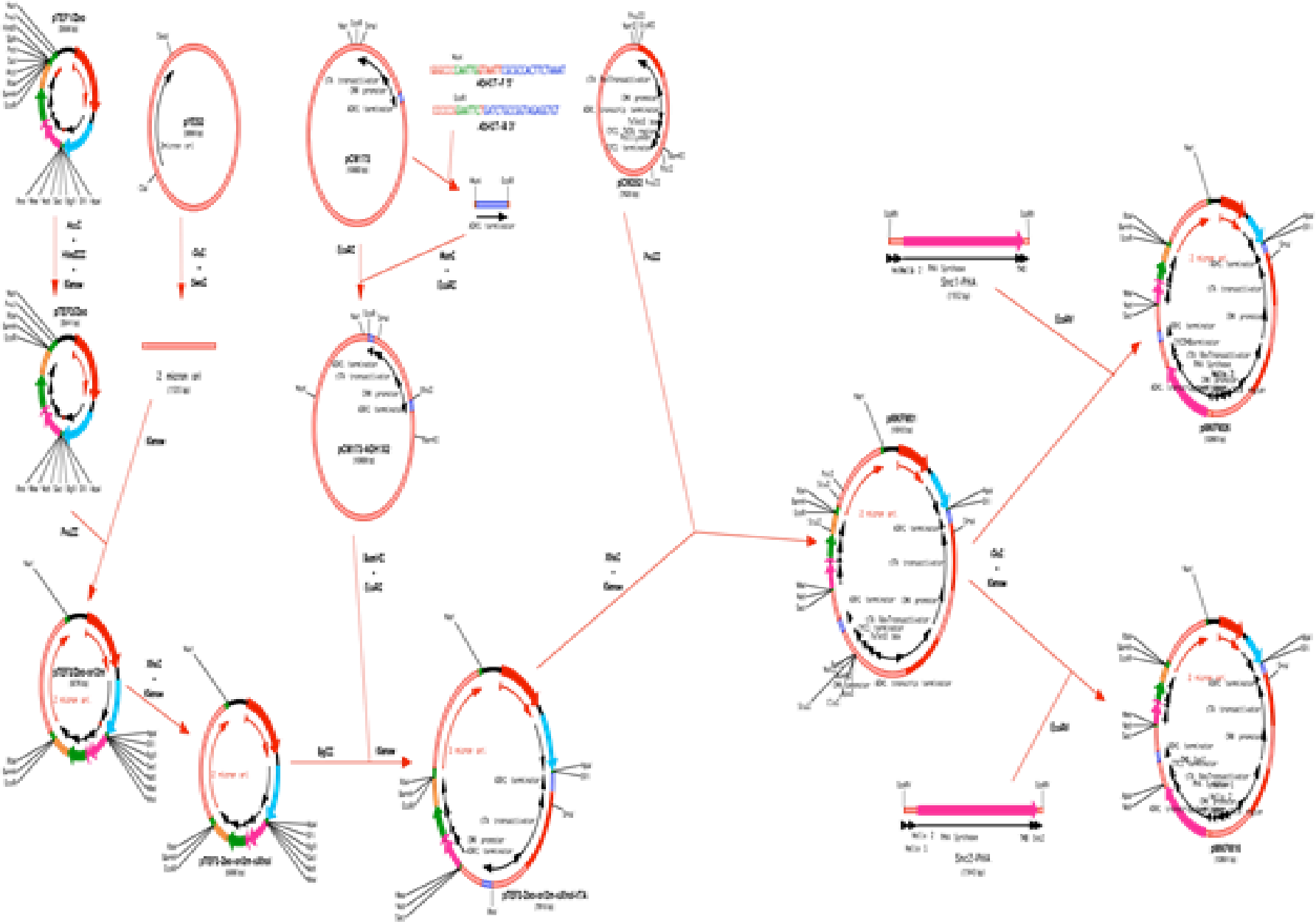


Figure 5 Sequential generation of plasmids to construct pMKFMJ01, pMKFMJ26 expression plasmids

VP16, a trans-activating domain) which would make a complete operon. This tTA operon was excised from pMKFMJK plasmid by digestion of EcoRI and BamHI. The resulting fragment of 2746bp was inserted to NotI filled PMKFMJP vector to make pMKFMJT plasmid. The reverse tetracycline trans-activator system with multiple cloning sites to insert the target gene was excised from pCM252 plasmid by the digestion of PvuII. A blunt ended fragment of 3094bp was inserted to XhoI-filled pMKFMJT vector to construct an empty expression vector pMKFMJ01. The vector constructed in the present study includes 2 micron origin of replication with zeocin resistance as a marker, two regulatory systems of expression (tTA and rtTA), a promoter system comprising TetO7 box fused to CYC1 TATA box regulated under the control of doxycycline antibiotic followed by multiple cloning sites and CYC1 terminator.

4.10 Construction of plasmid pMKFMJ26

To produce the *S. cerevisiae* expressing the fusion cassette, expression plasmid pMKFMJ26 was created. This vector carries a fusion PHA synthase gene in which $phaC_{Ps}$ gene was fused to the 54 amino acids (aas) nucleotide sequence derived from the snc2p gene of *S. cerevisiae* S288c that corresponds to helical loops at nitrogen terminal and 20 aas nucleotide sequence of trans-membrane domain of snc2 was fused at carboxy terminal. This fusion gene encoding a modified PHA synthase from *Pseudomonas putida* CA-3 along with the vesicle targeting signal peptides of *S. cerevisiae* was artificially synthesized and obtained from Genscript, USA. The gene fragment was obtained in the plasmid pUC18 exactly cloned at EcoRV recognition site. The EcoRV recognition sequence flanking the target gene fragment was digested from the plasmid pUC18 to obtain a fragment of 1943bp. This fragment was inserted immediately next to TetO7 Box of NotI filled expression vector pMKFMJ01 to obtain the plasmid pMKFMJ26.

4.11 Construction of plasmid pMKFMJ86

The plasmid construction design was focused to place the EGFP gene at the N-terminus of Chimeric Snc2-PHA synthase gene without any disturbance to the ORF allowing the expression of both functional genes. The construct design is illustrated in the Fig 6. The pEGFP-C1 plasmid was digested by EcoRI and refilled with Klenow fragment to have blunt ends. The blunt ended plasmid vector was phosphorylated with Shrimp Alkaline Phosphatase and gel purified. The Snc2-PHA synthase gene was extracted from pUC57-Snc2 by digestion of EcoRV enzyme and gel purified. The ligation was held at room temperature by T4 DNA ligase and transformant colonies were obtained.

4.11.1 Cloning of EGFP-Snc2-PHA in pMKFMJ01 expression plasmid

The NotI digested, blunt filled and phosphorylated pMKFMJ01 plasmid was used as a vector during cloning process. The insert EGFP-Snc2-PHA was derived from pEFGP-C1-Snc2-PHA using NheI and BamHI. This fragment was blunt filled by Klenow and ligated to the vector pMKFMJ01 by T4 DNA ligase.

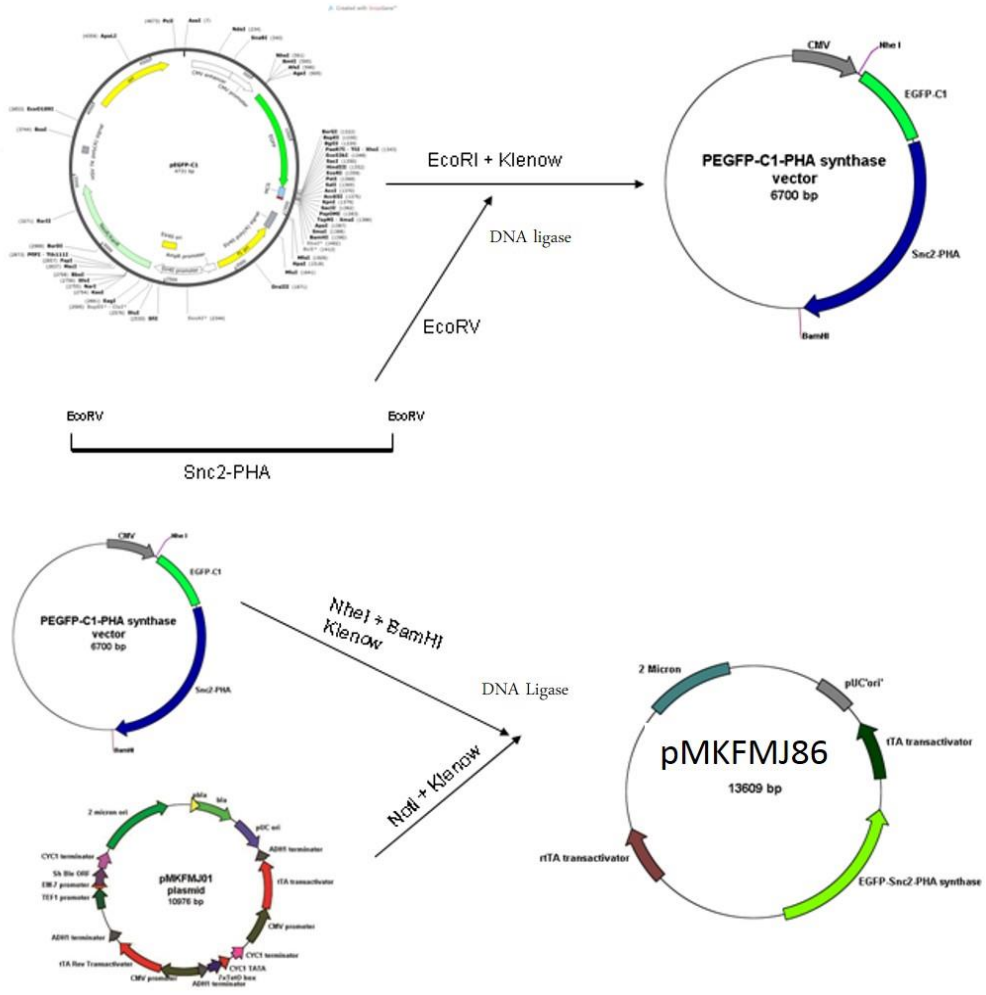


Figure 6 Sequential generation of plasmids to construct pMKFMJ86 expression plasmid carrying EGFP tagged chimeric PHA synthase **4.12**

Electroporation protocol of yeast *S. cerevisiae*

Electroporation methodology described for yeasts was used to transform plasmids in *S. cerevisiae*. Three weeks old colony of *S. cerevisiae* was taken and grown in a 100 ml of YPD medium. The cells were grown at 30°C until the optical density of cells reached 1.3-1.5 OD measured at 600 nm. The cells were collected and centrifuged in two falcon tubes for two times at 5000 rpm and washed with 100 ml of sterile cold water, centrifuged and washed with 50 ml water, centrifuged, suspended in 4 ml of 1M sorbitol (sterile, cold), centrifuged, suspended in 0.1 ml of 1M sorbitol (s, c). The prepared yeasts were aliquoted in 40 µl fraction, mixed with 8 µg of plasmid DNA (TE). The mix was let on ice for 4 min. The

apparatus (biorad, 165-2666) was set to 1.5kV, 25 µF (gene pulser) and 200 Ohms (pulse controller), pulse in a 2 mm chamber (time constant is 4.5 - 5 msec). Immediately, 1ml of ice cold sorbitol was added and poured on YPD plate containing 50 µg/ml of zeocin. The plates were left at 30°C for three days permitting the growth of yeast transformants.

4.13 Plasmid DNA isolation from *S. cerevisiae*:

The plasmidic DNA was isolated from yeast transformant, using the following protocol, was developed by Sobanski and Dickinson, (1985).

