INSTITUTO TECNOLÓGICO Y DE ESTUDIOS SUPERIORES DE MONTERREY

CAMPUS MONTERREY

DIVISIÓN DE INGENIERÍA Y ARQUITECTURA
PROGRAMA DE GRADUADOS EN INGENIERÍA

TECNOLÓGICO DE MONTERREY®

APLICACIÓN GENÉRICA DE SISTEMAS DE DOS FASES ACUOSAS PARA PROCESOS DE RECUPERACIÓN PRIMARIA DE COMPUESTOS BIOLÓGICOS

TESIS

PRESENTADA COMO REQUISITO PARCIAL PARA OBTENER EL GRADO ACADÉMICO DE:

DOCTOR EN CIENCIAS DE INGENIERÍA ESPECIALIDAD EN BIOTECNOLOGÍA

POR

JORGE ALEJANDRO BENAVIDES LOZANO

MONTERREY, N.L. **DICIEMBRE 2006**

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Diciembre 2006

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Resumen

En esta investigación se estableció la aplicación genérica de los sistemas de dos fases acuosas (SDFA) polietilénglicol (PEG) – solución salina para el desarrollo de bioprocesos que permiten la recuperación primaria de compuestos biológicos. Para este fin fueron utilizados cuatro modelos experimentales: B-ficoeritrina (BFE) producida por *Porphyridium cruentum*, C-ficocianina (CFC) producida por *Spirulina maxima*, pseudo-partículas 2/6 de rotavirus (dlRLP) producidas por células de insecto High Five™ y por último luteína producida por *Chlorella protothecoides*.

Se estudió la influencia de los parámetros de sistema de dos fases acuosas PEG – solución salina (peso molecular del polímero, PM PEG; longitud de línea de corte, LLC; relación de volumen, V_R; el pH del sistema, porcentaje de muestra, etc) sobre el comportamiento de partición de los modelos experimentales seleccionados. Se diseñaron procesos para la recuperación primaria de los productos de interés utilizando SDFA. Dichos procesos cuentan con un reducido número de etapas, lo cual favorece el rendimiento obtenido.

La influencia de los parámetros de sistema sobre el comportamiento de partición fue correlacionada con las características fisicoquímicas de los compuestos de interés. Se establecieron reglas heurísticas que permiten el desarrollo predictivo de procesos para la recuperación primaria de compuestos biológicos utilizando SDFA PEG – solución salina. Este diseño predictivo toma como base las características fisicoquímicas del producto de interés, así como las de los contaminantes presentes. Mientras mayor es el peso molecular del compuesto de interés menor debe ser el PM PEG utilizado para llevar a cabo la recuperación en la fase superior del sistema. El aumento del PM PEG y de la LLC genera un incremento en el volumen excluido de agua en la fase superior del sistema, lo cual favorece la partición de compuestos hidrofóbicos de bajo peso molecular hacia dicha fase. Es recomendable trabajar a valores de pH que brinden carga electroquímica negativa al compuesto de interés, de tal manera que su partición hacia la fase superior sea favorecida.

Las características fisicoquímicas de los sistemas experimentales seleccionados abarcan desde proteínas hidrofílicas de gran tamaño (dlRLP) hasta compuestos hidrofóbicos de bajo peso molecular (luteína). Esto permite suponer que el diseño predictivo de procesos de recuperación primaria utilizando sistemas de dos fases acuosas PEG – solución salina puede ser aplicado a un amplio espectro de compuestos biológicos (proteicos o no proteicos).

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Abreviatura	Significado
Abs	Absorbancia
BFE	B-ficoeritrina
CFC	C-ficocianina
DEAE	Dietil-aminoetil
dlRLP	Pseudo-partículas 2/6 de rotavirus (*double layered Rotavirus-Like Particles*)
EBA	Cromatografía de adsorción en cama expandida (*Expanded Bed Adsorption*)
EGFPVP2	Proteína de fusión GFP – VP2
GFP	Proteína verde fluorescente (*Green fluorescent protein*)
H/D	Relación Altura/Diámetro
HPLC	Cromatografía líquida a altas presiones (*High Pressure Liquid Chromatography*)
LLC	Longitud de línea de corte
nm	Nanómetro
OD_{Xnm}	Densidad óptica (*Optical density*) medida a X nm
PEG	Polietilénglicol
pI	Punto isoeléctrico
PM	Peso molecular
rpm	Revoluciones por minuto
SDFA	Sistemas de dos fases acuosas
V_R	Relación de volúmenes
X g	Gravedades

Capítulo 1. Introducción

1.1 Procesos biotecnológicos

El ser humano ha utilizado procesos biotecnológicos para su beneficio desde el principio de la civilización. El desarrollo tecnológico permitió generar el entendimiento necesario para comprender los fenómenos detrás de los procesos biotecnológicos que hasta ese momento se practicaban de forma empírica. En la actualidad es posible tomar un microorganismo, modificarlo genéticamente de ser necesario, y cultivarlo bajo condiciones controladas para producir compuestos de interés, utilizados en una gran variedad de aplicaciones. En muchas ocasiones no es necesario utilizar el microorganismo, sino solo una parte del mismo, como es el caso de los reactores enzimáticos.

Un proceso biotecnológico consta de dos etapas primordiales. Primeramente se tiene la etapa de producción, la cual tiene como función generar el producto de interés. Posteriormente se tiene la etapa de bioseparación, la cual a su vez consta de: a) recuperación primaria, y b) purificación. Durante la recuperación primaria la mezcla compleja resultante de la etapa de producción (la cual generalmente consta del compuesto de interés y una gran variedad de contaminantes) es procesada para recuperar el producto, removiendo a su vez la mayor cantidad de contaminantes posible. Durante esta etapa se lleva a cabo la liberación del producto, en caso de que el producto de interés sea intracelular. En la mayoría de los casos se llevan a cabo operaciones típicas, tales como centrifugación o filtración con la finalidad de remover partículas de gran tamaño y restos celulares. Posteriormente se busca recuperar la mayor cantidad de producto de interés, y al mismo tiempo remover la mayor cantidad de contaminantes. Esto se hace mediante técnicas como centrifugación, microfiltración, precipitación isoeléctrica, cromatografía en cama expandida, etc (Cisneros-Ruiz y Rito-Palomares, 2005). Se prosigue a la purificación del producto. Dependiendo de las características del compuesto de interés, las características de los contaminantes remanentes y del grado de pureza que se desee se diseña el proceso de purificación que brinde mejores resultados. Operaciones típicas de purificación son la

cromatografía líquida a altas presiones, electroforesis, precipitación escalonada, ultrafiltración, etc (Hagel, 2001; Cunha and Aires-Barros, 2002).

Los sistemas de dos fases acuosas (SDFA) son una técnica de recuperación primaria que puede actuar también para purificación en ciertos sistemas (Rito-Palomares, 2004; Cunha and Aires-Barros, 2002; Johansson and Walter, 2000; Cunha *et al*, 2003; Haraguchi *et al*, 2004; Kepka *et al*, 2003; Marcos *et al*, 2002; Reh *et al*, 2002; Shinomiya *et al*, 2003). En los últimos años esta técnica ha adquirido auge, porque tiene la capacidad de llevar a cabo la integración (sustitución de dos o mas operaciones unitarias por una sola) e intensificación (procesamiento de mayor cantidad de material biológico utilizando equipo de la misma capacidad) de procesos. A pesar de las numerosas ventajas que ha mostrado esta técnica, existe una pobre caracterización genérica para el desarrollo de procesos de recuperación primaria de compuestos biológicos utilizando sistemas de dos fases acuosas. Debido a esto se decidió estudiar la aplicación genérica de sistemas de dos fases acuosas polímero – solución salina para la recuperación de compuestos biológicos, eligiendo cuatro modelos experimentales (B-ficoeritrina, C-ficocianina, pseudo-partículas 2/6 de rotavirus y luteína). Se estudió la influencia de los parámetros de sistema sobre el comportamiento de partición de los modelos seleccionados, para obtener la información necesaria para generar reglar heurísticas que permitan el diseño predictivo de procesos de recuperación primaria mediante sistemas de dos fases acuosas.

1.2 Breve descripción de los sistemas de dos fases acuosas

Los primeros estudios realizados con sistemas de dos fases acuosas se remontan a década de los 50, cuando Albertsson (1956) demostró durante sus estudios doctorales, el potencial de esta técnica para separar y recuperar componentes celulares (organelos), así como pigmentos de algas y cianobacterias. Desde los trabajos de Albertsson hasta la fecha, se han realizado una gran cantidad de estudios acerca del comportamiento de partición de productos de interés comercial en sistemas de dos fases acuosas (Rito-Palomares, 2004; Cunha and Aires-Barros, 2002; Johansson and Walter, 2000).

Existen sistemas de dos fases acuosas polímero – polímero, los cuales están formados por dos fases acuosas poliméricas (polietilénglicol (PEG) – Dextrano, PEG – Polivinil alcohol, etc.). Este tipo de sistemas se han utilizado para separar células vegetales (Edahiro *et al*, 2005), para la producción y recuperación *in situ* de enzimas (Ivanova *et al*, 2001), en cromatografía a contracorriente (Shibusawa *et al*, 2006), etc. Sin embargo, el uso de estos sistemas está limitado por los altos costos de algunos polímeros (principalmente dextrano).

El polietilénglicol (PEG) forma sistemas de dos fases acuosas cuando es combinado con ciertas sales, resultando sistemas polímero – sal (PEG – fosfato de potasio, PEG – sulfato de sodio, etc.) (Albertsson *et al*, 1990). Debido a su bajo costo y corto tiempo de separación, estos sistemas son frecuentemente empleados (Srinivas *et al*, 2002; Esmanhoto and Vahan Kilikian, 2004). Los SDFA más utilizados y mejor caracterizados son los formados por PEG – fosfato de potasio (Rito-Palomares, 2004).

Las características de los productos biológicos, tales como peso molecular, punto isoeléctrico, hidrofobicidad, etc, influyen en su preferencia por cada una de las fases del sistema. Otro factor que es necesario considerar es la presencia de otros compuestos en el sistema de fases acuosas. La presencia de estos compuestos "contaminantes" en los SDFA puede afectar el comportamiento de partición del producto, ya sea generando saturación en las fases del sistema o bien interactuando directamente con el compuesto de interés. Los contaminantes, al interactuar de manera directa con del compuesto de interés, son capaces de enmascarar o modificar las propiedades fisicoquímicas del producto, lo cual altera su comportamiento de partición. La naturaleza de los contaminantes presentes esta íntimamente relacionada con el tipo de sistema de expresión que se este utilizando (bacterias, levaduras, células de mamífero, células de insecto, etc), de las condiciones particulares de cultivo, de si el producto es intracelular o extracelular, del método de ruptura celular utilizado (en caso que el producto sea intracelular), etc. Dependiendo de su función biológica y localización dentro de la célula el compuesto de interés puede encontrarse intrínsecamente ligado a contaminantes de una naturaleza específica (proteínas de membrana, pigmentos accesorio del sistema fotosintético, polisacáridos, etc). La

selección del método de ruptura celular que brinde mejores rendimientos de liberación del compuesto de interés en algunos casos puede ser una tarea laboriosa, ya que muchos sistemas de expresión (como por ejemplo las algas) presentan mucha resistencia ante el impacto mecánico y el tratamiento químico. El conocimiento de estos factores es de suma importancia para el desarrollo predictivo de procesos de recuperación primaria y purificación.

Adicionalmente los parámetros del sistema de dos fases acuosas juegan un papel de gran importancia durante la partición del producto de interés hacia una fase en particular. Entre estos parámetros se encuentran: tipo, peso molecular y concentración del polímero usado para construir el sistema, naturaleza y concentración de las sales utilizadas, diferencias de concentración de los componentes en cada una de las fases, pH del sistema, adición de moléculas con afinidad bioquímica, temperatura, etc (Albertsson *et al*, 1990; Sarubbo *et al*, 2000).

1.3 Parámetros y conceptos básicos en sistemas de dos fases acuosas

Curva binodal de los sistemas

La formación de las fases del sistema esta descrita por el diagrama de equilibrio o curva binodal de ese sistema en particular (Figura 1.1), la cual representa la frontera entre la región monofásica y bifásica. Las combinaciones polímero – sal que se encuentren por debajo de la curva formarán una sola fase heterogénea, mientras que las que se encuentren por arriba de la curva binodal formarán un sistema bifásico. El mecanismo de formación de fases esta relacionado con el balance de las fuerzas entálpicas y entrópicas involucradas en la hidratación de los solutos presentes (Huddleston *et al*, 1991 a).

Longitud de línea de corte (LLC)

La composición de un sistema de dos fases acuosas puede ser expresada en función de la concentración de los componentes en cada una de las fases del sistema a través de la longitud de la línea de corte (LLC; o bien TLL por su nombre en inglés, *Tie Line Length*). Tomando como ejemplo los sistemas en la curva binodal mostrada en la Figura 1.1, la línea

de corte de los sistemas X_1, X_2 y X_3 esta representada por la línea AC. Todos los sistemas que se encuentran sobre una misma línea de corte poseen composiciones idénticas en sus fases inferiores y superiores. Sin embargo estos sistemas difieren en su relación de volúmenes (Huddleston *et al*, 1991 a y b).

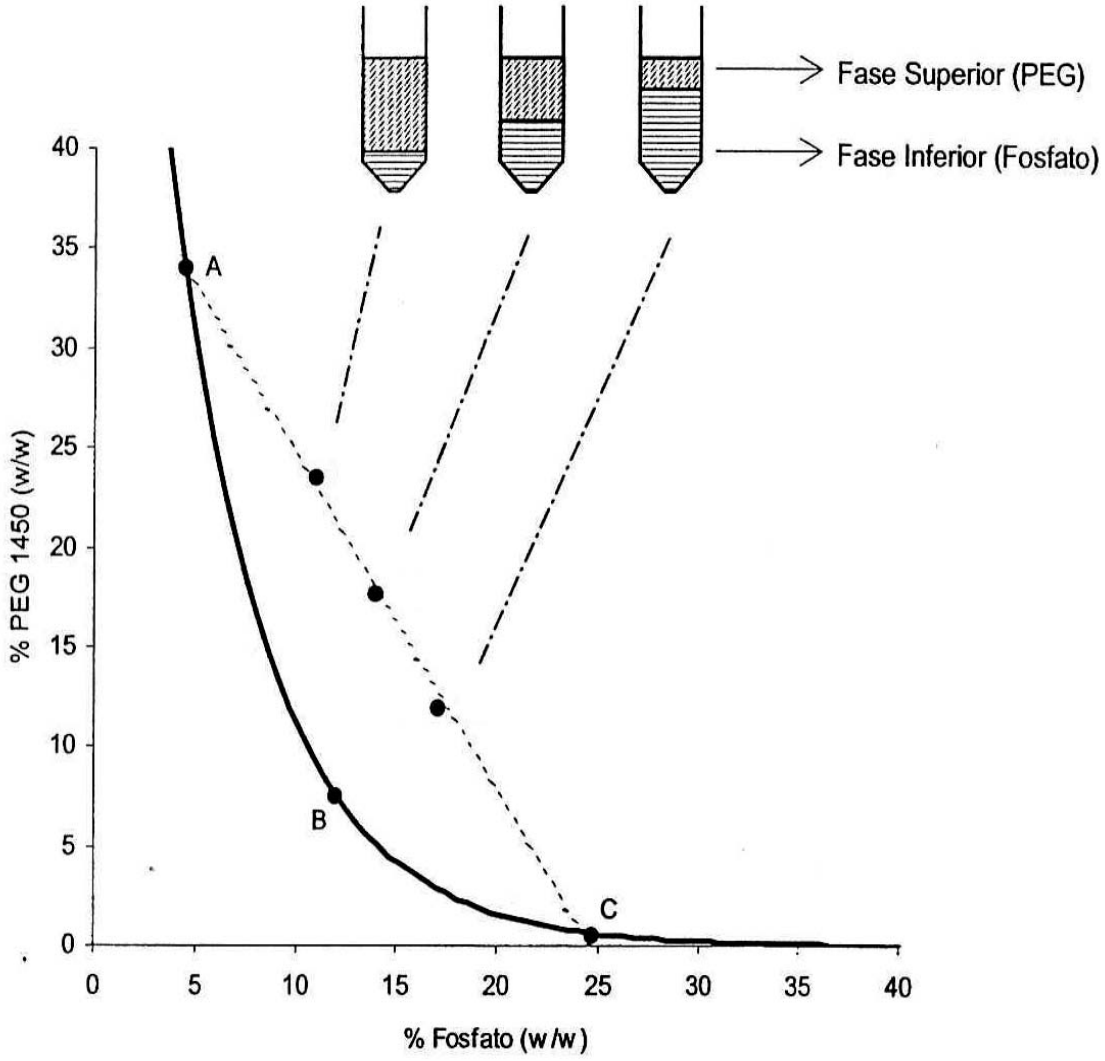

Figura 1.1. Ejemplo representativo de una curva binodal para los sistemas de dos fases acuosas PEG – fosfatos. Todas las combinaciones de PEG, en este caso particular 1450 g/gmol, y fosfato arriba de la curva ABC forman un sistema de dos fases acuosas, mientras que todas las combinaciones por debajo de dicha curva dan como resultado una sola fase heterogénea. Los sistemas X_1, X_2 y X_3 están construidos sobre la misma línea de corte (línea AC), y por lo tanto tienen la misma composición en sus fases superiores e inferiores, y dicha composición es definida por los puntos A (para la composición de la fase superior) y C (para la composición de la fase inferior). Los sistemas X_1, X_2 y X_3 difieren en sus relaciones de volúmenes (V_R). (Figura tomada de Núñez, 1999)

La longitud de la línea de corte (LLC) de un sistema de dos fases acuosas en particular puede ser calculada usando la ecuación 1.1:

$$LLC^2 = (\Delta PEG)^2 + (\Delta F)^2 \qquad \text{(Ecuación 1.1)}$$

donde: LLC = Longitud de línea de corte (% peso/peso, denotado % p/p)

ΔPEG = Diferencia de las concentraciones de PEG entre las fases (% p/p)

ΔF = Diferencia de las concentraciones de fosfato entre las fases (% p/p)

La LLC influye en los fenómenos de partición dentro de los sistemas de dos fases acuosas (Lin *et al*, 2003). Se ha reportado que el incremento en LLC se traduce en un aumento en la partición (Huddleston *et al*, 1991 b; Hernández, 1997; Rito-Palomares and Hernández, 1998) y explican este fenómeno en términos de variación de volumen libre de acuerdo a la teoría de Grossman y Gainer (1988).

Peso molecular del polímero

El peso molecular (PM) del polímero, principalmente PEG, utilizado para construir el sistema de dos fases acuosas desempeña un papel importante en el comportamiento de partición de los compuestos presentes hacia una u otra fase. Esto es debido a dos fenómenos: a) aumento de la hidrofobicidad en la fase polimérica, y b) aumento del volumen excluido (disminución del volumen libre) (Huddleston *et al*, 1991 a).

El aumento en la hidrofobicidad al usar polímeros de alto peso molecular es debido a la reducción estequiométrica de los grupos hidrofílicos terminales en las moléculas de polímero, lo cual disminuye la afinidad del polímero (y en general de la fase polimérica) por el agua (Huddleston *et al*, 1991 a). La mayoría de las proteínas son hidrosolubles, de tal forma que, al aumentar la hidrofobicidad de la fase tendrá menor afinidad hacia esta y tenderá a particionarse hacia la fase inferior o bien, a la interfase. Las cadenas (moléculas) de polímero interaccionan unas con las otras en la fase superior del sistema PEG – sal. El aumento de volumen excluido observado al aumentar el peso molecular del polímero se

debe a que dichas cadenas tienen mayor oportunidad de interaccionar unas con otras debido a su longitud, generándose de esta forma una red muy intrincada de cadenas poliméricas. Mientras mayor es la interacción de las moléculas, mayor es el volumen que estas ocupan, presentándose de esta forma un volumen excluido mayor al que se presenta con cadenas más cortas (peso molecular menor) de polímero. Este volumen excluido representa un obstáculo para las moléculas que intentan particionarse hacia la fase superior. El agua también se ve en cierto grado excluida de la fase polimérica, lo cual genera un aumento en la hidrofobicidad.

El grado de influencia que tiene el peso molecular del polímero sobre el comportamiento de partición de los compuestos biológicos de interés esta determinado hasta cierto punto por los pesos moleculares de los mismos. Los compuestos con altos pesos moleculares se ven influenciados en mayor grado por los cambios en peso molecular del polímero que aquellos con menor tamaño (Albertsson *et al*, 1990).

Relación de volúmenes entre las fases (V_R)

La relación de volúmenes de un sistema de dos fases acuosas esta definida como la relación del volumen de su fase superior entre el volumen de su fase inferior (V_R; Huddleston *et al*, 1991 a), como lo indica la ecuación 1.2. Retomando el ejemplo de los sistemas X_1, X_2 y X_3, mostrados en la Figura 1.1, aun cuando estos tres sistemas tienen la misma LLC difieren en su V_R. El sistema X_1 tiene un V_R mayor que X_2 y X_3 porque la composición global del sistema tiene un porcentaje peso más alto de PEG, lo cual genera una fase superior de mayor volumen. Siguiendo el mismo principio, el sistema X_3 tiene un V_R más pequeño que X_2 y X_1 debido a que el porcentaje de fosfatos en ese sistema es mayor, lo cual genera una fase inferior de mayor volumen.

$$V_R = \frac{V_S}{V_I} \qquad \text{(Ecuación 1.2)}$$

donde: V_R = Relación de volúmenes.

V_S = Volumen de la fase superior.

V_I = Volumen de la fase inferior.

Se ha observado que el volumen relativo de las fases es un parámetro que influye de manera directa sobre la partición de proteínas (Rito-Palomares *et al*, 2001) y especialmente sobre la distribución de la biomasa y de los restos celulares en el sistema de dos fases acuosas (Hustedt *et al*, 1985; Huddleston *et al*, 1991 b; Cueto-Gómez, 1999; Rito-Palomares and Cueto, 2000).

pH del sistema de dos fases acuosas

El pH del sistema de dos fases acuosas tiene influencia sobre la carga electroquímica superficial que adquieren los compuestos que se intentan particionar (Chen, 1992). Se ha comprobado mediante modificaciones químicas específicas que los sistemas de dos fases acuosas poseen la capacidad de separar diferencialmente proteínas homólogas que difieren en carga (Franco *et al*, 1996 a y b).

En los sistemas PEG – solución salina, la fase superior (rica en polímero) tiene carga positiva, y por lo tanto es de esperarse que mientras más negativa sea la carga electroquímica neta del compuesto de interés, mayor será su partición hacia la fase superior, debido al potencial que existe entre las moléculas de polímero (positivas) y el compuesto de interés (negativo). Una proteína adopta carga negativa neta cuando se encuentra por encima de su punto isoeléctrico (pI). El punto isoeléctrico es el valor de pH al cual la carga electroquímica superficial neta de un compuesto es igual a cero. Al aumentar el pH del medio en donde se encuentra el compuesto (proteico o no proteico), algunos grupos funcionales tienden a desprotonarse, adquiriendo carga negativa (o por lo menos neutra), de tal forma que cuando las cargas superficiales negativas rebasan en número a las cargas positivas, se dice que la proteína tiene una carga electroquímica neta negativa. Por esta razón es deseable al tratar de recuperar y purificar proteínas mediante sistemas de dos fases acuosas PEG – solución salina, trabajar por arriba del punto isoeléctrico del compuesto de interés. No solo la carga de las proteínas se ve afectada por el pH. La carga electroquímica superficial de toda molécula con grupos funcionales ionizables puede ser afectada por el pH del medio.

1.4 Breve descripción de la aplicación de los sistemas de dos fases acuosas para la recuperación de compuestos biológicos

En años recientes el interés sobre la aplicación de los sistemas de dos fases acuosas como método de recuperación primaria y purificación parcial de compuestos biológicos ha aumentado notablemente. Esta técnica ha demostrado ser aplicable en el desarrollo de procesos, sobre todo en el área biotecnológica.

Las principales ventajas que ofrecen los sistemas de dos fases acuosas son: a) su alta eficiencia, b) factibilidad de escalamiento, c) facilidad de manejo en cualquier escala de proceso, d) bajos costos de inversión y operación, e) pueden ser llevados a cabo en forma continua, y f) generalmente permiten la recuperación y purificación de compuestos biológicos en su forma nativa, lo cual es de gran valor, ya que al conservar la estructura se conserva al mismo tiempo la función especifica de las mismas.

Los sistemas de dos fases acuosas pueden ser usados en conjunto con otros procesos de separación sin afectar a las etapas previas o posteriores de recuperación (Rito-Palomares, 2002). Una de las aplicaciones más comunes de los sistemas de dos fases acuosas es como técnica de recuperación primaria, porque durante las etapas iniciales de separación lo que se desea es remover el mayor número de contaminantes posibles, sin que esto afecte significativamente el porcentaje de recuperación alcanzado. Han sido utilizados exitosamente en la recuperación y purificación de enzimas, proteínas virales, pigmentos de naturaleza proteica y no proteica, compuestos aromáticos, etc. A pesar de esto, la aplicación de esta técnica hasta el momento ha sido hasta cierto grado limitada debido a la escasa información acerca de los procesos y fenómenos involucrados en el comportamiento de partición (Rito-Palomares, 2004).

Con la finalidad de establecer la aplicación genérica de los sistemas de dos fases acuosas polímero – solución salina para la recuperación de productos biológicos en la presente investigación se presenta el desarrollo de procesos de recuperación utilizando cuatro modelos experimentales. Los modelos experimentales fueron: B-ficoeritrina (BFE)

producida por la microalga *Porphyridium cruentum*, C-ficocianina (CFC) producida por la microalga *Spirulina maxima*, pseudo-partículas 2/6 de rotavirus (*double layered Rotavirus Like-Particles*; dlRLP) producidas por células de insecto, y luteína producida por la microalga *Chlorella protothecoides*.

1.5 Características bioquímicas de los compuestos biológicos recuperados en la presente investigación y sus sistemas de expresión

Colorantes proteicos: B-ficoeritrina y C-ficocianina

Las ficobiliproteínas son un grupo de proteínas coloreadas encontradas en la naturaleza (algas rojas, cianobacterias, etc). Las ficobiliproteínas están ensambladas dentro de una estructura celular organizada llamada ficobilisoma (Gantt, 1980). Estos complejos absorben la luz dentro de un rango amplio de longitud de onda dentro del espectro visible, y transfieren la energía de excitación resultante de la absorción de luz a los centros de reacción en la membrana tilacoide, para de esta forma generar energía química (Glazer, 1985; Glazer, 1989). Como modelos de estudio representativos de proteínas coloreadas se eligieron a la B-ficoeritrina y a la C-ficocianina.