Pre-treatment

2 ml of yeast cells were taken and centrifuged at 12000 rpm for 1 min. To the collected cells, 200 µl of solution I (100 mM NaCI, 10 mM Tris-HCI pH 8.0, 1 mM EDTA, 0.1% SDS, w/v) was added in a 1.5 ml micro-centrifuge tube. Acid-washed glass beads (Sigma Aldrich) were added until just below the level of the liquid. The mix was incubated at 65°C for 1 min and vortexed for 30 sec at a maximum speed. Then, the mix was placed in liquid nitrogen for 1 min. This process was repeated for three times to facilitate the breakage of cells and the resulting mixture was used for plasmid DNA isolation.

Isolation protocol

Ice cold solution II (0.2M NaOH, 1% Triton X-100, w/v) (200 µl) was added to pre-treated cell suspension. After mixing by inversion, 150 µl of ice cold solution III (3M sodium acetate, pH 4.8) was added and the sample was again mixed thoroughly. Following a 5 min incubation on ice, the sample (still containing the glass beads) was treated with an equal volume of phenol chloroform-isoamyl alcohol (25:24:1). This mixture was briefly vortexed and centrifuged at 12,000 rpm for 2 min. The aqueous upper phase was transferred to a second tube and the phenol chloroform extraction was repeated. The aqueous layer was then placed in another micro-centrifuge tube and the nucleic acids precipitated with 2 volumes of ethanol (-20°C) plus 0.1 volume of 3 M sodium acetate, pH 6.0. After 30 min. incubation on

ice (or longer if desired) the DNA was pelleted by centrifugation at room temperature for 10 min. This pellet was washed with 1 ml 70% ethanol (v/v) and carefully dried. Plasmid DNA was re-suspended in 80 µl TE buffer (10mM Tris-HCI, 1mM EDTA, pH 8.0) and stored at 4°C. The isolated DNA was analysed by agarose gel electrophoresis.

4.14 Gene expression and polymer production

Expression of chimeric PHA synthase and secretion of nanoparticles by recombinant *S. cerevisiae* strain were carried out in 1000 ml flask with YPD medium at 30°C for 24h. For the maintenance of the plasmids, zeocin was added to the culture medium at a concentration of 50mg/L. In order to express the chimeric protein, the cells were grown in a medium without the inducer doxycycline *i.e.* constitutively regulated by the tTA system. The culture broth at the end of the cultivation was centrifuged to separate the cells and supernatant for the analysis of production and secretion of non-proteinaceous nanoparticles.

4.14.1 EGFP based localization studies

Cells expressing EGFP tagged chimeric constructs were grown in YPD medium at 30°C to mid-logarithmic phase. For the visualization of EGFP, the cells were fixed with 0.3% glutaraldehyde and mounted on glass slides for confocal microscopic studies. EGFP used in all these experiments were extracted from pEGFP-C1 plasmid (A gift from Villegas-Sepúlveda N, Department of Biomedicine Molecular) and it is characterized by red shifted variant of wild type GFP which has excitation maximum at 488nm and emission maximum at 507nm. Confocal microscopic analysis were made at 60X and 100X oil immersion objective using Olympus FV 1000 confocal microscopy and the images were processed with FV 10-ASW 4.0 viewer.

4.14.2 Nile red staining

The hydrophobic stain Nile Red (Sigma N-3013) was used to visualize, with confocal laser scanning microscopy, the polymer produced by the transformant *S. cerevisiae* pMKFMJ26

expressing the chimeric PHA synthase (Kacmar et al., 2005). The yeast cells grown in YPD medium for 24 h at 30°C were treated. To stain the hydrophobic polymeric inclusions, 25 µl of a 250 mg/L solution of Nile Red prepared in acetone were added to 2 mL of cell suspension. The solution was incubated at room temperature for approximately 15 min at dark before being analysed using Confocal microscope Leica TCS SP5 using an oil Ph3 CS objective. Nile Red was excited at 488 nm, and fluorescence was detected at a bandwidth of 555-620 nm.

4.15 Characterization of nanoparticles

4.15.1 Recovery of non-proteinaceous nanoparticles from supernatant

The extracellular polymeric nanoparticles were recovered from the supernatant. The particles were separated from cells and its debris, medium components and metabolites secreted by the organism.

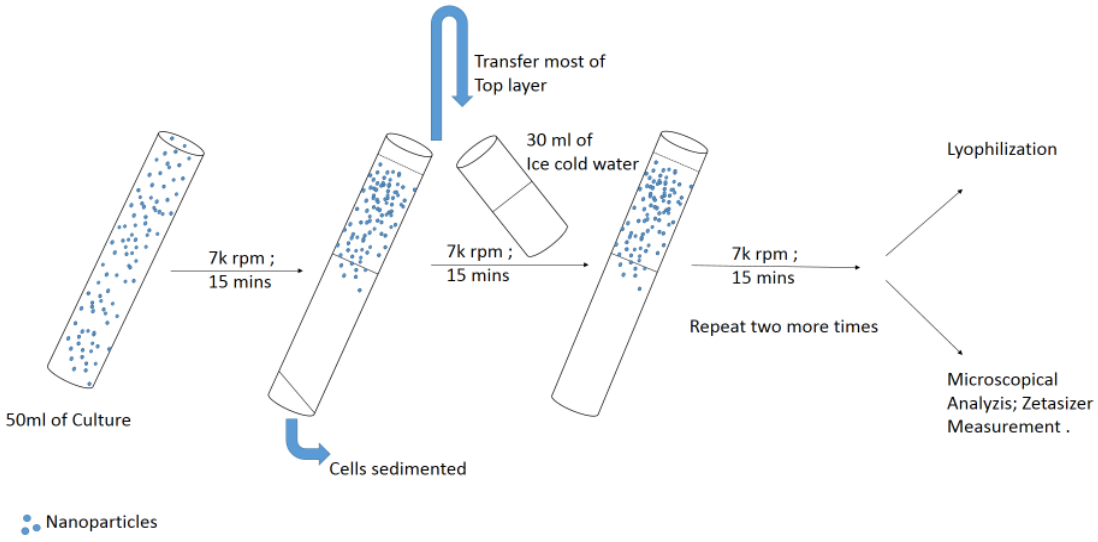


Figure 7 A schematic representation of the working principle and conditions in the separation of nanoparticles from the rest of the medium.

The protocol to separate the nanoparticles using centrifugation is depicted in the fig 7. The cells were centrifuged at 7,500 rpm for 15 min to have a complete separation of cells and

particles. The upper superficial layer of the supernatant containing the nanoparticles was recovered (containing particles) and washed sequentially for 3 times sequentially with ice cold water. The upper layer superficial phase was collected following in each every wash. The finally recovered superficial phase upper layer was taken to precede lyophilization. The materials (centrifuges tubes) used were previously sterilized and the recovered nanoparticles were stored at 4°C. All the procedures were done in aseptic conditions to avoid any contamination. Supernatants of the wild type yeast strain (which didn't carry the PHA synthase fusion protein) and the YPD medium used to grow the yeasts were used as controls. All controls were prepared by following the same protocol used to the transformant strain supernatant.

4.15.2 Morphological analysis

The presence and morphology of nanoparticles in supernatants of the transformant strain was observed by Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). Secreted nanoparticle morphology was investigated by means of scanning electron microscopy (SEM) using a JEOL LSM5600LV scanning electron microscope. The lyophilized nanoparticles were suspended in deionised water. A drop of the suspension was placed on the carbon and copper tapes, and dried overnight before SEM analysis.

4.15.3 Size measurement

To study the agglomeration in the nanoparticles, the secreted nanoparticles were suspended in the following three different liquid suspensions: Distilled Water, (2) Tris HCl (pH.7), (3) Phosphate buffered saline (pH.7)

The particle size distribution (average particle diameter) was determined by the light-scattering in the three different liquid suspensions. The particle size and zeta potential were measured by a laser light scattering Zetasizer Nano ZS of Malvern, UK. Plastic cuvettes

were used for size measurement. The supernatant (2 ml) was loaded to the respective sampler without any air bubbles. Triplicate measurements were made through 32 sweeps per sample. Water was used as reference for normalization.

4.15.4 Polymer Extraction

Supernatant was added with the $(1/4)^{th}$ volume of chloroform and mixed well by magnetic stirring. The chloroform extract was filtered and the filtrate was added directly to hexane (1:2) to precipitate the PHAs.

4.15.5 Fourier Transform –Infrared (FT-IR) spectroscopy

The functional groups and types of bonding present in the nanoparticles were determined by FT-IR using a spectrophotometer (PerkinElmer1600) in an exploration window from 350 to 4000cm^{-1}. The spectra were obtained through 32 sweeps per sample, whereas air was used as reference for normalization.

5. Results

5.1 Designing the Chimeric Cassette

The current investigation identified an appropriate fusion design for the single step immobilization of a heterologous protein PHA synthase to secretory vesicles (PGVs) of *S. cerevisiae*. The transport mechanism of a vesicular membrane protein called SNAREs was studied to design the fusion protein. A large number of membrane proteins had been identified in the post Golgi vesicles (PGVs) of *S. cerevisiae* (Guo et al., 1999; Gerst et al., 1992; Protopopov et al., 1993; Ossig et al., 1995; Jedd et al., 1997). Among them, Forksman et al., 2010 had shown that the characterized membrane proteins from secretory vesicles of *S. cerevisiae* are Ras/Rab Gtpases (Ypt31/32p, Sec4p, Sec2p) and SNAREs (Snc1p/2p and Sso1p/2p). It has been postulated that these proteins are anchored to the membrane surface of the vesicles by either TMD (SNAREs) or prenylation motif (Rab Gtpases) (Protopopov et al., 1993; Ossig et al., 1995; Jedd et al., 1997).

The literature had shown that the TMD domain allows SNARE proteins to maintain its contact with the vesicle permanently throughout the vesicle transport. Thus, SNAREs could be the appropriate candidate for demonstrating the immobilization of heterologous protein to PGVs. Vesicles SNARE (v-SNARE –Snc1p/Snc2p) and target SNARE (t-SNARE; plasma membrane; Sso1p/Sso2p) are the two types of reported SNAREs (Burri and Lithgow, 2004). The complete study of Snc1p and Snc2p in *S. cerevisiae* had shown role of two domains: the helical loop (acting with t-SNARE allowing vesicle fusion; ER to Golgi translocation) and trans-membrane domain (TMD; provides an anchorage to the protein) on translocation mechanism (Protopopov et al., 1993; Gerst, 1997). These two domains of Snc2p (v-SNARE) were chosen in the present work for immobilizing the PHA synthase to vesicles.

Additionally, the activity of the PHA synthase was considered essential in the fusion protein design. From the reports, focusing on the mutation studies on PHA synthase activity, it was concluded that the N-terminal part is not indispensable and the C-terminal part plays a significant role in the PHA production (Rehm et al., 2001 and 2002; Jahns and Rehm, 2009). Particularly, various fusion cassettes of PHA synthase with GFP and maltose binding protein (MBP) were used to study the function of both fused proteins. The hydrophobic N-termini of GFP and, MBP enhanced the activity of the PHA synthase which in turn influenced the production of PHA granules (Jahns and Rehm, 2009). Taking into account, in this work, the hydrophobic TMD of v-SNAREs is fused to the C-termini of PHA synthase, while the hydrophilic helical loop of v-SNAREs is fused with N-termini of PHA synthase. The C-termini fused TMD would provide the permanent anchorage to the PHA synthase once it is integrated to the vesicular membrane. The N-termini fused helical loops allow the PHA synthase protein to follow the translocation mechanism, incorporating them to surface of vesicles. Accordingly, a graphical representation of the design in placing the domains at right position along with the PHA synthase is presented in the Fig 8. The protein sequence of the selected regions in the v-SNAREs is tabulated in the Table 12 and the sequence of the full length protein is schematized in Fig 9.