La B-ficoeritrina (BFE) obtenida de *Porphyridium cruentum* (una microalga marina unicelular de color rojo) es una proteína formada por 13 subunidades: 6 subunidades α, cada una de las cuales tiene un peso molecular de 17,500 Da; 6 subunidades β, cada una de las cuales tiene un peso molecular de 17,500 Da; y una subunidad γ, con un peso molecular entre 29,000 y 30,000 Da (MacColl and Guard-Frair, 1987), lo cual le confiere un peso molecular total de 245,000 Da. El punto isoeléctrico (pI) de B-ficoeritrina, producida por *Porphyridium cruentum*, tiene un valor cercano a 4 (Hernández-Mireles and Rito-Palomares, 2006; Hernández-Mireles *et al*, 2006). BFE muestra tres picos de absorbancia en el espectro visible, a 498, 545 y 563 nm, siendo su pico máximo de absorbancia el de 545 nm (Bryant, 1982). BFE presenta fluorescencia, teniendo esta su pico máximo de emisión entre 575 y 578 nm (Bryant, 1982). Esta proteína es soluble en agua, es inodora y no es tóxica. Cuando se disuelve en agua forma una solución de color rosa intenso.

C-ficocianina (CFC) es una ficobiliproteína encontrada principalmente en cianobacterias (algas verde-azules). Al igual que B-ficoeritrina se trata de una proteína coloreada con estructura cuaternaria, conformada por 2 subunidades: una subunidad α y una subunidad β, con pesos de 20,500 y 23,500 Da respectivamente (Ciferri and Tiboni, 1985). Su pI se encuentra entre 4.7 y 5.1 (MacColl *et al*, 1971; Kao *et al*, 1975). CFC muestra un pico de absorbancia en el espectro visible a 620 nm, y presenta fluorescencia, teniendo su pico máximo de emisión alrededor de 650 nm (Minkova *et al*, 2003). Es extremadamente soluble en agua, es inodora y no tóxica (Lee, 1989), razón por la cual es utilizada como pigmento de origen natural en la industria alimentaria y de cosméticos (Bermejo *et al*, 2002; Bermejo *et al*, 2003). De igual forma, en alta pureza encuentra aplicación como marcador bioquímica en inmunoensayos, microscopía y citometría.

Para la producción de los dos modelos experimentales antes mencionados, BFE y CFC, se eligió a *Porphyridium cruentum* y *Spirulina maxima* como sistemas de expresión, respectivamente. *Porphyridium cruentum* ha demostrado ser eficiente para la producción de B-ficoeritrina y otras ficobiliproteínas, asi como de polisacáridos, ácidos grasos poli-insaturados y otros compuestos de interés comercial (Bermejo *et al*, 2002; Vonshak, 1990). Por este motivo *Porphyridium cruentum* fue seleccionado como organismo de trabajo para la producción de B-ficoeritrina (BFE). *Spirulina maxima* es una cianobacteria (alga verde-azul) con morfología helicoidal. Al igual que la mayoría de las algas y cianobacterias es una autótrofa obligada, la cual crece en cuerpos de agua dulce con pH alcalino (pH 8 – 11) dentro de un rango de temperatura de 32 – 35 ºC (Ciferri, 1983). Esta microalga cuenta con un elevado contenido proteico (60 - 70% de la masa seca es proteína), característica que la distingue de la mayoría de las algas (Clement, 1975).

Proteínas estructurales: pseudo-partículas 2/6 de rotavirus (*double layered Rotavirus Like-Particles*, dlRLP)

Las pseudo-partículas virales son estructuras idénticas o similares a los virus, pero que carecen del material genético nativo del virus. Debido a que la composición proteica y conformación estructural de las pseudo-partículas virales es similar a la de los virus nativos, se ha planteado y demostrado la utilidad de estas partículas como vacunas, ya que pueden

generar respuesta inmune en el organismo sin el riesgo de replicación ya que no cuentan con el material genético necesario para ello (Mena Méndez, 2004).

El rotavirus está conformado por RNA de cadena doble y por 6 proteínas estructurales (VP1, VP2, VP3, VP4, VP6 y VP7). De estas VP1 y VP3 están confinadas en el interior de la cápside. La cápside consta de 3 capas proteicas formadas por las proteínas VP2, VP4, VP6 y VP7 (Estes and Cohen, 1989; Kipikian and Chanock, 1996; Mathieu *et al*, 2001). La Figura 1.2 muestra la estructura simplificada del rotavirus.

Las pseudo-partículas 2/6 de rotavirus (dlRLP) están conformadas por las proteínas estructurales VP2 y VP6. Estas pseudo-partículas generan respuesta inmune contra el rotavirus, lo cual las hace candidatas para generar una vacuna a partir de ellas. La nucleocápside (capa proteica interna de la cápside, la cual esta en contacto con el centro del virus) esta formada por la proteína estructural VP2. La formación de la nucleocápside (la cual tiene un diámetro aproximado de 50nm) es dada por la interacción de 120 unidades de VP2 (Mena Méndez, 2004; Mena *et al*, 2005). La siguiente capa de la cápside esta conformada por 260 unidades de VP6. Dichas unidades son en realidad trímeros de dicha proteína, lo cual significa que la capa proteica está formada por 780 unidades de VP6 (Mena Méndez, 2004; Mena *et al*, 2005). El peso molecular total de las pseudo-partículas 2/6 de rotavirus (dlRLP) se encuentra alrededor de los 50,000 kDa.

A pesar de que las proteínas estructurales de diversas pseudo-partículas virales han sido expresadas en bacterias, levaduras y células de mamífero, el sistema de expresión más utilizado es el que involucra la infección de células de insecto con baculovirus, el cual contiene el material genético necesario para la expresión de las proteínas estructurales. Se sabe que las células de insecto son capaces de expresar y ensamblar in vivo cápsides virales conformadas por más de una proteína estructural. Adicionalmente, proporcionan un ambiente eucariótico el cual provee las condiciones óptimas para el ensamblaje, lo cual es reflejado en una alta productividad (comparada con células de mamífero). Una característica adicional que hace de las células de insecto un sistema de expresión ideal para

proteínas recombinantes es su capacidad de hacer modificaciones postraduccionales complejas (imposibles de llevar a cabo en bacterias y levaduras).

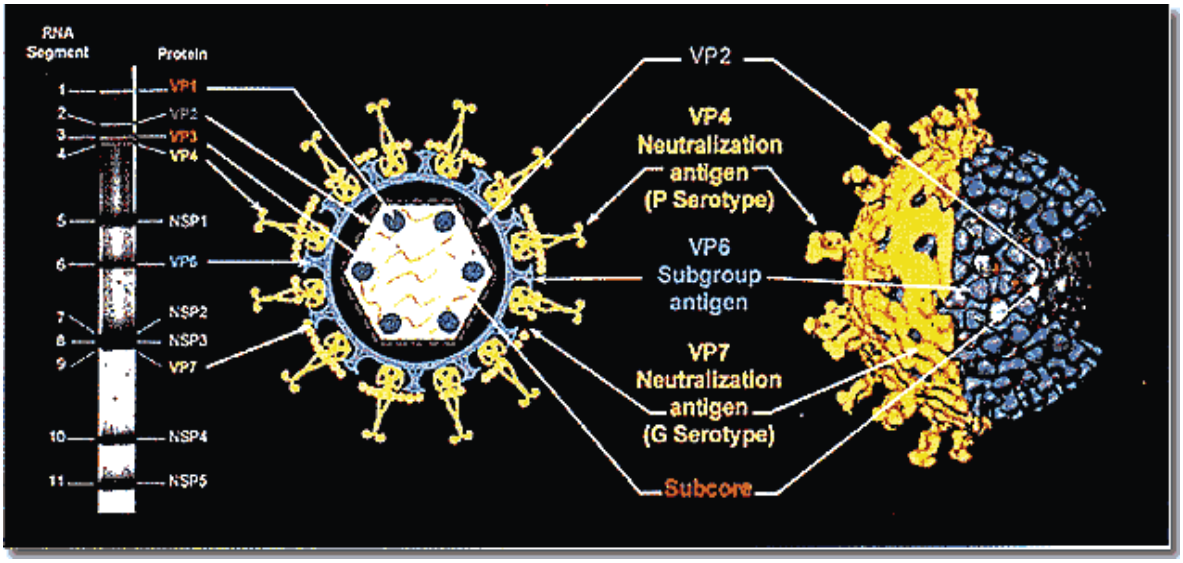

Figura 1.2 Estructura general de un rotavirus. El rotavirus esta formado por 6 proteínas estructurales diferentes, 4 de las cuales se encuentran en 3 capas proteicas que conforman la cápside. El ensamble de dos de estas proteínas (VP2 y VP6) da como resultado las pseudo-partículas 2/6 de rotavirus. Imagen obtenida de Estes, 1996.

Existen numerosas líneas celulares de insecto disponibles comercialmente, las cuales pueden ser cultivadas *in vitro* (bioreactor). Una de las más utilizadas es la Tn 5B1-4 disponible comercialmente con el nombre High Five™. Esta línea celular proviene del ovario de *Trichoplusia ni* (un lepidóptero conocido con el nombre de falso medidor del repollo). Debido a que la línea High Five™ es ampliamente utilizada para la expresión de proteínas recombinantes se decidió trabajar con ella.

Compuesto no proteico: luteína

La luteína es un carotenoide que puede ser encontrado en la naturaleza (vegetales de hoja verde, frutas, yema de huevo, microalgas, etc). Este compuesto tiene un peso molecular aproximado de 569 g/gmol (con fórmula química condensada $C_{40}H_{56}O_2$). La longitud de onda a la cual muestra su absorbancia máxima es 445 nm. La luteína es un compuesto lipofílico, por lo cual su solubilidad en agua es mínima. Se sabe que este compuesto brinda beneficios a la salud humana como antioxidante. Una dieta rica en

luteína puede prevenir la degeneración macular, enfermedad en la cual las células localizadas en la mácula sufren daño debido a la incidencia constante de luz altamente energética (Ahmed *et al*, 2005).

Chlorella protothecoides es una microalga de agua dulce que ha demostrado ser capaz de producir luteína en grandes cantidades bajo ciertas condiciones de cultivo. Se trata de una microalga unicelular, sin flagelos, de forma esférica, con aproximadamente 2-10 μm de diámetro. *Chlorella* incrementa su producción de luteína al entra en ciclo heterotrófico. Debido al potencial de *Chlorella* para la producción de luteína bajo ciertas condiciones y a que es fácilmente cultivable en bioreactor se eligió como sistema de producción (Shi *et al*, 2006; Shi *et al*, 2002; Shi *et al*, 2000; Shi *et al*, 1999).

1.6 Presente investigación y formato de la tesis

Se estudió el comportamiento de partición de los modelos experimentales, brevemente descritos en la sección anterior, en sistemas de dos fases acuosas: dos proteínas coloreadas (BFE y CFC), proteínas estructurales de virus (pseudo-partículas 2/6 de rotavirus; dlRLP) y por último un compuesto no proteico (luteína). La metodología detallada para realizar estos estudios, así como las técnicas analíticas empleadas se describen en el Capítulo 2. Explotando el conocimiento generado sobre el comportamiento de partición de estos compuestos biológicos se desarrollaron procesos de recuperación primaria considerando las condiciones de sistema que favorecieron la partición selectiva de los productos de interés.

De la investigación realizada se derivaron 5 artículos publicados en revistas internacionales con arbitraje. Como parte de la sección de Resultados y Discusión (Capítulo 3) del presente documento se incluyen dichos artículos, los cuales fueron numerados del I al V para su fácil referencia. Inicialmente se presenta un resumen de las investigaciones reportadas en los artículos. Las figuras o tablas de los artículos anexos se mencionan entre paréntesis con el número de dicha figura o tabla, seguido por el número del artículo (I-V) y su referencia.

En base al análisis y discusión de los resultados reportados en dichos artículos se establecerán reglas heurísticas que permitan el diseño predictivo de procesos prototipo para la recuperación primaria de compuestos biológicos en SDFA polietilénglicol (PEG) – solución salina. A continuación se muestra la lista de artículos publicados que se incluyen en el capítulo de Resultados y Discusión (Capítulo 3):

I **Bioprocess intensification: a potential aqueous two-phase process for the primary recovery of B-phycoerythrin from *Porphyridium cruentum***
Jorge Benavides and Marco Rito-Palomares
Journal of Chromatography B, 807 (2004) 33-38

II **Simplified two-stage method to B-phycoerythrin recovery from *Porphyridium cruentum***
Jorge Benavides and Marco Rito-Palomares
Journal of Chromatography B, 844 (2006): 39-44

III **Potential Aqueous Two-Phase Processes for the Primary Recovery of Colored Proteins from Microbial Origin**
Jorge Benavides and Marco Rito-Palomares
Engineering and Life Sciences, 5(3) (2005) 259-266

IV **Rotavirus-like particles primary recovery from insect cells in aqueous two-phase systems**
Jorge Benavides, Jimmy A. Mena, Mayra Cisneros-Ruiz, Octavio T. Ramírez, Laura A. Palomares and Marco Rito-Palomares
Journal of Chromatography B, 842 (2006) 48-57

V **Recovery in aqueous two-phase systems of lutein produced by the green microalga *Chlorella protothecoides***
Mayra Cisneros, Jorge Benavides, Carmen H. Brenes y Marco Rito-Palomares
Journal of Chromatography B, 807 (2004) 105-110

1.7 Objetivo de la investigación

El objetivo de esta investigación es establecer la aplicación genérica de sistemas de dos fases acuosas polímero (PEG) – solución salina para el desarrollo de bioprocesos que permitan la recuperación primaria de compuestos biológicos; utilizando los modelos experimentales de B-ficoeritrina (BFE) producida por *Porphyridium cruentum*, C-ficocianina (CFC) producida por *Spirulina maxima*, pseudo-partículas 2/6 de rotavirus (dlRLP) producidas por células de insecto High Five™ y luteína producida por *Chlorella protothecoides*.

Objetivos particulares

1. Establecer el efecto de los parámetros de sistema sobre el comportamiento de partición de los productos biológicos seleccionados en sistemas de dos fases acuosas (SDFA) polímero (PEG) – solución salina.

2. Desarrollar el diseño predictivo de procesos prototipo utilizando SDFA y etapas adicionales para la recuperación primaria de los productos biológicos seleccionados.