The developed fusion protein could hypothetically work in two ways: (1) In the *S. cerevisiae* vesicular transport system, the fusion protein itself could mimics the function of v-SNAREs and allows the vesicle fusion by interacting with t-SNAREs present in the plasma-membrane, (2) The SNARE anchored PHA synthase would be active to synthesize the polymer in the hydrophobic environment of vesicles. Then, this synthesized polymer, in the mobile hydrophobic lipid membranous vesicles environment, could be secreted as like as proteins through vesicles naturally.

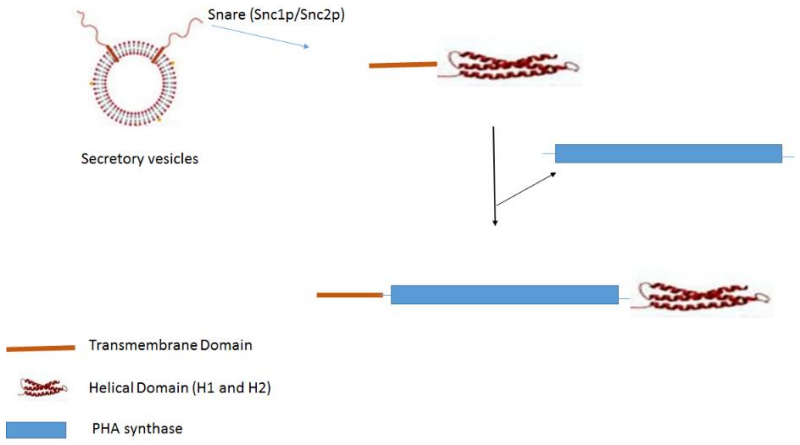


Figure 8 Schematic design of PHA synthase fusion protein for efficient translocation to secretory vesicles

Table 12 Selected SNAREs regions for immobilization of PHA synthase and its protein sequence

Domains	Regions	Snc1p	Snc2p
Helical Loops	**32-85**	LQAEIDDTVGIMRDNINK VAERGERLTSIEDKADN LAVSAQGFKRGANRVRK AM	RQEIDDTVGIMRDNINK VAERGERLTSIEDKADNL AISAQGFKRGANRVRKQ MW
Transmembrane Domain	**96-117**	LALVIIILLVVIIVPIAVHFS R	FLVVI ILLVVIIVPI VVHFS

atggcgagacaagagattgatgacacggtgggaataatgagagataatatcaacaaggtt
gctgaacgtggtgaaaggctaacatccattgaggacaaagctgataacttggctatctcc
M A R Q E I D D T V G I M R D N I N K V A E R G E R L T S I E D K A D N L A I S
gcacaaggattcaagagaggcgccaacagggtcagaaagcaaatgtggtggaaagatcta
atgactgaaaaaccaggtaaaggttctactactttgccagctactagaatgaatgttcaa
A Q G F K R G A N R V R K Q M W W K D LM T E K P G K G S T T L P A T R M N V Q
aatgctattttgggtttgagaggtagagatttgttgtctactttgagaaatgttggtaga
catggtttgagacatccattgcatactgctcatcatttgttggctttgggtggtcaattg
N A I L G L R G R D L L S T L R N V G RH G L R H P L H T A H H L L A L G G Q L
ggtagagttatgttgggtgatactccatatcaaccaaatccaagagatgctagattttct
gatccaacttggtctcaaaatccattttatagaagaggtttgcaagcttatttggcttgg
G R V M L G D T P Y Q P N P R D A R F SD P T W S Q N P F Y R R G L Q A Y L A W

caaaaacaaactagacaatggattgatgaatctcatttgaatgatgatgatagagctaga
gctcatttttttgtttaatttgattaatgatgctttggctccatctaattctttgttgaat
Q K Q T R Q W I D E S H L N D D D R A R A H F L F N L I N D A L A P S N S L L N
ccattggctgttaaagaattgtttaatactggtggtcaatctttggttagaggtgttgct
catttgttggatgatttgagacataatgatggtttgccaagacaagttgatgaaagagct
P L A V K E L F N T G G Q S L V R G V A H L L D D L R H N D G L P R Q V D E R A
tttgaagttggtgttaatttggctgctactccaggtgctgttgtttttagaaatgaattg
ttggaattgattcaatattctccaatgtctgaaaaacaacatgctagaccattgttggtt
F E V G V N L A A T P G A V V F R N E L L E L I Q Y S P M S E K Q H A R P L L V
gttccaccacaaattaatagattttatatttttgatttgtctgctactaattcttttgtt
caatatatgttgaaatctggtttgcaagtttttatggtttcttggtctaatccagatcca
V P P Q I N R F Y I F D L S A T N S F V Q Y M L K S G L Q V F M V S W S N P D P
agacatagagaatggggtttgtcttcttatgttcaagctttggaagaagctttgaatgct
tgtagatctatttctggtaatagagatccaaatttgatgggtgcttgtgctggtggtttg
R H R E W G L S S Y V Q A L E E A L N A C R S I S G N R D P N L M G A C A G G L
actatggctgctttgcaaggtcatttgcaagctaaaaaacaattgagaagagttagatct
gctacttatttggtttcttggttggatagaaaatttgaatctccagcttctttgtttgct
T M A A L Q G H L Q A K K Q L R R V R S A T Y L V S W L D R K F E S P A S L F A
aatgaacaaactattgaagctgctaaaagaagattttatcaaagaggtgttttggatggt
ggtgaagttgctagaatttttgcttggatgagaccaaatgatttgatttggaattattgg
N E Q T I E A A K R R F Y Q R G V L D G G E V A R I F A W M R P N D L I W N Y W
gttaataattatttgttgggtaaaactccaccagcttttgatattttgtattggaatgct
gattctactagattgccagctgctttgcatggtgatttgttggaatttttaaattgaat
V N N Y L L G K T P P A F D I L Y W N A D S T R L P A A L H G D L L E F F K L N
ccattgacttatgcttctggtttggaagtttgtggtactccaattgatttgcaacaagtt
aatattgattcttctactgttgctggttctaatgatcatattactccatgggatgctgtt
P L T Y A S G L E V C G T P I D L Q Q V N I D S S T V A G S N D H I T P W D A V
tatagatctgctttgttgttgggtggtgaaagaagatttgttttggctaattctggtcat
attcaatctattattaatccaccaggtaatccaaaagcttattatttggctaatccaaaa
Y R S A L L L G G E R R F V L A N S G H I Q S I I N P P G N P K A Y Y L A N P K
caatcttctgatccaagagcttggtttcatgatgctaaaagatctgaaggttcttggtgg
ccattgtggttgggttggattactgctagatctggtttgttgaaagctccaagaactgaa
Q S S D P R A W F H D A K R S E G S W W P L W L G W I T A R S G L L K A P R T E
ttgggtaatgctacttatccaccattgggtccagctccaggtacttatgttttgactaga
ttcttagttgttattattttactagtggtaattatcgttcctatcgtcgtccatttcagc
L G N A T Y P P L G P A P G T Y V L T R F L V V V I I L L V V V I I V P I V V H F S

Figure 9 Nucleotide and Amino acid sequence of the PHA synthase fusion protein.

5.2 Generation of expression vector

The cloning and expression of the PHA synthase fusion gene cassette requires an expression vector specific and functional in *S. cerevisiae* yeast. The existing widespread datas on the expression system of *S. cerevisiae* led us to develop the new expression vector pMKFMJ01, with multi-components as shown in Fig 10. The pTEF1/Zeo, a 2µ plasmid, is used as a platform to construct our expression vector with tTA and rtTA as means for regulating the gene expression. So, the expression of PHA synthase fusion gene is regulated by adding the tetracycline and its derivatives (Berens and Hillen, 2003).

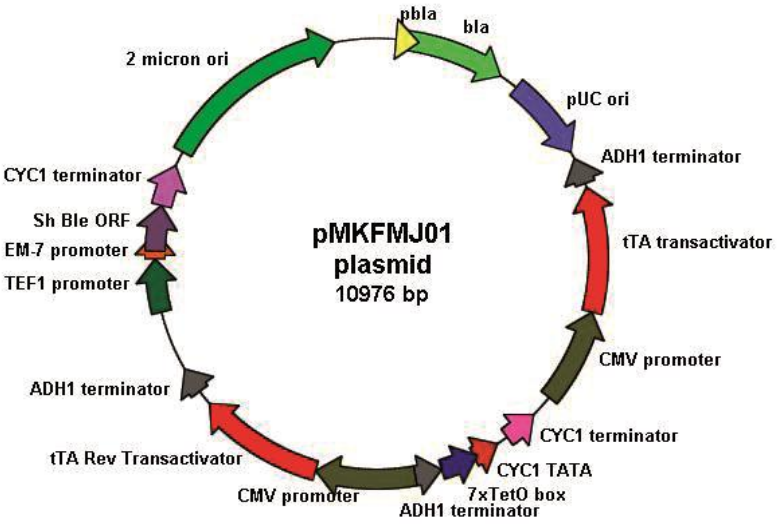


Figure 10 Schematic representation of components of the generated expression plasmid named pMKFMJ01 generated in this work

A series of plasmids was developed, as shown in the fig 5 and 6, to build the expression vector pMKFMJ01. All newly generated plasmids were confirmed by restriction analysis. Both linearization (single cut) and double digestion of the plasmids were made to determine the accuracy of the construction. The plasmid construction verification of the plasmids such as pMKFMJG, pMKFMJS, pMKFMJP, pMKFMJK, pMKFMJT were verified as shown in the Appendix IV. In the following sections, verifications of plasmids pMKFMJ01, pMKFMJ26, and pMKFMJ86 are presented.

The plasmid pMKFMJ01 clones were confirmed by restriction analysis using NotI and EcoRI. NotI digestion as shown in Fig 11 indicates the expected size of pMKFMJ01 plasmid. The use of EcoRI digestion have also ensured that the construction of pMKFMJ01 is complete and in the correct orientation. Fig 11 shows the restriction analysis of the plasmid extracted from *E. coli* DH5α cells.

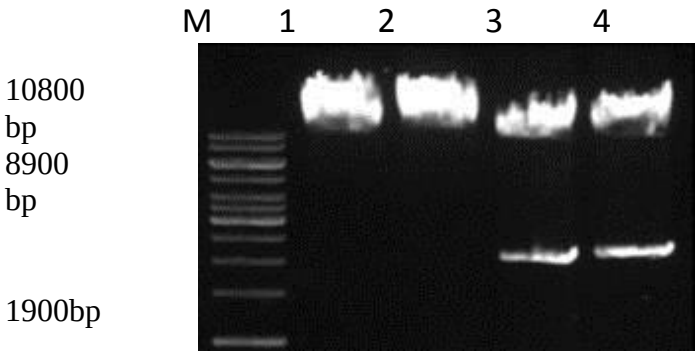


Figure 11 Restriction analysis of the pMKFMJ01 plasmid with EcoRI and NotI restriction enzymes. Lane 1 and 2- plasmid pMKFMJ01 digested with NotI enzyme; Lane 3 and 4 – plasmid pMKFMJ01 digested with EcoRI; M- 1kb DNA

The plasmid pMKFMJ26 carrying the PHA synthase fusion gene cassette, which was placed right next to TetO7 Box, was confirmed by PvuII digestion. Fig 12 shows the restriction pattern of pMKFMJ26 with PvuII.