3. Establecer reglas heurísticas que permitan el diseño predictivo de procesos prototipo para la recuperación primaria de compuestos de naturaleza biológica usando SDFA PEG – solución salina, tomando como información de partida las características fisicoquímicas del producto de interés.

1.8 Hipótesis

El estudio del comportamiento de partición de los compuestos biológicos seleccionados en sistemas de dos fases acuosas polímero (PEG) – solución salina, y la correlación de dicho comportamiento con las características fisicoquímicas del producto de interés, generarán el conocimiento necesario para establecer reglas heurísticas que permitirán el diseño predictivo de procesos prototipo para la recuperación primaria de compuestos biológicos.

Capítulo 2. Materiales y Métodos

2.1 Reactivos.

Los sistemas de dos fases acuosas PEG – solución salina fueron preparados con polietilénglicol de peso molecular 400, 600, 1000, 1450, 3350 y 8000 g/gmol obtenidos de SIGMA Chemical Co. y fosfato de potasio monobásico (KH_2PO_4), dibásico (K_2HPO_4), sulfato de amonio ($(NH_4)_2SO_4$) y sulfato de sodio (Na_2SO_4) fueron obtenidos de Desarrollo y Especialidades Químicas (Monterrey, México). Para la construcción de los sistemas de dos fases acuosas no se utilizaron las sustancias en forma sólida, en su lugar soluciones estándar fueron previamente preparadas.

El pH de los sistemas se ajustó utilizando ácido ortofosfórico (85% v/v) o hidróxido de potasio (1 N) (para sistemas PEG – fosfato), ácido sulfúrico (2 N) o hidróxido de amonio (1 N) (para sistemas PEG – sulfato de amonio), o bien ácido sulfúrico (2N) o hidróxido de sodio (1N) (para sistemas PEG – sulfato de sodio) según fue necesario, hasta alcanzar el pH deseado en el sistema. La cantidad de solución adicionada para ajustar el pH del sistema nunca excedió el 5% v/v del total del sistema.

Para los experimentos con sistemas de dos fases acuosas modelo (definidos como aquellos en donde se alimenta un extracto purificado del producto de interés) se utilizaron estándares de B-ficoeritrina y C-ficocianina disponibles comercialmente (ProZyme Inc). En lo que respecta al extracto purificado de pseudo-partículas 2/6 de rotavirus (dlRLP) este se obtuvo mediante la técnica de gradiente de cloruro de cesio (Mena Méndez, 2004). En el caso de luteína no fueron probados sistemas modelo.

2.2 Técnicas analíticas.

Cuantificación de proteína total mediante la técnica de Bradford.

El método de Bradford (1976) es uno de los más utilizados para determinar la concentración de proteína total debido a su gran sensibilidad. Se basa en la unión del

colorante azul brillante de Coomasie G-250 a los residuos básicos (principalmente arginina) y aromáticos de las proteínas, generándose de esta forma un complejo coloreado que tiene su pico máximo de absorbancia a una longitud de onda de 595 nm. Es precisamente esta longitud de onda la que se toma para realizar las lecturas de absorbancia para determinar la concentración de proteína total.

Para la determinación de proteína total se agregaron 1.5 ml del reactivo de Bradford (SIGMA Chemical Co., Bradford reagent, B-6916) a 50 µl de la solución "problema", agitando la mezcla por inversión en repetidas ocasiones y dejándola reposar un mínimo de 10 minutos (tiempo suficiente para que toda la proteína presente reaccione con el reactivo Bradford y se estabilice el color obtenido) y un máximo de 50 minutos (tiempo tras el cual el complejo proteína – colorante se torna inestable y como resultado de esto empieza a degradarse, causando esto una pérdida en el color), según instrucciones proporcionadas por el proveedor del producto (Sigma Chemical Co.). El ensayo se realizó a temperatura ambiente. Se leyó absorbancia a 595 nm en un espectrofotómetro Beckman DU 650 (Beckman Instruments). Las lecturas de absorbancia se tradujeron en concentraciones de proteína total mediante la ecuación de la línea de tendencia de la curva de calibración generada con este fin. La curva de calibración se generó preparando soluciones de concentración conocida de proteína total (0.1 – 1.2 mg/ml) a partir de una solución 5 mg/ml de Albúmina de Suero Bovino (Sigma Chemical Co., Bovine Serum Albumin, B-4287).

Determinación de pureza de B-ficoeritrina y C-ficocianina mediante relaciones de absorbancias.

Todas las proteínas tienen por lo menos dos regiones de absorbancia máxima en la región del espectro ultravioleta, una a 280 nm, debido a los residuos de los aminoácidos aromáticos (tirosina y triptófano) y otra a 180 nm debido a la absorbancia generada por los enlaces peptídicos de la proteína. Es debido a esto que es común expresar pureza de proteínas en forma de relaciones de absorbancia. En el caso específico de BFE y CFC se sabe que sus absorbancias máximas se encuentran a 545 nm y 620 nm respectivamente (Bermejo *et al*, 2002; ProZyme Inc., 2003; Bryant, 1982), por lo tanto, al obtener la relación de absorbancia A545 / A280 y A620 / A280 se puede estimar el grado de pureza

de la proteína de interés (BFE y CFC respectivamente) con respecto a otras proteínas. Una relación de absorbancias menor a 1.0 significa una gran cantidad de impurezas (proteínas contaminantes presentes), mientras que una relación mayor a 4.2 es considerada un grado muy alto de pureza (ProZyme Inc.). Las lecturas de absorbancia a 280 nm (ultravioleta), 545 nm y 620 nm (visible) fueron realizadas en un espectrofotómetro Beckman DU 650 (Beckman Instruments, CA, USA).

Determinación de la concentración de B-ficoeritrina (BFE), R-ficocianina (RFC) y aloficocianina (AFC) mediante sistema de ecuaciones y mediciones espectrofotométricas.

La concentración de ficobiliproteínas BFE, RFC y AFC de *Porphyridium cruentum* se obtuvo mediante el uso de absorbancias a 565, 620 y 650 nm, y un sistema de ecuaciones reportado previamente (Bermejo *et al*, 2002; Bennett and Bogorad, 1973). A continuación se muestran las ecuaciones que conforman el sistema:

$$RFC\,(mg\,ml^{-1}) = \frac{(OD_{620nm} - 0.7\,OD_{650nm})}{7.38} \qquad \text{(Ecuación 2.1)}$$

$$AFC\,(mg\,ml^{-1}) = \frac{(OD_{650nm} - 0.19\,OD_{620nm})}{5.65} \qquad \text{(Ecuación 2.2)}$$

$$BFE\,(mg\,ml^{-1}) = \frac{(OD_{565nm} - 2.8\,(RFC) - 1.34\,(AFC))}{12.7} \qquad \text{(Ecuación 2.3)}$$

donde: RFC, AFC y BFE son las concentraciones (en mg/ml) de R-ficocianina, aloficocianina y B-ficoeritrina respectivamente y OD_{Xnm} es absorbancia (densidad óptica) a la longitud de onda X. Como se puede observar, el cálculo de la concentración de BFE requiere determinar previamente las concentraciones de RFC y AFC utilizando las ecuaciones 2.1 y 2.2. Posteriormente la concentración de BFE puede ser calculada mediante la ecuación 2.3. Las lecturas de absorbancia a 565, 620 y 650 nm fueron realizadas en un espectrofotómetro Beckman DU 650 (Beckman Instruments).

Cuantificación de pseudo-partículas 2/6 de rotavirus (dlRLP) mediante cromatografía líquida de exclusión y detector de fluorescencia

La cuantificación de las pseudo-partículas 2/6 de rotavirus fue determinada mediante cromatografía líquida a altas presiones (High Pressure Liquid Chromatography, HPLC) de exclusión acoplado a un detector de fluorescencia. La exclusión de tamaño separa las moléculas presentes basándose en su peso molecular. Ya que las pseudo-partículas 2/6 tienen un elevado peso molecular (50,000 kDa), la técnica de exclusión de tamaño resulta ideal para su purificación.

Debido a que ninguna de las dos proteínas estructurales (VP2 y VP6) que conforman las dlRLP tienen por si mismas una longitud de onda máxima de absorción en el espectro visible fue necesario modificar las pseudo-partículas de tal manera que su detección y cuantificación se llevará a cabo de una forma rápida y confiable. Por tal motivo se trabajó con la proteína de fusión EGFPVP2, la cual consiste en la proteína estructural VP2 unida a la proteína verde fluorescente (green fluorescent protein; GFP). La expresión de esta proteína de fusión se realizó infectando las células de insecto con el baculovirus recombinante bac EGFPVP2, donado por el Dr. Jean Cohen (INRA, Francia). La concentración de EGFPVP2, y por lo tanto la concentración de pseudo-partículas 2/6 de rotavirus (dlRLP) fue determinada por comparación con una curva estándar generada con soluciones de GFP a concentraciones conocidas contra fluorescencia (área bajo el pico). Mediante la relación másica GFP – EGFPVP2 fue posible determinar la concentración de EGFPVP2 a partir de la curva generada con GFP. Se sabe que 15.27 g de pseudo-partículas 2/6 de rotavirus contienen 1 g de GFP (Mena Méndez, 2004).

Antes de ser inyectadas al sistema, las muestras fueron ultrafiltradas mediante centricones (10 y 300 kDa de tamaño de corte), con la finalidad de remover el polímero (PEG) y las sales presentes. La muestra fue colocada en los centricones, los cuales a su vez fueron centrifugados a 15,000 X g por 15 min. El retenido fue resuspendido en un volumen conocido de agua mQ. Una vez ultrafiltrada y resuspendida la muestra, esta fue inyectada al cromatógrafo. Los componentes que conformaban el sistema cromatográfico fueron: sistema de bombas Waters 626, controlador Waters 600S, columna de exclusión de tamaño

Waters Ultrahydrogel 500 y 2000 (7.8 mm X 300 mm), detector de fluorescencia 2475, y software Millenium 32 (Waters, MA, USA). Las condiciones de corrida fueron las siguientes: flujo isocrático de 0.9 ml/min con fase móvil Tris-EDTA (10mM pH 8). En cuanto al detector de fluorescencia, la excitación de la proteína GFP (fusionada a VP2, EGFPVP2) fue llevada a cabo a 484 nm, mientras que la emisión fue medida a 510 nm).

Determinación de pureza de pseudo-partículas 2/6 de rotavirus mediante cromatografía líquida de exclusión de tamaño y detector de arreglo de fotodiodos

La pureza de las pseudo-partículas 2/6 de rotavirus con respecto a otras proteínas fue determinada mediante un detector de arreglo de fotodiodos (Waters 929), acoplado en serie al detector de fluorescencia.

La absorbancia a 280 nm (longitud de onda a la cual absorben todas las proteínas) fue medida a lo largo del tiempo de la corrida. En el cromatograma generado, el área del pico correspondiente a las pseudo-partículas 2/6 fue calculada y dividida entre el área total bajo la curva (correspondiente a todas las proteínas presentes). De esta manera se pudo calcular la fracción que correspondía a las pseudo-partículas 2/6, y por lo tanto la pureza de las mismas respecto a otras proteínas. Las condiciones de corrida fueron las mismas que las especificadas en la sección anterior.

Cuantificación de luteína mediante espectrofotometría

La cuantificación de luteína en cada una de las fases de los SDFA fue llevada a cabo mediante la medición de absorbancia a 445 nm utilizando un espectrofotómetro (Beckman DU 650). Como blanco se utilizaron las fases de los sistemas sin luteína. La concentración de luteína fue expresada como equivalentes de luteína utilizando un coeficiente de extinción $E^{1\%}_{1cm}$ de 2550 en etanol y un peso molecular de 569 g/gmol.

Estimación de la hidrofobicidad (Kyte-Doolittle) de BFE, CFC y dlRLP

Existen varias escalas para estimar la hidrofobicidad de una proteína o fracción de la misma, entre las cuales se encuentran: Kyte-Doolittle, Engelman, Elsenberg, Hoop-Woods, Cornett, Rose, Janin, etc. La estimación de la hidrofobicidad de las proteínas B-ficoeritrina

(BFE), C-ficociaina (CFC) y pseudo-partículas 2/6 de rotavirus (dlRLP) se llevó a cabo utilizando la escala Kyte-Doolittle y el programa computacional CLC Protein Workbench (Versión 2.0.2, Demo). Esta técnica se basa en el análisis de la secuencia de aminoácidos. Dependiendo de la hidrofobicidad de los aminoácidos es posible determinar las regiones hidrofóbicas e hidrofílicas a lo largo de la secuencia. De tal manera, las proteínas citoplásmicas (expuestas a un ambiente acuoso) generalmente tienen una mayor proporción de regiones hidrofílicas. Por el contrario, las proteínas transmembranales (expuestas a un ambiente no acuoso) presentan una mayor proporción de regiones hidrofóbicas. La hidrofobicidad se representa mediante una escala de color a lo largo de la secuencia de aminoácidos (generalmente las regiones hidrofílicas de la proteína se presentan en color azul mientras las regiones hidrofóbicas en rojo) y/o gráficamente. Las secuencias de aminoácidos para las subunidades α y β de BFE de *Porphyridium cruentum*, las subunidades α y β de CFC de *Galdieria sulphuraria*, y las proteínas estructurales VP2 y VP6 del grupo C de rotavirus fueron obtenidas del Nacional Center for Biotechnology Information (NCBI). A lo largo de las secuencias de aminoácidos se muestran en color azul las regiones hidrofílicas, mientras que en rojo se muestran las regiones hidrofóbicas. De esta manera es posible determinar (de manera cualitativa) la hidrofobicidad relativa de la proteína mediante la proporción de regiones hidrofílicas e hidrofóbicas.

2.3 Cultivo de sistemas de expresión.

Cultivo de *Porphyridium cruentum*

Se trabajó con una cepa (no axénica) de *Porphyridium cruentum* obtenida del CINVESTAV (CDBB-A-001002 CINVESTAV IPN, D.F., México). El medio de cultivo utilizado para la fermentación fue el Medio Algal (MA), el cual fué sugerido por el Centro de Investigaciones Biológicas del Noroeste (CIBNOR, La Paz, México), preparado en agua de mar artificial. Se siguió la especificación estándar para agua de mar artificial de la ASTM (American Society for Testing and Materials, ASTM Designation D1141-75, 1975). El cloruro de estroncio ($SrCl_2$), el fluoruro de sodio (NaF) y los demás elementos traza, fueron omitidos. En la Tabla 2.1 se muestra la composición detallada (compuestos

utilizados y cantidades de los mismos para preparar un litro) del agua de mar artificial utilizada para cultivar *Porphyridium cruentum*.

Tabla 2.1. Composición del agua de mar artificial utilizada para preparar el Medio Algal para *Porphyridium cruentum*.

Nombre del compuesto	Fórmula	Cantidad (por litro)
Cloruro de sodio	NaCl	24.53 g
Cloruro de magnesio	$MgCl_2$	5.20 g
Sulfato de sodio	Na_2SO_4	4.09 g
Cloruro de calcio	$CaCl_2$	1.16 g
Cloruro de potasio	KCl	0.70 g
Bicarbonato de sodio	$NaHCO_3$	0.20 g
Bromuro de potasio	KBr	0.10 g
Ácido bórico	H_3BO_3	0.03 g

ASTM (American Society for Testing and Materials, ASTM Designation D1141-75, 1975)

Se pesó la cantidad de sales requeridas en una balanza analítica para preparar el volumen de agua de mar artificial deseado y una vez hecho esto, se agregaron al volumen de agua bidestilada necesario. Posteriormente se agitó a temperatura ambiente hasta obtener una solución homogénea. En la Tabla 2.2 se muestra la composición detallada (compuestos utilizados y cantidades de los mismos para preparar un litro) del medio de cultivo utilizado, conocido como Medio Algal (MA), el cual se preparó en agua de mar artificial (Tabla 2.1). Se pesaron las cantidades requeridas de sales utilizando una balanza analítica y se agregaron al agua de mar artificial. Posteriormente se agitó a temperatura ambiente hasta lograr una solución homogénea. El pH del medio de cultivo (sin las vitaminas) fué ajustado a 7.6 con ácido clorhídrico (1 N) o hidróxido de sodio (1 N) según se requirió, para después ser esterilizado en una autoclave. Posteriormente en condiciones de esterilidad (campana de flujo laminar de The Baker Company, Inc. SterilchemGARD 4-TX) se agregaron las soluciones estándar de vitaminas, las cuales fueron esterilizadas previamente por filtración (filtros Millipore 0.22 μm).

Tabla 2.2. Composición del medio de cultivo utilizado para la fermentación de *Porphyridium cruentum* (preparado en agua de mar artificial).

Nombre del compuesto	Fórmula	Cantidad (por litro)
Nitrato de sodio	$NaNO_3$	170.00 mg
Fosfato monobásico de sodio	NaH_2PO_4	13.80 mg
Cloruro de zinc	$ZnCl_2$	0.14 mg
Cloruro manganoso	$MnCl_2$	0.20 mg
Molibdato de sodio	Na_2MoO_4	0.24 mg
Cloruro de cobalto	$CoCl_2$	0.01 mg
Sulfato de cobre	$CuSO_4$	0.03 mg
EDTA sal disódica	$Na_2C_{10}H_{14}N_2O_8$	9.90 mg
Citrato de fierro	$Fe_3(C_6H_5O_7)_2$	4.50 mg
Tiamina	$C_{12}H_{17}N_4OS$	35 μg
Biotina	$C_{10}H_{16}N_2O_3S$	5 μg

Medio de cultivo sugerido por el Centro de Investigaciones Biológicas del Noroeste (CIBNOR)

Porphyridium cruentum fue cultivada con el medio antes descrito en una modalidad tipo lote en matraces Erlenmeyer con volumen de trabajo de 1 L, a temperatura ambiente (22 ºC – 25 ºC). La aireación del cultivo fue proporcionada por una bomba para aire ELITE799, con un flujo volumétrico de aire de aproximadamente 9.6 cm^3/seg. La aireación proporcionada a los matraces generaba agitación en el cultivo. El inóculo al matraz fue aproximadamente 100 ml (con 0.5 gramos de biomasa en base húmeda). El alga fue crecida por 30 días (alcanzando una concentración de 35 – 40 g de biomasa húmeda por litro de cultivo), después de los cuales las células fueron recuperadas mediante centrifugación a 2,000 X g por 5 minutos (Centrífuga IEC HN-SII).

Cultivo de *Spirulina maxima*

Para los experimentos de recuperación de C-ficocianina se trabajó con una cepa de *Spirulina maxima* amablemente proporcionada por el Laboratorio de Ecología Microbiana del Centro de Investigaciones y Estudios Avanzados IPN (Dr. Juan José Peña Cabriales).

Spirulina maxima fue cultivada utilizando el medio descrito por Herrera *et al* (1989). El medio de cultivo fue hecho mediante la combinación de 3 soluciones que fueron pesadas, disueltas y esterilizadas de forma independiente: una solución con macro-nutrientes, una solución con micro-nutrientes (elementos traza) y una solución de fosfato de potasio dibásico (K_2HPO_4) con una concentración de 50 g/L. Todas las soluciones fueron hechas usando agua bidestilada. La Tabla 2.3 muestra la composición de la solución con macro-nutrientes.

Tabla 2.3. Composición de la solución con macro-nutrientes para el medio de cultivo de *Spirulina maxima*.

Nombre del compuesto	Fórmula	Cantidad (por litro)
Bicarbonato de sodio	$NaHCO_3$	16.8
Nitrato de sodio	$NaNO_3$	2.5 g
Sulfato de potasio	K_2SO_4	1.0 g
Cloruro de sodio	NaCl	1.0 g
Sulfato de magnesio	$MgSO_4\ 7H_2O$	200 mg
EDTA sal disódica	$Na_2C_{10}H_{14}N_2O_8$	80 mg
Cloruro de calcio	$CaCl_2\ 2H_2O$	40 mg
Sulfato de fierro	$FeSO_4\ 7H_2O$	10 mg

Composición detallada del medio obtenida de Mondragón (2001)

Se pesaron las cantidades requeridas de los compuestos utilizando una balanza analítica, se disolvieron en 950 ml de agua bidestilada, se agitó a temperatura ambiente hasta lograr una solución homogénea y posteriormente se aforó a 1 L. El pH de la solución de macro-nutrientes fue ajustado a 9.0 con ácido clorhídrico (1 N) o hidróxido de sodio (1 N) según se requirió. La Tabla 2.4 muestra la composición de la solución con micro-nutrientes. Se pesaron las cantidades requeridas de los compuestos, se disolvieron en 980 ml de agua bidestilada, se agitó a temperatura ambiente hasta lograr una solución homogénea y posteriormente se aforó a 1 L. Las 3 soluciones (macro-nutrientes, micro-nutrientes y fosfato de potasio dibásico (50 g/L)) fueron esterilizadas por calor (121ºC por 15 min). Las

soluciones necesitan ser esterilizadas por separado para evitar la precipitación de las sales al experimentar calentamiento.

Tabla 2.4. Composición de la solución con micro-nutrientes para el medio de cultivo de *Spirulina maxima.*

Nombre del compuesto	Fórmula	Cantidad (por litro)
Ácido bórico	H_3BO_3	2.9 g
Cloruro de manganeso	$MnCl_2\ 4H_2O$	1.8 g
Molibdato de sodio	$Na_2MoO_4\ 2H_2O$	400 mg
Nitrato de amonio	NH_4NO_3	230 mg
Sulfato de zinc	$ZnSO_4\ 7H_2O$	222 mg
Sulfato de cromo y potasio	$K_2Cr_2(SO_4)_4\ 24H_2O$	96 mg
Sulfato de cobre	$CuSO_4\ 5H_2O$	79 mg
Nitrato de cobalto	$Co(NO_3)_2$	49 mg
Sulfato de niquel	$NiSO_4\ 7H_2O$	48 mg
Sulfato de titanio	$Ti_2(SO_4)_3$	40 mg
Tungstanato de sodio	$Na_2WO_4\ 2H_2O$	18 mg

Composición detallada del medio obtenida de Mondragón (2001)

Una vez a temperatura ambiente y en condiciones de esterilidad (campana de flujo laminar de The Baker Company, Inc. SterilchemGARD 4-TX) las soluciones fueron mezcladas de la siguiente manera: a 1 L de solución con macro-nutrientes se le agregaron 10 ml de la solución de fosfato de potasio dibásico (50 g/L) y 1 ml de solución con micro-nutrientes. La medio de cultivo resultante se agitó hasta homogeneidad y fue vertido en matraces Erlenmeyer de 500 ml (con 250 ml volumen de trabajo), para ser inoculado con *Spirulina maxima*. El inóculo fue 10% del volumen total del cultivo, con una densidad óptica de 0.7 (a 560 nm; Ravishankar *et al*, 1999).

Spirulina maxima fue cultivada con el medio antes descrito en una modalidad tipo lote en matraces Erlenmeyer de 500 ml con volumen de trabajo de 250 ml, a temperatura ambiente (22 ºC – 25 ºC). La agitación del cultivo fue proporcionada por un agitador

reciproco (Lab-Line, 80 rpm). El inóculo al matraz fue de aproximadamente 25 ml (con 0.1 gramos de biomasa en base húmeda). El alga fue crecida por 12 días (alcanzando una densidad óptica de 0.7 a 560 nm), después de los cuales las células fueron recuperadas mediante centrifugación a 2,000 X g por 10 minutos (Centrífuga IEC HN-SII).

Cultivo de células High Five™

Los experimentos relacionados con la recuperación de pseudo-partículas 2/6 de rotavirus (dlRLP) fueron desarrollados utilizando la línea celular BTI–Tn–5B1–4 (Invitrogen, USA), conocida como High Five™, la cual es derivada del ovario del lepidóptero *Trichoplusia ni*.

Se utilizó un medio de cultivo libre de suero, SF-900-II (producido por GIFCO-Invitrogen, USA), el cual esta comercialmente disponible en forma líquida y estéril. La composición exacta del medio se guarda en confidencialidad por la compañía. Sin embargo, en diversos estudios llevados a cabo (Schlaeger, 1996; Radford *et al*, 1997; Hu and Bentley, 1999; Doverskog *et al*, 2000; Mena Méndez, 2004) se han determinado por lo menos algunos de los compuestos presentes en el medio, entre los cuales se encuentran: glucosa (10g/L), sacarosa (2 g/L), maltosa (1.2 g/L), glutamina, aminoácidos libres, etc (Mena Méndez, 2004). En condiciones de esterilidad se vertió el medio de cultivo estéril en matraces Erlenmeyer de 250 ml (con volumen de trabajo de 60 ml) previamente esterilizados en autoclave (121ºC por 15 min).

Los matraces con medio estéril fueron inoculados a una densidad de $5X10^5$ células/ml (viabilidad del 95% y en estado exponencial de crecimiento). Simultáneamente se llevó a cabo la infección con 2 baculovirus recombinantes: bac EGFPVP2, el cual codifica para la proteína de fusión GFP-VP2, que es la unión de la proteína estructural VP2 y la proteína verde fluorescente (GFP, green flourescent protein), y bac VP6, el cual codifica para la proteína estructural VP6. bac EGFPVP2 fue donado por el Dr. Jean Cohen (INRA, Francia), mientras que bac VP6 fue donado por la Dra. Susana López (UNAM, México). Para asegurar la infección de la mayoría de las células presentes en el cultivo se utilizó una multiplicidad de infección de 5 unidades formadoras de placa/ml por cada baculovirus. Los cultivos fueron mantenidos en agitación a 110 rpm y 27ºC por 48 horas. Una vez

transcurrido el tiempo de cultivo se recuperaron las células mediante centrifugación a 15,000 X g por 10 min (Beckman, UK).

Cultivo de *Chlorella protothecoides*

Se trabajó con una cepa de *Chlorella protothecoides* Kruger obtenida de la ATCC (American Type Culture Collection), con número ATCC 30420. *Chlorella protothecoides* fue cultivada utilizando el medio descrito por Shi *et al* (1999 y 2000). La Tabla 2.5 muestra la composición del medio de cultivo algal basal.

Tabla 2.5. Composición del medio de cultivo basal para *Chlorella protothecoides*.