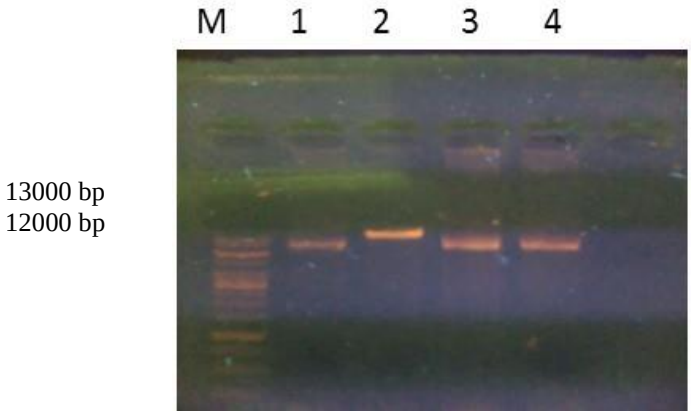


Figure 12 Restriction analysis of the pMKFMJ26 and pMKFMJ16 plasmid with PvuII enzyme. Lane 1, 3 and 4- pMKFMJ16 (carry Snc1- PHA synthase fusion gene); Lane 2 – plasmid pMKFMJ26 (carry Snc2-PHA synthase fusion gene); M- 1kb DNA ladder

The plasmid pMKFMJ86 carrying the EGFP tagged PHA synthase fusion gene cassette, which was placed right next to TetO7 Box, was confirmed by PstI digestion. Fig 13 shows the restriction analysis of pMKFMJ86 with PstI.

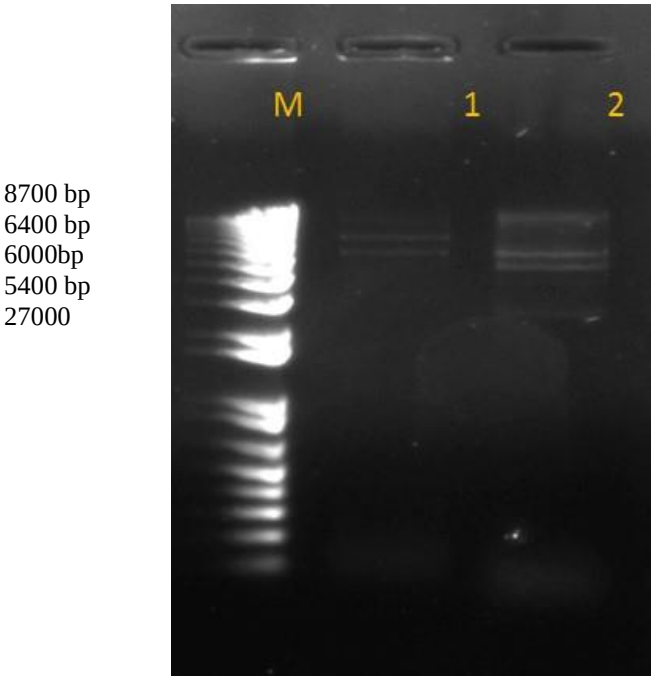


Figure 13 Restriction analysis of the pMKFMJ86 plasmid with PstI enzyme. Lane 1- plasmid pMKFMJ86 (negative orientation); Lane 2- plasmid pMFKMJ86 (positive orientation); M – 1kb DNA ladder

5.3 Localization of the PHA synthase fusion protein in the *S. cerevisiae* transformants

The yeast S. cerevisiae cells were electroporated with generated plasmids and obtained transformants were named as follows – *S. cerevisiae* pMKFMJ86 (expressing EGFP tagged fusion protein), *S. cerevisiae* pMKFMJ87 (expressing EGFP alone; untargeted) and *S. cerevisiae* pMKFMJ26 (expressing PHA synthase fusion protein alone without EGFP). The gene expression was done constitutively in all three strains *i.e.* in absence of the essential inducer molecules such as tetracycline or its analogs (doxycycline, anhydro-tetracycline).

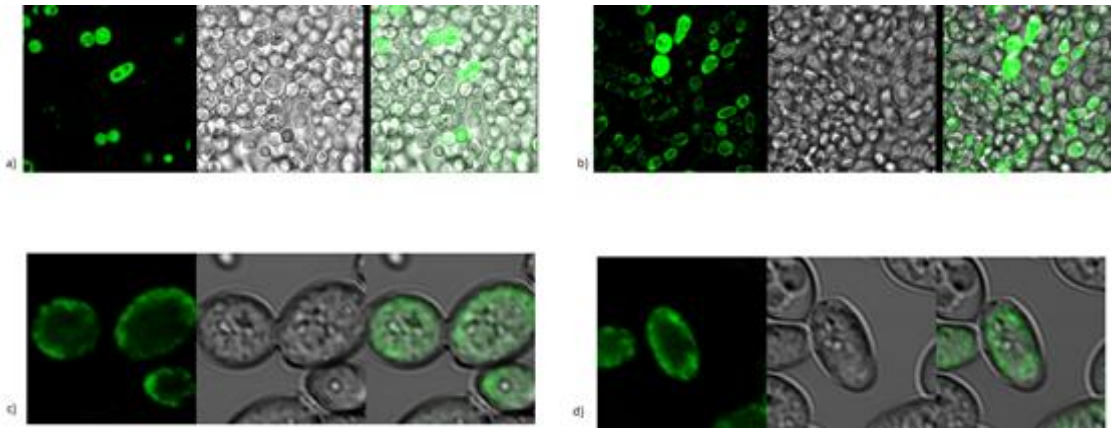
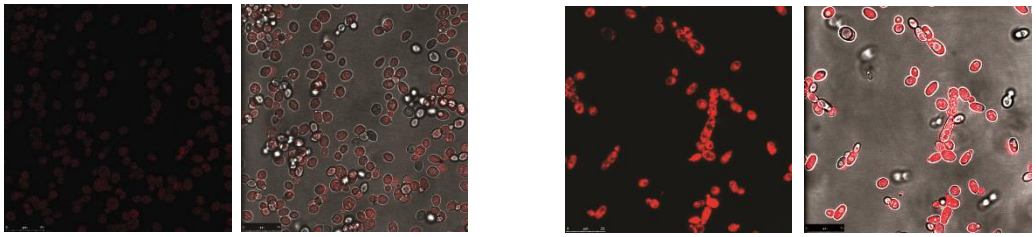


Figure 14 Cellular localization of the EGFP and EGFP with the PHA synthase fusion protein. EGFP-tagged fusion proteins were expressed in yeast and viewed by confocal laser scanning microscopy. Three images of the same cells are shown: EGFP fluorescence (left), bright-field (middle) and merged (right). (a) Cells expressing EGFP alone show an intense fluorescence in the cytosol and (b) Expression of EGFP- PHA synthase fusion protein in *S.cerevisiae* pMKFMJ86. (c) and (d) show the fluorescence of EGFP fused chimeric construct labelling the plasma-membrane with cytoplasmic spots in *S. cerevisiae* pMKFMJ86 cells. (Adapted from Muniasamy and Perez-Guevara, 2014)

The confocal microscopy reveals a distinct subcellular localization of fluorescence in cells expressing the EGFP in *S. cerevisiae*. In cells expressing EGFP, due to the absence of signal domains, the fluorescence accumulated was observed in the cytoplasm (Fig. 14 a). In contrast, *S. cerevisiae* pMKFMJ86 having the fusion construct shows the fluorescence labelling more strongly all through the plasma membrane and in some cytoplasmic regions (Fig 14 b-d) (Muniasamy and Perez-Guevara, 2014). The observations of *S. cerevisiae* pMKFMJ86 strain agree well with the labelling pattern as described earlier for GFP-Snc2p expression (Gurunathan et al., 2000; Lewis et al., 2000). The notable significance in the fluorescence demonstrates that the fusion proteins are translocated and immobilized to the vesicles.

5.4 Functional analysis of PHA synthase fusion protein using Nile Red

After confirming the membrane localization of the EGFP fused PHA synthase fusion protein, the *S. cerevisiae* pMKFMJ26 strain expressing PHA synthase fusion protein was analyzed for the production of polymer. Preliminary evidence of polymer production was obtained by Nile red staining, which is able to disclose hydrophobic lipid bodies (Kacmar et al., 2005). The staining was carried out to wild type and transformant strain pMKFMJ26. The confocal microscopy imaging shows notable differences between the strains. The Fig. 15 shows intense fluorescence from transformant pMKFMJ26 suggests the production of polymer, no significant fluorescence was seen in the wild type strain (lacking the PHA synthase gene). These images indicate that the PHA synthase fusion protein is active to synthesize polymers in the vesicle bound state.



(a) (b)

Figure 15 Photographs of two *S. cerevisiae* strains obtained with the confocal microscopical imaging. Nile Red is a hydrophobic dye that is able to stain hydrophobic bodies. (a) Wild Type ATCC 9763 cells, control lacking PHA synthase gene, appears without fluorescence. (b) *S. cerevisiae* pMKFMJ16, which expresses PHA synthase fusion protein, show fluorescence upon Nile red staining.

5.5. Characterization of secreted polymeric nanoparticles

5.5.1 FESEM (Field Emission-SEM) and TEM microscopy

The preliminary characterization of secreted polymeric nanoparticles was made by FESEM. The microscopical imaging had shown the presence of nanoparticles in the washed supernatant of pMKFMJ26 samples (Fig16, 2C). Contrastingly, there were no particles

present in the samples of washed medium and in the supernatant of ATCC 9763 cultures (Fig 16, 2A and 2B). Figure 16 (1A and 1B) shows the extracellular polymer particles of the transformant strain pMKFMJ26. This demonstrated the capability of synthesizing and secreting nanoparticles by the transformant strain pMKFMJ26. The extracellular occurrence of the nanoparticles was explained to be due to the activity of immobilized PHA synthase fusion protein in the vesicles.

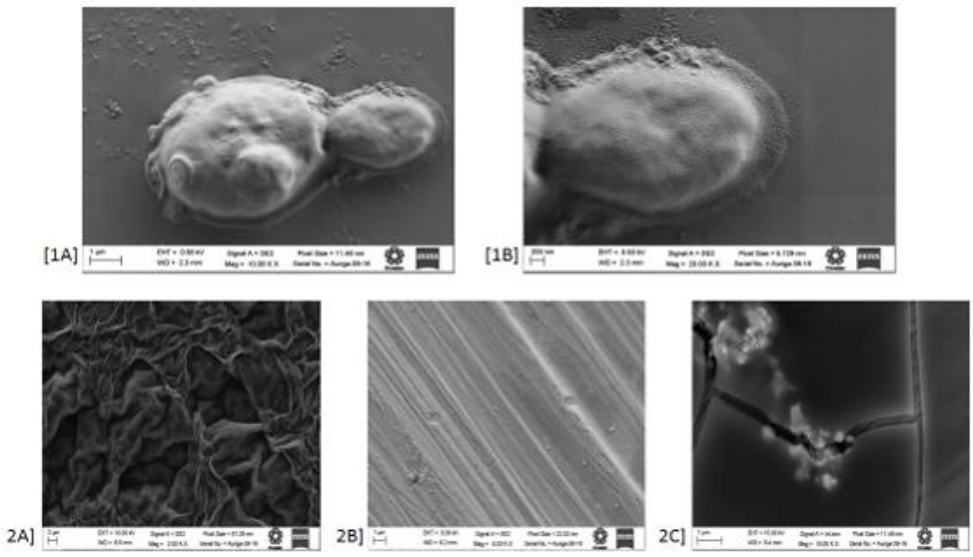


Figure 16 FESEM microscopical images [1A] and [B] shows the extracellular nanoparticles of the transformant *S. cerevisiae* pMKFMJ26. [2A, and 2B] No nanoparticles can be seen in the samples of washed medium and supernatant of the ATCC 9763 strain. Image [2C] confirms the presence of nanoparticles in supernatant of the transformant pMKFMJ26.