Nombre del compuesto	Fórmula	Cantidad (por litro)
Glucosa	$C_6H_{12}O_6$	9.0 g
Nitrato de potasio	KNO_3	1.25 g
Fosfato de potasio monobásico	KH_2PO_4	1.25 g
Sulfato de magnesio	$MgSO_4\ 7H_2O$	1.0 g
EDTA sal disódica	$Na_2C_{10}H_{14}N_2O_8$	500 mg
Ácido bórico	H_3BO_3	115 mg
Cloruro de calcio	$CaCl_2\ 2H_2O$	110 mg
Sulfato de zinc	$ZnSO_4\ 7H_2O$	90 mg
Sulfato de fierro	$FeSO_4\ 7H_2O$	50 mg
Sulfato de cobre	$CuSO_4\ 5H_2O$	16 mg
Cloruro manganoso	$MnCl_2\ 4H_2O$	14 mg
Oxido de molibdeno	MoO_3	7 mg
Nitrato de cobalto	$Co(NO_3)_2\ 6H_2O$	5 mg

Como se puede observar, las fuentes de carbono, nitrógeno y fosfato en el medio fueron glucosa, nitrato de potasio y fosfato monobásico de potasio respectivamente. Ha sido reportado que suplementar el medio con glucosa adicional, así como urea favorece la producción de luteína (Shi *et al*, 1999 y 2000). De tal forma en lugar de usar 9 g/L de glucosa se utilizaron 40 g/L (como esta reportado en Shi *et al*, 2000). En lo que respecta a

la urea, se suplementó al medio de cultivo para alcanzar una concentración de 3.6 g/L. Se pesaron las cantidades requeridas de los compuestos utilizando una balanza analítica. Todos los compuestos (a excepción de la urea) fueron disueltos en 900 ml de agua bidestilada. Por su parte la urea fue disuelta por separado en 100 ml de agua bidestilada. Se agitó a temperatura ambiente hasta lograr una solución homogénea. El pH de ambas soluciones fue ajustado a 6.6 con ácido clorhídrico (1 N) o hidróxido de sodio (1 N) según se requirió. Las soluciones fueron colocadas en botes de borosilicato, para posteriormente ser esterilizadas en una autoclave (121ºC por 15 min). Una vez esterilizadas, bajo condiciones de esterilidad (campana de flujo laminar de The Baker Company, Inc. SterilchemGARD 4-TX), ambas soluciones fueron mezcladas una vez que alcanzaron temperatura ambiente (22 – 25ºC) para obtener un volumen total de medio de 1 L. Posteriormente el medio fue inoculado con *Chlorella protothecoides* (10% v/v).

Chlorella prototecoides fue cultivada en una modalidad tipo lote en matraces Erlenmeyer de 500 ml con volumen de trabajo de 250 ml, a 28ºC, con agitación enérgica (180 rpm) cubriendo los matraces con papel aluminio para proteger de la luz. La agitación y obscuridad generan las condiciones de estrés necesarias para que *Chlorella* produzca luteína. El control de temperatura y la agitación fueron dados por una incubadora con agitación (Innova 4000, New Brunswick Scientific).

Se decidió trabajar a una escala mayor de cultivo para de esta manera obtener toda la biomasa necesaria para llevar a cabo los experimentos de partición de luteína en sistemas de dos fases acuosas. Para esto se utilizó un fermentador BioFlo III (New Brunswick) con un volumen de trabajo de 2.5 L. Las condiciones de cultivo fueron las siguientes: pH 6.6, 28ºC, agitación 480 rpm (la cual fue proporcionada por el agitador del reactor) y concentración de oxigeno disuelto de 50% respecto a saturación.

El alga fue crecida por 4 - 5 días (alcanzando una concentración aproximada de 15 gramos de biomasa húmeda por litro de cultivo), después de los cuales las células fueron recuperadas mediante centrifugación a 2,000 X g por 5 minutos (Centrífuga IEC HN-SII).

2.4 Ruptura celular de sistemas de expresión.

Maceración manual

Debido a que tanto B-ficoeritrina (BFE) como C-ficocianina (CFC) son productos intracelulares, es necesario llevar a cabo ruptura celular para liberarlos al medio. Se realizó la ruptura celular de *Porphyridium cruentum* y *Spirulina maxima* mediante maceración manual utilizando un mortero, el cual se mantuvo a bajas temperaturas durante todo el proceso mediante el uso de hielo. El mortero previamente enfriado fue adicionado con biomasa húmeda.

En el caso de *Porphyridium cruentum*, por cada gramo de biomasa utilizada, se agregaron 4.3 ml de agua bidestilada (lo cual significa un porcentaje de 23.25% p/v, no considerando el vidrio molido) y 0.98 g de polvo de vidrio. El tiempo de maceración se estimó considerando la cantidad de biomasa utilizada, siendo este de 15 minutos por gramo de biomasa húmeda. Las condiciones para la ruptura manual de *Porphyridium cruentum* fueron establecidas tomando como base los condiciones reportadas para *Spirulina maxima* (Mondragón, 2001), llevando a cabo modificaciones en las mismas que permitieran una mayor liberación de BFE. Al finalizar el tiempo estimado de maceración, se observó que la suspensión resultante adquirió una apariencia lechosa. Mediante observación al microscopio (Olympus CK2, objetivo 100X) se pudo comprobar la ruptura celular, al ver como las algas habían perdido su morfología y color característicos (esféricas y rojas), para convertirse en un agregado de color gris-verdoso.

En el caso de *Spirulina maxima*, por cada gramo de biomasa utilizada, se agregaron 10 ml de una solución 0.1 M de $CaCl_2$ (lo cual significa un porcentaje de 10% p/v, no considerando el vidrio molido) y 0.98 g de polvo de vidrio. El tiempo de maceración se estimó considerando la cantidad de biomasa utilizada, siendo este de 10 minutos por gramo de biomasa húmeda. Las condiciones optimas de ruptura manual fueron establecidas en un estudio previo (Mondragón, 2001). Al finalizar el tiempo estimado de maceración, se observó que la suspensión resultante adquirió una apariencia lechosa. Mediante observación al microscopio (Olympus CK2, objetivo 100X) se pudo comprobar la ruptura

celular, al ver como las algas habían perdido su morfología y color característicos (helicoidales y verdes), para convertirse en un agregado de restos celulares de color grisáceo.

La remoción de los restos celulares en ambos casos, cuando fue necesaria, se realizó mediante centrifugación a 12,000 X g por 10 min (Eppendorf 5415 C).

Sonicación por inmersión

Se hicieron experimentos para determinar la eficiencia de liberación de BFE de *Porphyridium cruentum* utilizando estos dos métodos de ruptura diferentes: maceración manual (descrita en la sección anterior) y sonicación por inmersión. En esta técnica cavitaciones ultrasónicas impactan la membrana celular desestabilizando su estructura. Esto genera la ruptura de la membrana y la liberación del material intracelular.

La ruptura celular mediante sonicación por inmersión se realizó en un tubo de vidrio con capacidad máxima de 50 ml y diámetro de 2.2 cm. Biomasa húmeda de *Porphyridium cruentum* y agua destilada (4 ml/g de biomasa húmeda) fueron agregados al tubo de vidrio, el cual fue sumergido en el sonicador (Branson 1510). El tiempo óptimo de sonicación fue determinado experimentalmente (10 min por cada gramo de biomasa). Tiempos mayores a 10 min no generaron un aumento en la concentración de BFE liberada. Debido a que la biomasa de *Porphyridium* tiende a precipitar (su densidad es mayor a la del agua) fue necesario mezclar constantemente el contenido del tubo. Una vez transcurrido el tiempo de sonicación se verificó la ruptura celular mediante el uso de un microscopio óptico (Olympus CK2). Se pudo observar como las algas habían perdido su morfología y color característicos (esféricas y rojas), para convertirse en un agregado de color gris-verdoso.

Sonicación con punta metálica

Una fracción (aproximadamente el 60%; Mena *et al*, 2005) de las pseudo-partículas 2/6 de rotavirus (dlRLP) producidas por las células High Five^{TM} son liberadas al medio de cultivo (por acción lítica del virus) y son recuperadas sin necesidad de llevar a cabo ruptura celular. En este caso el medio de cultivo gastado puede ser alimentado directamente a los

sistemas de dos fases acuosas, o bien puede llevarse a cabo una concentración opcional del mismo.

Sin embargo, la fracción restante (aproximadamente el 40% de las dlRLP; Mena *et al*, 2005) permanece en células que no experimentaron lisis. La fracción intracelular es significativa, por lo tanto es deseable recuperarla para poder aumentar el rendimiento global de recuperación. Para la ruptura de las células High Five™ se utilizó sonicación con punta (o sonda) metálica. El principio de esta técnica es idéntico al de sonicación por inmersión, sin embargo en este caso las cavitaciones ultrasónicas son generadas por una sonda metálica que es sumergida directamente en la muestra que se desea homogenizar.

En el caso particular de las células High Five™, estas fueron recuperadas por centrifugación (15,000 X g por 10 min) del medio de cultivo, en tubos cónicos de plástico para centrifuga con capacidad máxima de 50 ml. La pastilla celular formada fue resuspendida en 40 ml de solución amortiguadora de lisis (10 mM Tris-HCl, 1 mM EDTA y deoxicolato de sodio 2% p/v). La resuspensión se sonicó durante 3 minutos con intervalos de 10 seg (con periodos de 5 seg de descanso). La viscosidad del homogeneizado aumenta después de ruptura celular, principalmente debido a la liberación de ácidos nucleicos al medio. El homogeneizado resultante se alimentó directamente a los sistemas de dos fases acuosas (incluyendo restos celulares).

2.5 Obtención del extracto concentrado de B-ficoeritrina (BFE) mediante precipitación con sulfato de amonio.

Cuando se utilizó la ruptura celular mediante maceración manual de *Porphyridium cruentum* se obtuvo una concentración muy baja de B-ficoeritrina liberada al medio. Por lo tanto fue necesario concentrar el extracto generado. En esta investigación, esta concentración se realizó mediante precipitación con sulfato de amonio (Stryer, 1992). A pesar de que esta técnica se utiliza generalmente para purificar proteínas (precipitación escalonada), en este caso solo se utilizó la precipitación con sulfato de amonio para concentrar proteínas (un solo paso de precipitación).

La metodología experimental que se siguió fue la siguiente. El suspendido obtenido de la maceración (el cual contenía B-ficoeritrina, compuestos contaminantes y restos celulares) fue centrifugado a 2,000 X g por 5 minutos (Centrifuga IEC HN-SII) para de esa forma eliminar restos celulares. El sobrenadante fue recuperado en tubos Eppendorf para microcentrífuga (volumen máximo de 1.8 ml), y centrifugado a 15,000 X g por 2 minutos (Centrifuga Eppendorf 5415C). El volumen total del sobrenadante obtenido fue registrado. Por cada mililitro de sobrenadante se agregaron las siguientes cantidades de sales: sulfato de amonio 0.47 gramos (que representa aproximadamente el 80% v/v a temperatura ambiente, porcentaje de sulfato de amonio al cual prácticamente todas las proteínas precipitan), fosfato de potasio monobásico 9 mg y azida de sodio 0.4 mg. Una vez agregadas las sales se agitó en vortex (Vortex Genie 2, VWR Scientific) hasta que las sales se disolvieron totalmente. Se centrifugó en tubos Eppendorf a 15,000 X g por 10 minutos (Centrifuga Eppendorf 5415C).

Después de la centrifugación se pudo observar la formación de un precipitado de color rosa intenso, se retiró el sobrenadante, y el precipitado se resuspendió en un reducido volumen de solución amortiguadora de sulfato de amonio (60% v/v) en fosfato de potasio 50 mM (pH 7) y azida de sodio 5 mM (ProZyme Inc.), con la finalidad de tener una alta concentración de la proteína. Al concentrado resultante se le llamó extracto concentrado de BFE. Se guardó el extracto cubriéndolo con papel aluminio (para protegerlo de la luz) a 4 ºC (ProZyme, Inc., 2003) hasta su posterior uso.

2.6 Recuperación y purificación de pseudo-partículas 2/6 de rotavirus mediante gradiente de CsCl.

Para los experimentos iniciales con sistemas de dos fases acuosas para la recuperación de pseudo-partículas 2/6 de rotavirus fue necesario contar con pseudo-partículas purificadas a fin de definir los sistemas modelo. Esta purificación fue llevada a cabo mediante la técnica de gradiente de cloruro de cesio (Mena Méndez, 2004). Las dos fracciones de pseudo-partículas (intracelulares y extracelulares) se procesaron por separado.

Para la fracción intracelular, las células High Five™ fueron recuperadas mediante centrifugación (15,000 X g por 10 min, Beckman, UK). La pastilla celular fue resuspendida y sonicada según se especifica en la sección de sonicación con punta metálica. El homogenizado resultante fue centrifugado a 15,000 X g por 10 min para de esa forma remover los restos celulares. El sobrenadante fue centrifugado y distribuido en tubos para ultracentrífuga (Beckman, UK). A cada tubo se agregó 4.5 ml de sobrenadante, al cual a su vez se adicionó 1.89 g de CsCl (0.42 g de CsCl por ml de sobrenadante). Se agitó mediante vortex hasta lograr la total disolución de la sal. Se centrifugó durante 18 hr a 148,000 X g. Una vez terminada la ultracentrifugación se retiraron los tubos del rotor teniendo cuidado de no perturbar las bandas de gradiente formadas. La banda de las pseudo-partículas 2/6 de rotavirus fue recuperada utilizando para esto una jeringa con aguja. La banda fue almacenada a -20 ºC para su posterior uso y análisis.

En lo que respecta a la fracción extracelular, el medio de cultivo gastado fue ultracentrifugado a 80,000 X g por 2 hr a través de un colchón de sacarosa de alta pureza (GIBCO, USA) al 35% p/v en una solución con 1.58 g/L Tris-HCl y 8.18 g/L NaCl a pH 7.5. Posteriormente la pastilla generada fue recuperada y purificada mediante la técnica de gradiente de CsCl, descrita anteriormente.

2.7 Extracción de luteína de *Chlorella protothecoides.*

Una vez terminado el cultivo de *Chlorella protothecoides*, la biomasa fue recuperada mediante centrifugación (2,000 X g por 5 min, Eppendorf 5415 C). La pastilla celular formada fue resuspendida en etanol absoluto (30% masa de biomasa húmeda por unidad de volumen). La adición de etanol a la biomasa generó el aumento de la permeabilidad de la membrana celular de *Chlorella*, lo cual dio como resultado la liberación de luteína al medio. La remoción de los restos celulares se realizó mediante centrifugación (2,000 X g por 5 min, Eppendorf 5415 C). El sobrenadante fue almacenado y posteriormente utilizado para el estudio del comportamiento de partición de luteína en sistemas de dos fases acuosas.