The microscopical observation of nanoparticles prepared in three different liquid suspensions demonstrates the presence of dispersed and agglomerated nanoparticles (Figures 17 and 18). A comparison of particles size measured in zetasizer is presented in the Table 13. Due to interference of salts in the imaging and size measuring, water was used as the liquid suspension for the separation of the particles.

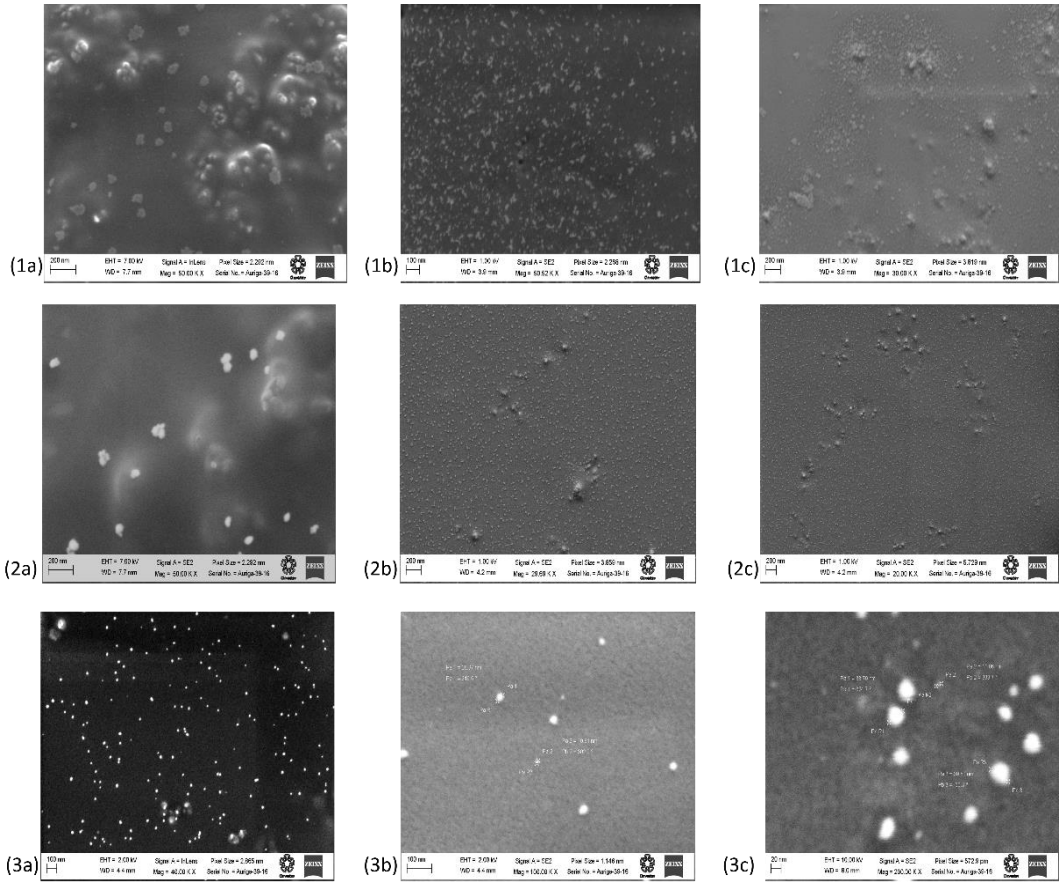
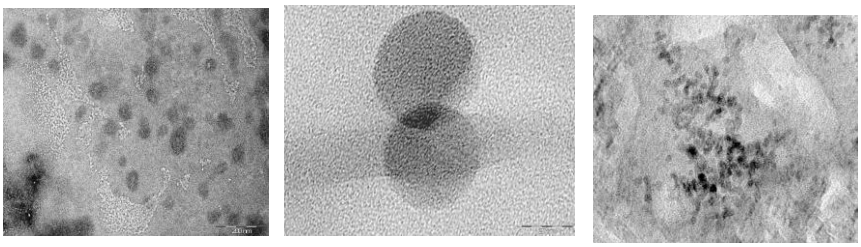


Figure 17 FESEM images of extracellular secreted nanoparticles at different scales ranging from 20 – 200 nm (1a-1c) water washed; (2a-2c) Tris HCl washed and (3a-3c) PBS washed. In all cases, the nanoparticles were obtained from the supernatant of the transformant pMKFMJ26.



(A) (B) (C)

Figure 18 TEM images showing water washed (A and B) and PBS washed (C) extracellular nanoparticles. In all cases, the nanoparticles were obtained from the supernatant of the transformant pMKFMJ26.

Table 13 Size distribution of extracellular secreted nanoparticles in different liquid suspension measured in zetasizer.

Samples	Size (nm)
Tris HCl	128.5 139.1, 21.88 250.8
Water	138.6, 26.95 210.5 207.7
PBS	140.1 159.4, 24. 27, 9.69 249.3, 49.04

5.6 Preliminary Chemical Characterizations secreted nanoparticles

As a result of chloroform extraction, unlike PHAs, no polymer film was obtained. Hence, the lyophilized nanoparticles obtained were used for analysis of the chemical composition. The identification of the chemical structure of these particles is under process. However, the FT-IR and Energy dispersive X-ray spectroscopy studies (Appendix V) suggested the presence of nitrogen in the polymer backbone. Probably, this could be a significant reason for the insoluble nature of secreted nanoparticles in chloroform. Moreover, a region corresponding -C-O-C- (1100 cm^{-1} region) was identified from the FT-IR studies. Therefore, to identify structure of the unknown secreted polymer, a broad research on studies of chemical characterization will be carried out in the future.

6. Discussion

PHA synthase, the soluble cytoplasmic free enzyme, of different organisms, are expressed in a way to translocate them to the specific cellular organelles with the respective signal peptides as shown in Table 4. The translocated PHA synthase enhanced the production of PHAs inside the targeted organelles of eukaryotic organisms. However, there are both *in vivo* and *in vitro* studies describing the immobilization of PHA synthase. The already existed single step strategy reported in immobilizing the variety of industrial enzymes in the natural and artificial materials was an advantage to the present work (Table 1). The heterologous proteins immobilization on PHA granules and magnetosomes has demonstrated the use of identified proteins and domains as anchors present on their surface.

Using this background knowledge, the present work had shown a single step immobilization of the PHA synthase to the secretory vesicles (PGVs) of *S. cerevisiae* using the domains of the Snc2p, a v-SNARE protein present in the membrane surface of PGVs. Previous fusion studies had shown the production and translocation of proteins to PGVs using these domains. For example, Ossig et al., 1995 demonstrated that the Ypt1p, a Rab Gtpase, could be anchored to the membrane of vesicles using the TMD of v-SNAREs instead of native prenylation motif. Grote et al., 2000 have also examined the localization of v-SNAREs to PGVs, upon replacement of transmembrane domain with the geranylgeranyl lipid anchor. Similarly, our work had shown the transfer of the PHA synthase to the surface of PGVs with the membrane anchorage provided by TMD of Snc2p. Besides, helical domains of Snc2p, carrying the significant information code of translocation, was also added to the N-terminal of PHA synthase for the successful translocation.

EGFP tagged version of the PHA synthase fusion protein was used to verify the protein translocation to vesicles. According to the previous authors (Gurunathan et al., 2002; Lewis et al., 2000), the EGFP protein gene was tagged in the N-terminus of the PHA synthase

fusion gene cassette. The EGFP tagged PHA synthase fusion protein cassette was cloned into an expression plasmid pMKFMJ86 under the control of $TetO_7$ promoter regulated by tTA and rtTA components. A control plasmid carrying only EGFP protein (pMKFMJ87) was developed to discriminate the fluorescence pattern among the strains. The expression pattern of these strains analyzed through confocal microscopy revealed a major difference in fluorescence distribution. *S. cerevisiae* pMKFMJ86 had shown a pattern of fluorescence along the plasma membrane (Fig. 14), a natural feature of snc2p in *S. cerevisiae* organisms, which is discussed below. On the other side, fluorescence was seen all through the cell of *S. cerevisiae* pMKFMJ87.

The fluorescent pattern observed in the pMKFMJ86 strain could be explained by the behavior of Snc2p protein in the wild type organism. During the vesicle fusion, a strong interaction occurs between v-SNAREs (Snc1p/Snc2p) and t-SNAREs (Sso1p/Sso2p) in the plasma membrane. The α-helical domains of v-SNAREs and t-SNAREs interact with each other to form a stable, high affinity complex which initiates the vesicle fusion (Burri and Lithgow, 2004). Taking this into account, as like v-SNAREs, during vesicles fusion, the PHA synthase fusion protein (vesicle) carrying helical domain holds the capability to interact and fuse with the t-SNAREs (plasma membrane). The interaction of PHA synthase fusion protein with t-SNARE permits the latter to stay at membrane for a short period of time, before it was recycled to Golgi complex (TGN) (Lewis et al., 2000). Thus, before recycling to TGN through endocytosis, the PHA synthase fusion protein was seen in the plasma membrane (cis-SNARE) apart from vesicles membrane (trans- SNARE) (Gurunathan et al., 2002; Lewis et al., 2000).

With this fluorescent studies, the integrity of fusion protein translation can be argued. The fluorescence pattern observed suggested that the sequence of snc2p domains and EGFP utilized were placed in the exact position to localize PHA synthase onto the secretory

vesicles. Otherwise, if either component of the sequence was not placed at the expected position, the translated protein would be misplaced and the visualized fluorescence would be similar to the control i.e., spread through the cytoplasm. Hence, the usage of snc2p domains has efficiently transferred the PHA synthase fusion protein to membrane of PGVs as like as natural protein Snc2p.

Cytoplasmic puncture spots were seen in pMKFMJ86 fluorescence pattern (Fig. 14). These spots are due to use of multi copy plasmids in this study, as in the case of Curwin et al., 2013 report. They have presented the expression of GFP-Snc1p utilizing a high copy 2µ plasmid and expressed fusion protein GFP-Snc1p was accumulated as cytoplasmic punctuate spots in live cells. Contrastingly, use of centromeric plasmids usually limited the expression and permits the localization of v-SNARE proteins (Snc1p & Snc2p) to almost exclusively on plasma-membrane (Gurunathan et al., 2002; Lewis et al., 2000). As expected with the usage of 2µ plasmid, the EGFP tagged PHA synthase fusion protein was found coating the plasma membrane with cytoplasmic spots all through the cell in the present work (Muniasamy and Perez-Guevara, 2014).

In addition to usage of 2µ plasmid, the level of gene expression generated by the regulatory system also played a significant role in the distribution of GFP-Snc1/2p. Previously, it has been demonstrated that the expression level produced by Tet system is higher than the level obtained with homogenous promoters (Tef, Gal, and Leu) on *S. cerevisiae* (Belli et al., 1998; Wishart et al., 2005). In the present work, the gene expression was made constitutive (tTA; tet off system) i.e., active in absence of inducer molecules (dox, tet). Many reports have shown that the maximum expression reaches within the period of 12 hours in *S. cerevisiae* using the Tet system (Belli et al., 1998; Wishart et al., 2005). Such maximal expression of the PHA synthase fusion protein was seen in the pMKFMJ86 strain which resulted in the cytoplasmic spots throughout the cell (Fig. 14). In some cases, the fluorescence was seen in

endosome like structures in the cells of pMKFMJ86. So these spots were seen because of the recycling nature of the fusion protein similar to endogenous Snc2p through endocytosis. Moreover, the recycling process of PHA synthase fusion protein has to be studied detail in future works. This fluorescent distribution observed in *S. cerevisiae* pMKFMJ86 also explained clearly that the Snc2p regions of fusion protein exhibited functional activity in trans-locating themselves to vesicles and later to plasma-membrane during fusion (Muniasamy and Perez-Guevara, 2014).