2.8 Construcción de los sistemas de dos fases acuosas PEG – sal.

Los sistemas de dos fases acuosas se construyeron utilizando soluciones estándar de polietilénglicol (PEG) y solución salina. Para preparar las soluciones estándar de PEG 50% p/p se pesaron 200 gramos del PEG del peso molecular requerido (1000, 1450, 3350 u 8000 g/gmol) y se agregaron 200 gramos de agua bidestilada, la mezcla fue mantenida en constante movimiento utilizando un agitador magnético hasta lograr una solución homogénea transparente. En el caso del PEG 1000 y PEG 1450 esto se logró después de 3 o 4 horas de agitación a temperatura ambiente, mientras que para el PEG 3350 y PEG 8000 fue necesario dejar agitando por lo menos 6 horas bajo las mismas condiciones. En el caso de los sistemas con PEG 400 o 600 g/gmol estos fueron construidos utilizando PEG 100% p/p, ya que el PEG 400 y 600 en forma pura son líquidos a temperatura ambiente, lo cual hace innecesaria la tarea de generar una solución diluida de los mismos.

Para elaborar la solución estándar de fosfato de potasio 40% p/p se utilizó fosfato de potasio monobásico (KH_2PO_4), así como dibásico (K_2HPO_4), en una proporción 7:18 respectivamente. De esta forma para preparar 400 gramos de solución fue necesario pesar 240 gramos de agua bidestilada, y posteriormente se le agregaron 115.2 gramos de fosfato de potasio dibásico, después de lo cual fue necesario dejar agitando hasta lograr la disolución total de la sal. Una vez logrado esto se añadieron 44.8 gramos de fosfato de potasio monobásico, y se agitó hasta lograr la disolución total de la sal. Como paso final se ajustó el pH de la solución, con ácido ortofosfórico (85% v/v) o hidróxido de potasio (1 N) según fue necesario, dependiendo del pH al cual fuera a ser construido el sistema de dos fases acuosas.

Para elaborar la solución estándar de sulfato de amonio ($(NH_4)_2SO_4$) 40% p/p (utilizada solo en los sistemas probados para la recuperación de pseudo-partículas 2/6 de rotavirus) se siguió básicamente la misma metodología que en el caso de la solución de fosfatos. De esta forma para preparar 400 gramos de solución fue necesario pesar 240 gramos de agua bidestilada, y posteriormente se le agregaron 160 gramos de sulfato de amonio, después de lo cual fue necesario dejar agitando hasta lograr la disolución total de la sal. Como paso

final se ajustó el pH de la solución, con ácido sulfúrico concentrado (98% v/v) o hidróxido de amonio (1 N) según fue necesario, dependiendo del pH al cual fuera a ser construido el sistema de dos fases acuosas.

Los sistemas fueron construidos pesando las soluciones estándar previamente elaboradas en tubos cónicos para microcentrífuga (con capacidad máxima de 1.8 ml) para los sistemas de 1 gramo o bien en tubos cónicos de 15 o 50 ml para los sistemas de mayor peso. Posteriormente se agregó agua bidestilada y por último la muestra con el compuesto de interés. Para la construcción de los sistemas blanco (llamados así debido a que sus fases superiores e inferiores desempeñar la función de blancos espectrofotométricos) se sustituyó la muestra con el compuesto de interés por agua bidestilada.

Los valores de longitud de línea de corte (LLC) fueron calculados directamente de las curvas binodales (Zaslavsky, 1995), mediante el uso de la ecuación 1.1. Una vez construidos, los sistemas de dos fases acuosas se agitaron en vortex por 10 min en un rotor (Scientific Industries, Inc. Multi-purpose rotator modelo 151) para de esta forma lograr mezclar los compuestos. Posteriormente los sistemas se centrifugaron (1500 – 2000 X g por 10 – 20 min) para de esa forma acelerar el equilibrio de fases y la partición de los compuestos en el sistema (Rito-Palomares *et al*, 2001). Posteriormente se procedió a obtener el V_R (relación de volumen entre la fase superior y la inferior) del sistema, calculando para esto el volumen de la fase superior e inferior de los sistemas. El volumen de cada fase se estimó utilizando tubos de centrífuga graduados (tubos de 1.8, 15 o 50 ml).

Muestras representativas fueron obtenidas de cada fase para su posterior análisis. La fase superior del sistema fue removida con la ayuda de una micropipeta, teniendo cuidado de no tomar fase inferior ni perturbar la interfase, para evitar contaminación. Posteriormente, con la ayuda de una micropipeta se tomaron muestras de la fase inferior, teniendo cuidado de perturbar lo menos posible la interfase del sistema. Cuando asi fue necesario (con las pseudo-partículas 2/6 de rotavirus), se tomaron muestras de la interfase. En este caso la interfase se definió como el volumen restante después de haber recuperado el mayor volumen posible de fase superior e inferior teniendo cuidado de no alterar ni

perturbar de manera significativa la interfase. Se llevaron a cabo análisis de las fases recolectadas, según el compuesto de interés recuperado (sección 2.2). El número de repeticiones para cada sistema nunca fue inferior a dos. El porcentaje de error siempre estuvo entre ± 10%.

Capítulo 3. Resultados y Discusión

Este capítulo consta de 3 secciones principales. La primera (sección 3.1) es un resumen de las investigaciones realizadas y publicadas en los artículos científicos, así como el vínculo que existe entre ellas. La segunda (sección 3.2) incluye consideraciones generales, reglas heurísticas deducidas de los resultados obtenidos para los modelos experimentales seleccionados: dos proteínas coloreadas (ficobiliproteínas; BFE y CFC), proteínas estructurales (pseudo-partículas 2/6 de rotavirus; dlRLP) y un compuesto no proteico (luteína). La tercera sección (3.3) incluye una estrategia secuencial que permite el diseño predictivo de procesos de recuperación primaria de compuestos biológicos en sistemas de dos fases acuosas polietilénglicol (PEG) – solución salina. Al final de este capítulo se anexan los 5 artículos publicados, relacionados con la investigación presentada en este documento.

3.1 Estudio del comportamiento de partición y desarrollo de procesos para la recuperación primaria de los modelos experimentales seleccionados.

Desarrollo de un proceso prototipo para la recuperación primaria de B-ficoeritrina (BFE) producida por *Porphyridium cruentum* (artículos I y II)

Se desarrolló un proceso prototipo para la recuperación primaria de BFE producida por *Porphyridium cruentum*. Primero se estudió el comportamiento de partición de BFE en sistemas de dos fases acuosas PEG – fosfato de potasio. La influencia de los parámetros de sistema (peso molecular del polímero, longitud de línea de corte, pH del sistema y relación de volúmenes) sobre el comportamiento de partición de BFE fue establecida (artículo I). Consecuentemente fue posible determinar bajo que condiciones B-ficoeritrina se recuperaba selectivamente (concentrándose en la fase superior del sistema, mientras que los contaminantes se concentraban en la fase inferior o interfase del sistema). Se observó una obvia afinidad de BFE por la fase superior del sistema, particularmente en los sistemas con PEG de bajo peso molecular.

De esta manera, fue posible establecer los parámetros de sistema bajo los cuales se favorece la partición de BFE hacia la fase superior de sistemas de dos fases acuosas PEG – fosfato de potasio: PEG de bajo peso molecular (1000 – 1450 g/gmol) (Tabla 1 y 4, I; Benavides and Rito-Palomares, 2004), LLC alto (> 40% p/p) (Tabla 1, I; Benavides and Rito-Palomares, 2004), pH 7-8 (Tabla 2, I; Benavides and Rito-Palomares, 2004) y V_R mayor a 1 (Tabla 3, I; Benavides and Rito-Palomares, 2004). En general los resultados obtenidos demostraron el gran potencial de los sistemas de dos fase acuosas PEG – fosfato de potasio para llevar a cabo la recuperación primaria de BFE (77%), al obtener elevados porcentajes de recuperación y lograr una purificación primaria de BFE (Abs545nm/Abs280nm = 2.9, contra un valor de 0.7 en el extracto de BFE) (artículo I; Benavides and Rito-Palomares, 2004). La existencia de áreas de oportunidad quedó expuesta al momento de diseñar un proceso simplificado de recuperación primaria, con el número mínimo de etapas posibles. En un intento de integrar etapas e intensificar el proceso, estudios adicionales fueron realizados (artículo II; Benavides and Rito-Palomares, 2006). En los estudios previos se utilizó como muestra un extracto concentrado de BFE, obtenido mediante maceración manual de la biomasa de *Porphyridium cruentum*, remoción de los restos celulares mediante centrifugación y precipitación con sulfato de amonio para de esa manera concentrar BFE en la muestra (artículo I; Benavides and Rito-Palomares, 2004).

Se realizaron estudios de ruptura celular utilizando dos métodos diferentes: maceración manual y sonicación por inmersión. La ruptura celular mediante sonicación fue mucho más efectiva para la liberación de BFE, logrando obtener una concentración 5 veces mayor (Figura 1, II; Benavides and Rito-Palomares, 2006). Debido a que la sonicación permite obtener un extracto concentrado de BFE (sin la necesidad de recurrir a la precipitación con sulfato de amonio) se eligió como la técnica a utilizar para llevar a cabo la ruptura celular de *Porphyridium cruentum*. De esta forma se evitaron etapas de concentración innecesarias en el proceso. Se probó la factibilidad de procesar extracto crudo de BFE, obtenido directamente de ruptura celular (sin remoción de los restos celulares) en los sistemas de dos fases acuosas. La presencia de los restos celulares en los sistemas de fases acuosas no

afectó la pureza de BFE en la fase superior. Se estudió la influencia del incremento en la concentración de extracto crudo de BFE (de 10 a 40% p/p) sobre el comportamiento de partición de BFE (Tabla 1, II; Benavides and Rito-Palomares, 2006). En todos de los sistemas probados el porcentaje de recuperación fue superior al 90%. Mientras mayor el porcentaje de extracto crudo de BFE mayor fue la pureza del producto de interés.

Posteriormente se estudió la cinética de formación y estabilización de fases utilizando diferentes geometrías de sistema, caracterizadas como la relación entre la altura de sistema (H) y el diámetro interno del contenedor (D) (Tabla 2, II; Benavides and Rito-Palomares, 2006). Las geometrías que requirieron menores tiempos de estabilización fueron aquellas con relación H/D menor a 1 (Figura 2, II; Benavides and Rito-Palomares, 2006). De esta manera se logró establecer las condiciones y parámetros de sistema óptimos para generar un proceso a nivel comercial para la recuperación primaria de BFE producida por *Porphyridium cruentum* en sistemas de dos fases acuosas PEG – fosfato de potasio. Estos parámetros fueron: PEG 1000 g/gmol, LLC 45% p/p, pH 7, V_R 4.5, 40% p/p extracto crudo de BFE y H/D menor a 1.

Bajo estas condiciones se logró una concentración de BFE de 110 mg/L en la fase superior del sistema. Esta concentración demuestra el potencial que tiene esta técnica para la recuperación primaria de BFE. De esta manera se recupero el 92% de BFE en la fase superior del sistema con una pureza (Abs545nm/Abs280nm) de 3.2. Otras técnicas de recuperación primaria han sido estudiadas por otros grupos de investigación para el caso particular de BFE.

Cromatografía en cama expandida (EBA) ha sido utilizada para la recuperación primaría de BFE (Bermejo *et al*, 2006) logrando una recuperación del 78% y una pureza (Abs545nm/Abs280nm) > 3 utilizando una resina Streamline-DEAE (dietil-aminoetil, GE Healthcare). El porcentaje de recuperación obtenido con SDFA es ligeramente mayor, mientras que las purezas de BFE obtenidas por ambas técnicas son similares. Sin embargo la capacidad de adsorción de la resina utilizada para la cromatografía en cama expandida se ve fuertemente afectada por la presencia de proteínas contaminantes, mientras que los

SDFA han mostrado ser robustos ante la presencia de contaminantes. La Figura 3.1 muestra el diagrama simplificado de los procesos de recuperación primaria de BFE desarrollados (artículos I y II; Benavides and Rito-Palomares, 2004 y 2006), los cuales cuentan con un número reducido de etapas y pueden ser escalados sin dificultad técnica.