From the literature reports, it was known that the overexpression of vesicle trafficking proteins resulted in the impairment of growth in *S. cerevisiae*. For example, overexpression of Sec15p from the strong GAL/ promoter interferes with the transport of the secretory vesicles at a stage between the Golgi apparatus and the plasma membrane, causing the vesicles to aggregate. Interference with the secretory pathway is generally associated with a growth defect (Novick et al., 1980). But, as SNARE proteins are the rate limiting components in the secretion machinery, there is not any such impairment of growth in the SNARE overexpression *S.cerevisiae* strains. Ruohonen et al., 1997 confirmed non growth defect in the *S.cerevisiae* cells when the t-SNAREs (sso1p/2p) were overexpressed for the enhancement of protein secretion. Similarly, the overexpression of PHA synthase fusion protein in *S. cerevisiae* PMKFMJ26 and PMKFMJ86 does not have any observable effect in the growth. The growth rate of both the wild type and transformant strains were found to consistent (unpublished data). Thus, the overexpression of SNAREs was confirmed as non-harmful to the growth of *S.cerevisiae* and a key factor for increasing the secretion process.

Recently, Kiyono et al., 2011 had demonstrated the expression the SYP111 (homolog of Sso 1/2p) fused bacterial MerC protein resulted in the localization at the plasma membrane. They have also examined that the localization of active MerC protein increased the accumulation of the mercury inside the cell. In the present work, as a result of expression

and immobilization, PHA synthase of fusion protein had found to exhibit the activity of synthesizing and secreting a polymer by *S. cerevisiae*. This was confirmed by the significant difference in fluorescence between the transformant pMKFMJ26 and wild type strain after staining with Nile red (Fig. 15). As a consequence of the immobilization, the synthesized polymer was found extracellular e.g., in the supernatant of the pMKFMJ26 culture. The secretion of the polymer particles were demonstrated by the microscopical analysis such as FESEM, TEM. The transformant strain pMKFMJ26 have shown to secrete number of non-proteinaceous polymeric particles, when there are not any particles found in the wild type (Fig. 16).

The precise role of the PHA synthase in production and secretion of the particles remains open and to be worked out. But, this process of secretion could be explained based on the concept of the vesicular transport system. The PHA synthase was unaltered and found functionally active in the immobilized fusion protein. Under these circumstances, the active PHA synthase fusion protein would polymerizes the monomer(s) available in the cytoplasm and prepares granules as nanoparticles, very probably, at the hydrophobic membrane of vesicles. The vesicles fusion with the plasma membrane allows granular polymeric particles pass to the extracellular surface along with the secretory proteins.

The size of the particles measured in the microscopy studies was in the range of 4 – 100 nm. Further, dynamic light scattering gave information on the nanoparticle agglomeration. Particles dispersed in each suspension had shown different degrees of agglomeration (Figures 17 and 18). To understand the state of agglomerations, the chemical interaction between the nanoparticles should be established. But unfortunately, only preliminary tests were made to identify the chemical structure of the secreted particles of the transformant pMKFMJ26. Most of the subsequent research would be focused to identify the chemical composition of the polymer. The insolubility nature of the particles in solvents such as

chloroform, dichloromethane and presence of the nitrogen in the backbone of the polymer (Appendix V), suggests strongly that the secreted polymer was not a polyhydroxyalkanoate and it has to be studied in detail. This suggests that the immobilized PHA synthase fusion protein polymerizes a polymer which is certainly not belonging to group of PHAs.

Rahman et al., 2013 had reported the secretion of PHA granules via type1 secretion in *E. coli*. This study expressed the active PHA synthase in the cytoplasmic region to accumulate PHA granules, which were later exported by bacterial Type I secretion systems. They demonstrated the fusion of PhaP1 to Hly signal peptides secrete PHA granules through the secretion channel of size ranging from 3.5-14 nm. But, in the present work, the PHA synthase was immobilized to a mobile vesicular organelles rather than expressing it in cytoplasm. The substrate specificity of immobilized PHA synthase fusion protein in PGVs seems to be unpredictably different. This was evident in the presence of nitrogen in the so far identified chemical composition of the secreted polymer synthesized by the immobilized PHA synthase fusion protein. The substrate specificity of the PHA synthase in the immobilized state has to be studied in detail.

Most of the conclusions demonstrating the transport behavior of PHA synthase fusion protein are derived from the Snc2p wild-type protein. It was evident from the present work that the membrane anchors of vesicular proteins could be a targeting moiety in the single step immobilization. These studies make evident that the domains of Snc2p are capable of displaying the heterologous protein to surface of the PGVs in *S. cerevisiae.* In addition, this process facilitates the heterologous protein to retain the functional activity in the immobilized state. This was clear in the present work that the impact of immobilizing PHA synthase fusion protein had resulted in the production of the non-proteinaceous particles to the extracellular milieu.

Combining together the localization and particles secretion studies, the usage of v-SNAREs to immobilize an active bacterial protein PHA synthase was developed and it was demonstrated by using the fluorescent marker EGFP protein.

7. Conclusions

In the present study, we have shown that the immobilization of active PHA synthase onto membrane of vesicles in *S. cerevisiae* is practiable. The immobilization of PHA synthase onto vesicles was confirmed by fluorescent microscopy observation. The activity of immobilized PHA synthase in secretory vesicles was validated *in vivo* by the microscopical observation of polymeric nanoparticles in the culture supernatant of transformant pMKFMJ26.

Despite unresolved questions on the characterization of the nanoparticles, this work shows PGVs are a practical support for immobilization and surface display of enzymes. The advantageous part represents the option for the exploitation of the secretory vesicles for immobilization process rather than the utilizing them for secretion of the recombinant proteins. The present work also highlighted that active PHA synthase bound onto vesicles can be used to produce and secrete the polymeric particles. Therefore, the strategy to immobilize heterologous proteins onto vesicles was experimentally established and validated in this work.

8. Perspectives

Several motivating outcomes from the immobilization of PHA synthase to the secretory vesicles, as follows, has to be addressed in future research studies:

1. Reconstitution of PHA synthase to vesicles/liposomes – *in vitro* PHA synthase fusion protein activity

   Expression and purification of PHA synthase fusion protein from *E. coli*,
   Preparation of the liposomes integrating the PHA synthase fusion protein to its surface,
   Measuring the PHA synthase fusion protein activity,
   Microscopical imaging of the polymer particles – TEM, SEM and Atomic Force Microscopy,
   Characterization - Size exclusion chromatography, gas chromatography.

2. Production of the polyhydroxyalkanoates by the expression of PHA synthase fusion protein

   Transformation and expression of the PHA synthase fusion gene to the following hosts – *R. eutropha* PhaC1 negative strain and *E. coli fad* mutant strains,
   Localization of PHA synthase fusion protein during the course of the growth and PHA production – confocal microscopical analysis,
   PHA production with different substrates,
   Characterization – Gas chromatography, NMR.

3. Activity of the PHA synthase fusion protein onto the secretory vesicles of *S. cerevisiae*

   Use of late *sec* mutants to isolate the vesicles with the PHA synthase fusion protein on its surface,
   Measuring the PHA synthase fusion protein activity,
   Microscopical imaging of the polymer particles – TEM, SEM and AFM,
   Characterization - Size exclusion chromatography, gas chromatography.

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Appendices
Appendix I- Proposed models in the biosynthesis of PHA

Three models for granule biogenesis are elucidated in the Figure A.1. The first one is the "micelle" model, which based on the assumption that the PHA synthase is present in the cell as a soluble enzyme, more or less randomly distributed in the cytoplasm. Once polymerization starts, the nascent polyester chain converts the initially soluble enzyme into an amphipathic molecule and the increasing hydrophobic PHA chains aggregate into a micelle-like structure. In this model, the constituents of the boundary layer, that is, phospholipids and other GAPs apart from the synthase, would gradually become incorporated as the self-assembled PHA inclusion increases in size (Jossek et al., 1998: Rehm et al., 2002). The second model is the more recent "budding" model, which suggests that the PHA synthase localizes to the inner face of the cytoplasmic membrane (Fig. A.1), either inherently or as soon as a PHA chain emerges from the enzyme. In this case, biosynthesis of the polyester would be directed into the inter-membrane space where the extending chains would accumulate until eventually PHA inclusions surrounded by a phospholipid monolayer would bud off the membrane (Tian et al., 2005a). The third model (*Scaffold Model*) assumes that PHB synthase of nascent PHB granules is or becomes attached to a yet unknown scaffold molecule within the cell. In this case, subcellular localization of PHB granules would depend on the nature and localization of the scaffold of the PHB accumulating cell. Contributions of Sinskey/Stubbe have suggested that PHB granule formation most likely proceeds via a scaffold mechanism with the bacterial nucleoid being the scaffold in *R. eutropha*.

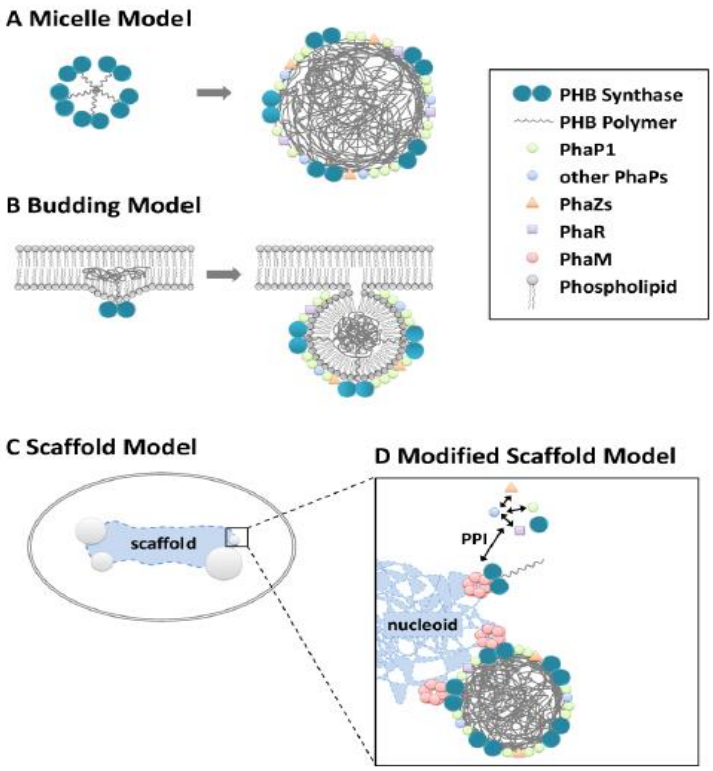

Figure A.1 Proposed models for the formation of polyhydroxyalkanoate granules.

Appendix II- DNA coding sequences of enzymes used in this study

Nucleotide Sequence of phaC2 Polymerase of *Pseudomonas putida* CA-3 (Genbank_id=”AAU44815.1”)

ATGACTGAAAAACCAGGTAAAGGTTCTACTACTTTGCCAGCTACTAGAATGAA
TGTTCAAAATGCTATTTTGGGTTTGAGAGGTAGAGATTTGTTGTCTACTTTGAG
AAATGTTGGTAGACATGGTTTGAGACATCCATTGCATACTGCTCATCATTTGTT
GGCTTTGGGTGGTCAATTGGGTAGAGTTATGTTGGGTGATACTCCATATCAACC
AAATCCAAGAGATGCTAGATTTTCTGATCCAACTTGGTCTCAAAATCCATTTTA
TAGAAGAGGTTTGCAAGCTTATTTGGCTTGGCAAAAACAAACTAGACAATGGA
TTGATGAATCTCATTTGAATGATGATGATAGAGCTAGAGCTCATTTTTTGTTTA
ATTTGATTAATGATGCTTTGGCTCCATCTAATTCTTTGTTGAATCCATTGGCTGT
TAAAGAATTGTTTAATACTGGTGGTCAATCTTTGGTTAGAGGTGTTGCTCATTT
GTTGGATGATTTGAGACATAATGATGGTTTGCCAAGACAAGTTGATGAAAGAG
CTTTTGAAGTTGGTGTTAATTTGGCTGCTACTCCAGGTGCTGTTGTTTTTAGAA
ATGAATTGTTGGAATTGATTCAATATTCTCCAATGTCTGAAAAACAACATGCTA
GACCATTGTTGGTTGTTCCACCACAAATTAATAGATTTTATATTTTTGATTTGTC
TGCTACTAATTCTTTTGTTCAATATATGTTGAAATCTGGTTTGCAAGTTTTTATG
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GTTCAAGCTTTGGAAGAAGCTTTGAATGCTTGTAGATCTATTTCTGGTAATAGA
GATCCAAATTTGATGGGTGCTTGTGCTGGTGGTTTGACTATGGCTGCTTTGCAA
GGTCATTTGCAAGCTAAAAAACAATTGAGAAGAGTTAGATCTGCTACTTATTT
GGTTTCTTGGTTGGATAGAAAATTTGAATCTCCAGCTTCTTTGTTTGCTAATGA
ACAAACTATTGAAGCTGCTAAAAGAAGATTTTATCAAAGAGGTGTTTTGGATG
GTGGTGAAGTTGCTAGAATTTTTGCTTGGATGAGACCAAATGATTTGATTTGGA
ATTATTGGGTTAATAATTATTTGTTGGGTAAAACTCCACCAGCTTTTGATATTTT
GTATTGGAATGCTGATTCTACTAGATTGCCAGCTGCTTTGCATGGTGATTTGTT
GGAATTTTTTAAATTGAATCCATTGACTTATGCTTCTGGTTTGGAAGTTTGTGG
TACTCCAATTGATTTGCAACAAGTTAATATTGATTCTTCTACTGTTGCTGGTTCT
AATGATCATATTACTCCATGGGATGCTGTTTATAGATCTGCTTTGTTGTTGGGT
GGTGAAAGAAGATTTGTTTTGGCTAATTCTGGTCATATTCAATCTATTATTAAT
CCACCAGGTAATCCAAAAGCTTATTATTTGGCTAATCCAAAACAATCTTCTGAT
CCAAGAGCTTGGTTTCATGATGCTAAAAGATCTGAAGGTTCTTGGTGGCCATTG
TGGTTGGGTTGGATTACTGCTAGATCTGGTTTGTTGAAAGCTCCAAGAACTGAA
TTGGGTAATGCTACTTATCCACCATTGGGTCCAGCTCCAGGTACTTATGTTTTG
ACTAGA

Nucleotide Sequence of Snc2p of *Saccharomyces cerevisiae* S288c (locus_tag="YOR327C")

ATGTCGTCATCAGTGCCATACGATCCATATGTGCCTCCAGAGGAGAGTAACTC
AGGCGCAAACCCAAATTCCCAAAACAAGACTGCTGCTTTGAGACAAGAGATTG
ATGACACGGTGGGAATAATGAGAGATAATATCAACAAGGTTGCTGAACGTGGT
GAAAGGCTAACATCCATTGAGGACAAAGCTGATAACTTGGCTATCTCCGCACA
AGGATTCAAGAGAGGCGCCAACAGGGTCAGAAAGCAAATGTGGTGGAAAGAT
CTAAAAATGAGAATGTGTTTATTCTTAGTTGTTATTATTTTACTAGTGGTAATT
ATCGTTCCTATCGTCGTCCATTTCAGCTAA

Appendix III- Solutions molecular biology protocols

Components of Fiche technique

TFB-1 solution

30 mM potassium acetate	0.294g/100ml
100 mM rubidium chloride	1.21g
10 mM $CaCl_2$	0.147g
50 mM $MnCl_2$	0.99g
15% glycerol	

Adjust pH to 5.8 with 0.2 M Acetic Acid, Don't adjust with KOH.

TFB-2 solution

10 mM MOPS	0.209g/100mL
75 mM $CaCl_2$	1.102g
10 mM Rubidium Chloride	0.121g
15% Glycerol	

Adjust the pH to 6.5 with KOH. After adjusting the pH to both the solutions, it has to be filtered and stored at 4^0C.

Components of 'Quick plasmid mini-prep kit-Invitrogen technologies'

Resuspension buffer - (R3; 50 mM Tris-HCl, pH 8.0; 10 mM EDTA)

Lysis buffer - (L7; 200 mM NaOH, 1% w/v SDS)

Precipitation buffer - 3 M NaoAC pH to 4.8 with glacial acetic acid

Wash buffer - 10 mM Tris-HCl pH 7.5, 80% ethanol

Appendix IV – Restriction verification of plasmids

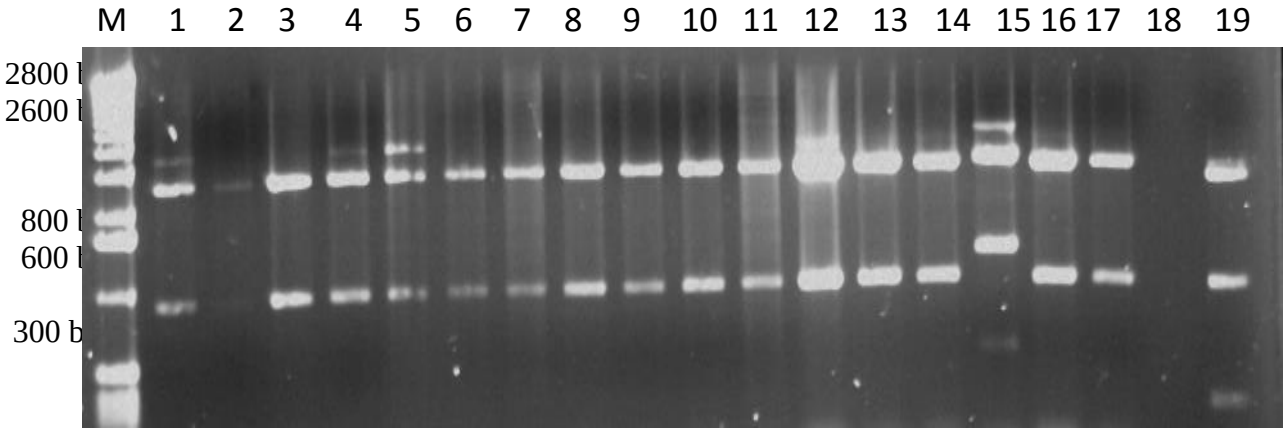


Figure A.2 Restriction digestion of pMKFMJG with PstI and EcoRV. Lane 1-6, 17 – pMKFMJS ; Lane 19 – pTEF1/Zeo

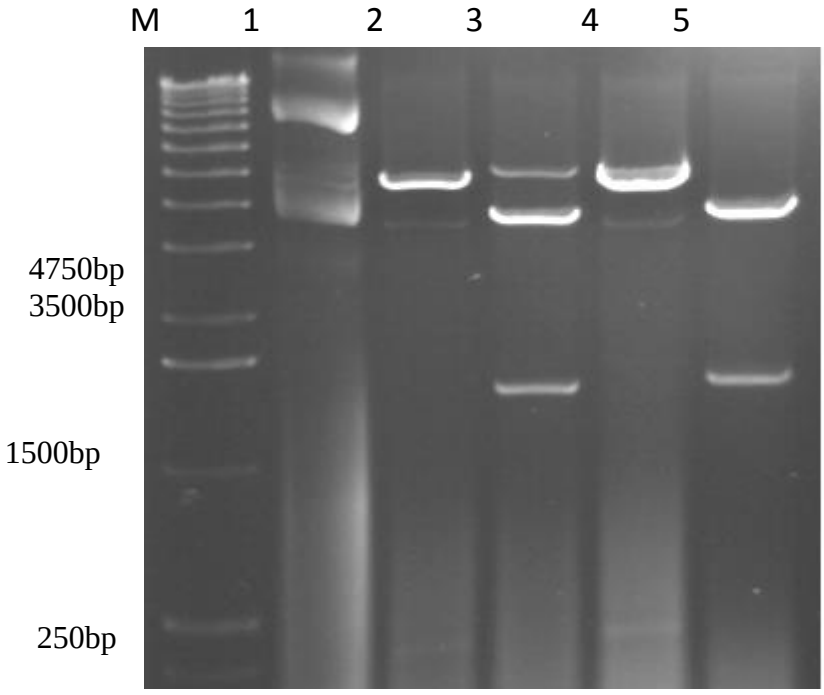


Figure A.3 Restriction digestion of pMKFMS with PstI and EcoRI. Lane 1 – pMKFMJS; Lane 2,4 – pMKFMJP (positive orientation) ; Lane 3,5 – pMKFMJP (negative orientation)

The PCR conditions followed to obtain ADH1 terminator from PCM173 plasmids were as follows:

Initial Denaturation	- 94^{o}C for 3 min
Denaturation	- 94^{o}C for 30 sec
Annealing	- 50^{o}C for 1 min
Extension	- 60^{o}C for 3 min
Cycles	- 35
Cool at 4^{o}C	

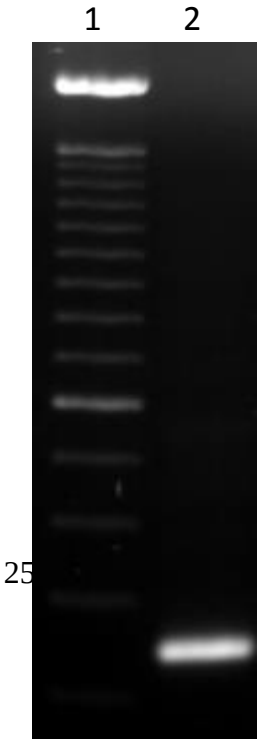


Figure A.4 PCR product of ADH1 Terminator from pCM 173 Lane 1 – 1kb ladder; Lane 2 – 250 bp PCR product

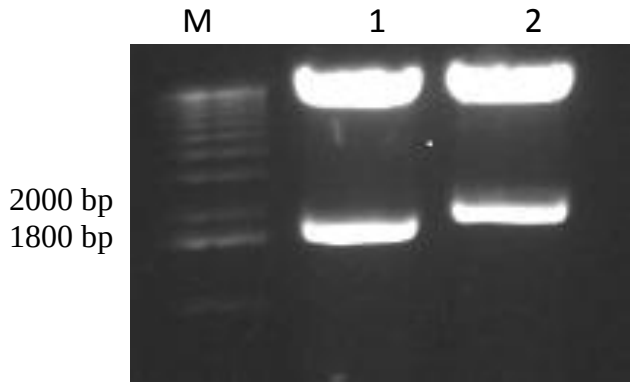
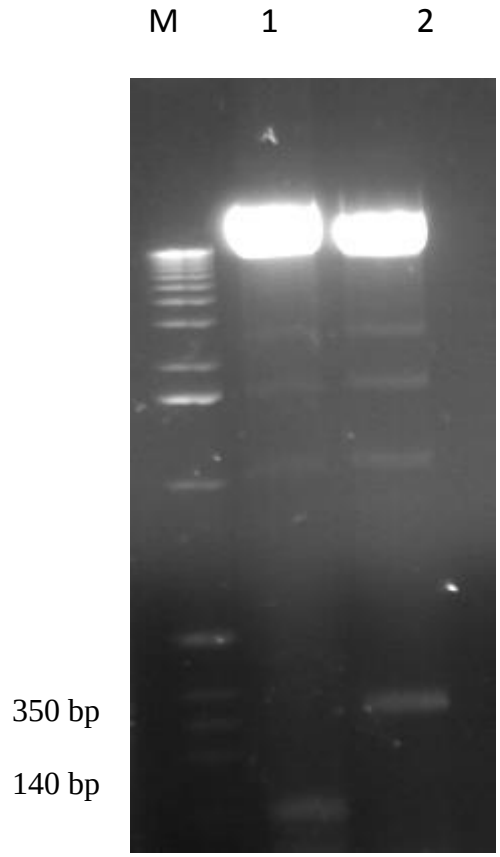


Figure A.5 Restriction digestion of pCM173 and pMKFMJK with EcoRI and XhoI. Lane 1- pCM173 plasmid; Lane 2 – plasmid pMKFMJK



gure A.6 Restriction digestion of pCM173 and pMKFMJK EcoRI and SmaI. Lane 1 – plasmid pCM173; Lane 2 – plasmid pMKFMJK

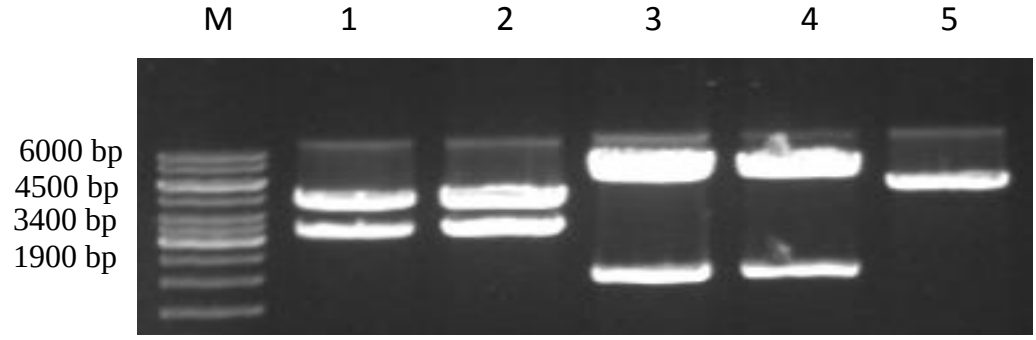


Figure A.7 Restriction digestion of pMKFMJT with EcoRI and XhoI. Lane 1, 2 – plasmid pMKFMJT (negative orientation); Lane 3, 4 – plasmid pMKFMJT (positive orientation); Lane 5 – plasmid pMKFMJK

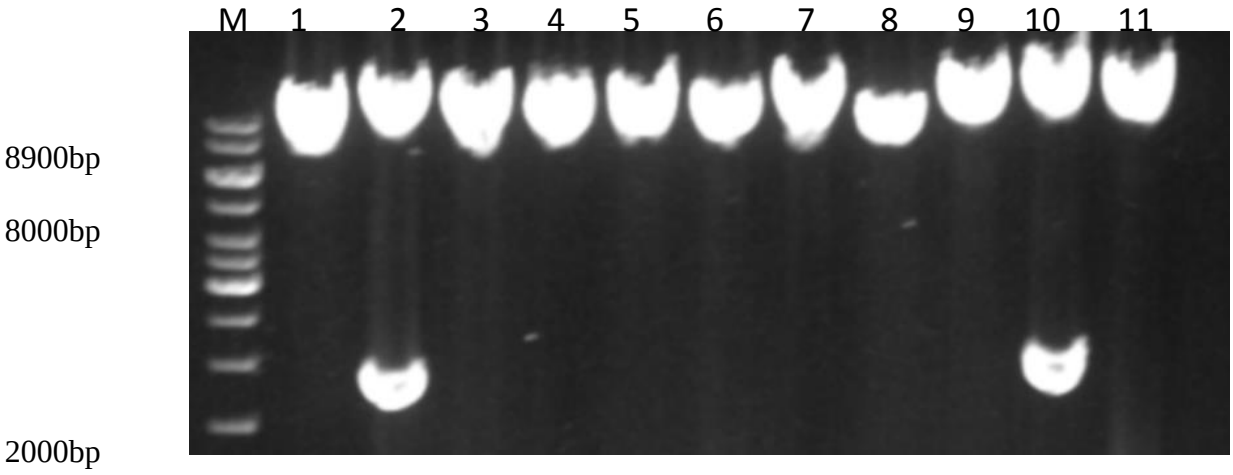


Figure A.8 Restriction digestion of pMFKMJ01 and pMKFMJwith EcoRI. Lane 1, 2-9, 11 – pMKFMT; Lane 2, 10 – pMKFMJ01

Appendix V-Chemical characterization of nanoparticles

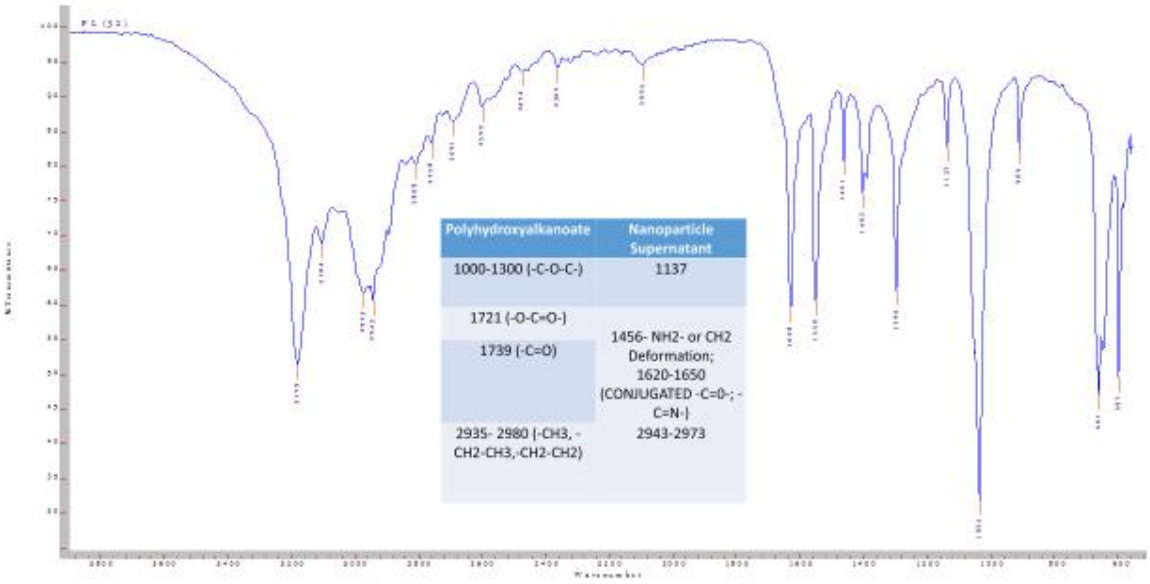
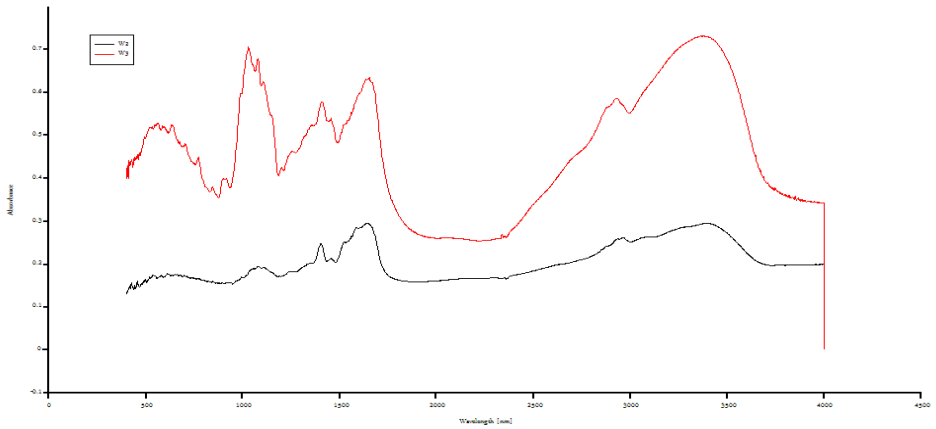
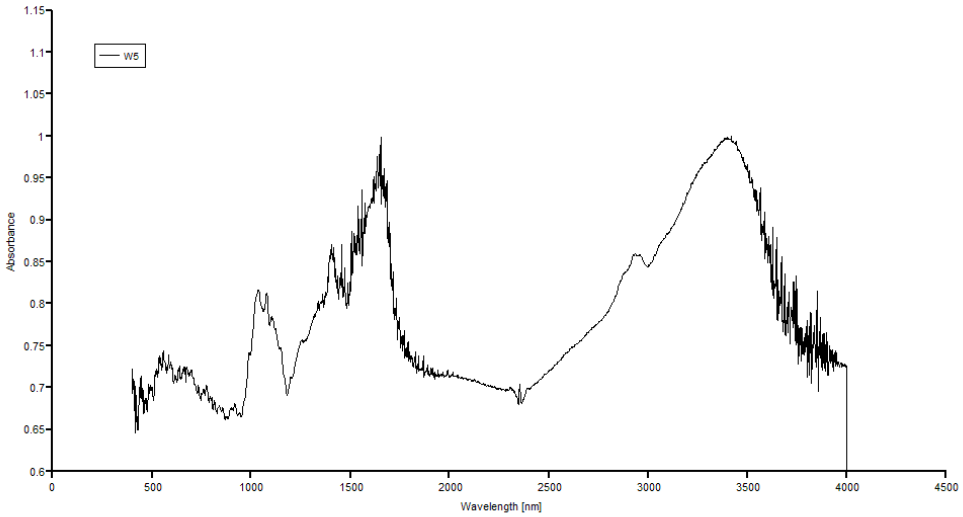


Figure A.9 FT-IR spectra obtained from the lyophilized (three times washed supernatant) samples of transformant pMKFMJ26. The spectra was compared with the known PHB and it was tabulated.

[A]





[B]

Figure A.20 FT-IR spectra demonstrating the role of subsequent washes in the reduction of the impurities

Spectrum: spectra

Element	Series	unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
Carbon	K-series	1.79	25.92	33.96	0.36
Chlorine	K-series	0.55	7.91	3.51	0.05
Nitrogen	K-series	0.47	6.75	7.58	0.17
Oxygen	K-series	3.34	48.25	47.45	0.53
Sulfur	K-series	0.00	0.00	0.00	0.00
Phosphorus	K-series	0.00	0.00	0.00	0.00
Sodium	K-series	0.74	10.69	7.32	0.07
Potassium	K-series	0.03	0.47	0.19	0.03
	Total:	6.91	100.00	100.00	

Figure A.11 EDX Table representing the distribution of the atoms in the nanoparticles and it confirms the presence of nitrogen molecule in the backbone of the polymer.