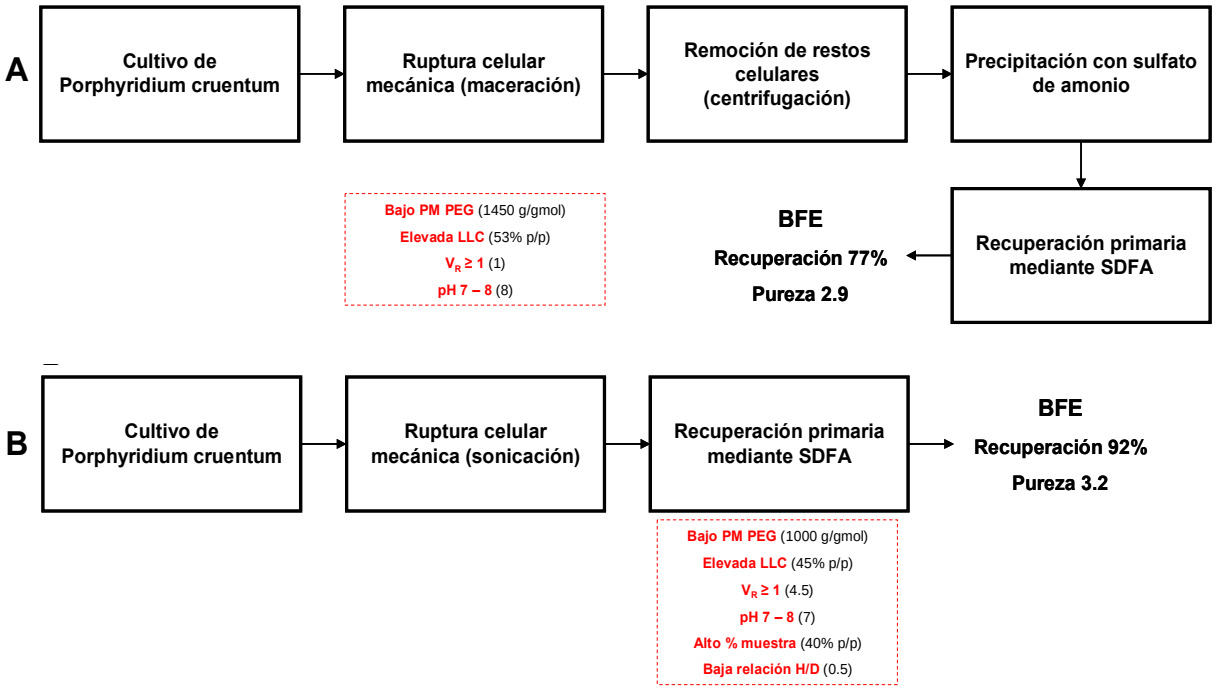

Figura 3.1 Comparación simplificada de los procesos desarrollados para la recuperación primaria de B-ficoeritrina (BFE) producida por *Porphyridium cruentum* utilizando sistemas de dos fases acuosas (SDFA) PEG – fosfato de potasio. El uso de maceración como método de ruptura general genera extractos de BFE diluidos, por lo cual es necesario introducir en el proceso etapas de concentración (A, artículo I). El uso de sonicación como método de ruptura celular permite obtener un extracto concentrado de BFE, eliminando de esta manera la necesidad de etapas de concentración adicionales en el proceso (B, artículo II). El proceso integrado e intensificado (B) no tan solo permite eliminar etapas innecesarias de proceso, sino que adicionalmente reporta mayor pureza y porcentaje de recuperación de BFE. El sistema de dos fases acuosas (PEG 1000 g/gmol, LLC 45% p/p, pH 7, V_R 4.5 y H/D menor a 1) se adicionó con 40% p/p de extracto crudo de BFE.

Es importante mencionar que tomando como base la investigación reportada en estos dos artículos científicos (I y II), Hernández-Mireles y Rito-Palomares (2006) diseñaron un proceso para la recuperación y purificación de BFE, agregando una etapa de precipitación

isoeléctrica previa a los sistemas de dos fases acuosas. Dicho proceso permitió una recuperación del 72% y una pureza de BFE (Abs545nm/Abs280nm) de 4.1, valor de pureza considerado grado analítico.

Desarrollo de un proceso prototipo para la recuperación primaria de proteínas coloreadas de origen microbiano (artículo III)

Con el fin de demostrar la aplicación genérica de los sistemas de dos fases acuosas (SDFA) polímero – solución salina para la recuperación primaria de proteínas coloreadas, particularmente ficobiliproteínas, se realizó un estudio comparativo del comportamiento de partición de B-ficoeritrina (BFE) producida por *Porphyridium cruentum* y C-ficocianina (CFC) producida por *Spirulina maxima*.

Se realizó una comparación de la influencia de los parámetros de sistema sobre el comportamiento de partición de BFE y CFC a fin de determinar bajo que condiciones las proteínas de interés y los contaminantes presentes se concentraban en fases opuestas del sistema. El análisis comparativo de los resultados obtenidos para ambas proteínas permitió correlacionar la influencia de los parámetros de sistema y las características fisicoquímicas de los productos de interés sobre su comportamiento de partición en SDFA.

En el caso de CFC los parámetros de sistema que promovieron la partición del producto de interés hacia la fase superior fueron: PEG 1450 g/gmol (Tabla 1 y Figura 3, III; Benavides and Rito-Palomares, 2005), LLC 34% p/p (Tabla 1, III; Benavides and Rito-Palomares, 2005), V_R 0.3 (Figura 2, III; Benavides and Rito-Palomares, 2005) y pH 7 (Figura 1, III; Benavides and Rito-Palomares, 2005). Bajo estas condiciones se logró un porcentaje de recuperación del 98% y una pureza de CFC (definida como la relación Abs620nm/Abs280nm) de 2.1.

En el caso de BFE los parámetros que optimizaron la partición del producto de interés hacia la fase superior fueron: PEG 1000 g/gmol (Tabla 1 y Figura 3, III; Benavides and Rito-Palomares, 2005), LLC 50% p/p (Tabla 1, III; Benavides and Rito-Palomares, 2005), V_R 1.0 (Figura 2, III; Benavides and Rito-Palomares, 2005) y pH 7 (Figura 1, III;

Benavides and Rito-Palomares, 2005). Bajo dichas condiciones se logró un porcentaje de recuperación del 82% y una pureza de BFE (definida como la relación Abs545nm/Abs280nm) de 2.8.

Desarrollo de un proceso para la recuperación primaria de pseudo-partículas 2/6 de rotavirus (dlRLP) (artículo IV)

La técnica de gradiente de cloruro de cesio (Mena Méndez, 2004) permite recuperar solamente el 2% de las pseudo-partículas 2/6 de rotavirus (dlRLP), con una pureza del 9% (con respecto a otras proteínas). Generar el gradiente de cloruro de cesio requiere ultracentrifugación a 148,000 X g por 18 horas. Si se deseara recuperar y purificar pseudo-partículas 2/6 de rotavirus para su comercialización sería deseable utilizar una técnica alternativa de recuperación y purificación primaria. En esta parte de la investigación se evaluó el comportamiento de partición de las dlRLP en sistemas de dos fases acuosas PEG – solución salina. El entendimiento de este modelo de estudio no solo permitió diseñar un proceso de recuperación primaria para estas pseudo-partículas, sino que teóricamente los resultados obtenidos pueden ser extrapolados a partículas proteicas con geometría esférica, hidrofílicas y muy elevado peso molecular.

Se estudió la influencia de los parámetros de sistema sobre el comportamiento de partición de dlRLP en SDFA. Posteriormente ambas fracciones, intracelular y extracelular (40 y 60% del total de dlRLP, respectivamente), de las dlRLP fueron recuperadas con estrategias independientes. La fracción intracelular de las dlRLP fue liberada mediante ruptura celular con sonicación con punta metálica. Para recuperar la fracción intracelular se diseñó un proceso de recuperación constituido por dos etapas de extracción PEG – fosfato de potasio en serie (PEG 400 g/gmol, V_R 13.0, pH 7 y LLC 35% p/p), logrando recuperar el 90% de las dlRLP intracelulares en la fase superior del sistema (Tabla 6, IV; Benavides *et al*, 2006), concentrando los restos celulares en la interfase. La concentración promedio de dlRLP en la fase superior del sistema fue de 300 μg/L. En lo que respecta a la recuperación de la fracción extracelular se diseñó una estrategia que involucra una sola extracción con SDFA PEG – fosfato de potasio (PEG 3350 g/gmol, V_R 1.0, pH 7 y LLC 17% p/p), logrando recuperar el 82% de las dlRLP extracelulares en la interfase (Tabla 6, IV;

Benavides *et al*, 2006). La concentración promedio de dlRLP en la interfase del sistema fue de 820 μg/L. El volumen de la interfase es reducido (aproximadamente representa el 10% del volumen total del sistema), lo cual permite concentrar el producto, lo cual representa un beneficio adicional al evitar la introducción de etapas posteriores para la concentración del producto de interés.

De esta manera se logró un rendimiento global del 85% y una pureza similar a la obtenida con el gradiente de cloruro de cesio (6 – 11%) (Figura 3, IV). La estrategia propuesta es más simple, económica y rápida que la técnica de gradiente de cloruro de cesio para la recuperación de pseudo-partículas 2/6 de rotavirus.

Desarrollo de un proceso para la recuperación primaria de luteína (artículo V)

En la naturaleza existen una gran variedad de compuestos de naturaleza no proteica con propiedades nutraceúticas (carotenoides, antocianinas, isoflavonas, etc). Dichos compuestos despiertan día a día el interés de los sectores industriales y la población en general debido a sus propiedades benéficas sobre la salud humana. Estos compuestos comparten la característica de tener un peso molecular inferior al de la mayoría de las proteínas (< 3000 g/gmol). Se eligió la luteína como compuesto modelo para estudiar el comportamiento de partición de productos no-proteicos en sistemas de dos fases acuosas PEG – fosfato de potasio. Los resultados obtenidos pueden extrapolarse para predecir el comportamiento de partición de compuestos con características fisicoquímicas similares (bajo peso molecular e hidrofobicidad).

Se estudió la influencia de los parámetros de sistema (peso molecular del polímero, longitud de línea de corte y concentración del producto) sobre el comportamiento de partición de luteína en sistemas de dos fases acuosas PEG – fosfato de potasio. La adición de etanol al sistema de dos fases acuosas fue necesaria para lograr la disolución de la muestra. Se evaluó el efecto de la adición de etanol sobre el V_R de los sistemas (Tabla 1, V; Cisneros-Ruiz *et al*, 2004). El sistema de fases acuosas PEG – fosfato de potasio con PEG 8000 g/gmol, LLC 49.4% p/p, pH 7 y V_R 2.9 (V_R 1.0 para el sistema sin etanol) proporcionó las mejores condiciones para la partición de luteína hacia la fase superior,

mientras que los restos celulares se concentraron en la fase inferior del sistema (Tabla 2, V; Cisneros-Ruiz *et al*, 2004). Bajo estas condiciones se logró una recuperación de luteína en la fase superior del 81%, con una concentración de 30 mg/L. Los resultados obtenidos demuestran la potencial aplicación genérica de los SDFA polímero – solución salina para la recuperación de compuestos biológicos de naturaleza no proteica.

3.2 Consideraciones generales para la recuperación primaria de compuestos biológicos en sistemas de dos fases acuosas PEG – solución salina

Considerando las propiedades fisicoquímicas de los compuestos que se han estudiado, así como la información recabada a lo largo de la investigación relacionada a su recuperación primaria en sistemas de dos fases acuosas PEG – solución salina, es posible establecer consideraciones generales que permitan anticipar el comportamiento de partición de compuestos biológicos en sistemas de dos fases acuosas a fin de facilitar el desarrollo de sistemas de recuperación. La Tabla 3.1 sumariza las principales características fisicoquímicas de los compuestos que se recuperaron utilizando sistemas de dos fases acuosas.

Los pesos moleculares de los compuestos, así como los puntos isoeléctricos de la BFE y CFC fueron tomados de literatura (Hernández-Mireles and Rito-Palomares, 2006; MacColl *et al*, 1971; Kao *et al*, 1975). La estimación del punto isoeléctrico de VP2, VP6 y GFP se realizó mediante el uso del programa computacional CLC Protein Workbench (Versión 2.0.2, Demo), utilizando para el cálculo la secuencia de aminoácidos de VP2 y VP6 del grupo C de rotavirus, así como la de la proteína verde fluorescente (GFP). En lo que respecta a su hidrofobicidad, se sabe por literatura que BFE, CFC (Lee, 1989) y dlRLP son proteínas hidrofílicas. Se utilizaron las secuencias de aminoácidos de las subunidades de las proteínas y el programa computacional CLC Protein Workbench (Versión 2.0.2, Demo) para determinar la proporción relativa de regiones hidrofóbicas e hidrofílicas usando la escala Kyte-Doolittle (Kyte and Doolittle, 1982). Se encontró que existe una mayor

proporción de regiones hidrofílicas en las proteínas estudiadas. Las representaciones Kyte-Doolittle de BFE, CFC y dlRLP se muestran en el Anexo (Fig. A.1 – A.4).

Tabla 3.1 Características fisicoquímicas de los compuestos recuperados mediante sistemas de dos fases acuosas.

Compuesto	Peso molecular (g/gmol)	Hidrofobicidad	pI
Luteína	569	Hidrofóbica	NA
C-ficocianina (CFC)	44,000	Hidrofílica	4.7-5.1
B-ficoeritrina (BFE)	245,000	Hidrofílica	4
Pseudo-partículas 2/6 de rotavirus (dlRLP)	50,000,000	Hidrofílica	6.5 - 6.6*

* Punto isoeléctrico (pI) para VP2 - VP6, estimado utilizando el programa computacional CLC Protein Workbench (Versión 2.0.2, Demo). El pI de la GFP se estimó en 6.1. NA: No aplicable

Peso molecular

Una de las características fisicoquímicas del compuesto a recuperar que tiene mayor impacto sobre su comportamiento de partición en sistemas de dos fases acuosas PEG – solución salina es su peso molecular. De los resultados obtenidos se puede deducir que si se desea recuperar un compuesto de elevado peso molecular en la fase superior del sistema (fase polimérica) es necesario utilizar polímero de bajo peso molecular.

La B-ficoeritrina (245 kDa) y la fracción intracelular de las pseudo-partículas 2/6 de rotavirus (50,000 kDa) fueron recuperadas en la fase superior del sistema utilizando PEG 1000 y 400 g/gmol respectivamente. Si se desean recuperar compuestos de mediano peso molecular es posible trabajar con PEG de bajo a mediano peso molecular. La C-ficocianina (44 kDa), siendo más pequeña que la B-ficoeritrina, fue recuperada en la fase superior del sistema utilizando PEG 1450 g/gmol. Por último para recuperar compuestos de bajo peso molecular (< 5000 g/gmol) es posible trabajar con una amplia variedad de pesos moleculares de polímero (400 – 20000 g/gmol). Tomando como ejemplo la luteína (569 g/gmol), esta fue recuperada en la fase superior del sistema utilizando PEG de alto peso molecular (8000 g/gmol). La Figura 3.2 muestra las mejores condiciones de sistema para la

recuperación y purificación primaria de los modelos estudiados (BFE, CFC, dlRLP y luteína), así como la relación entre el peso molecular del polímero y del compuesto de interés. Mientras mayor el peso molecular del compuesto de interés, menor el peso molecular del polímero con el cual se logró la recuperación óptima en la fase superior del sistema.

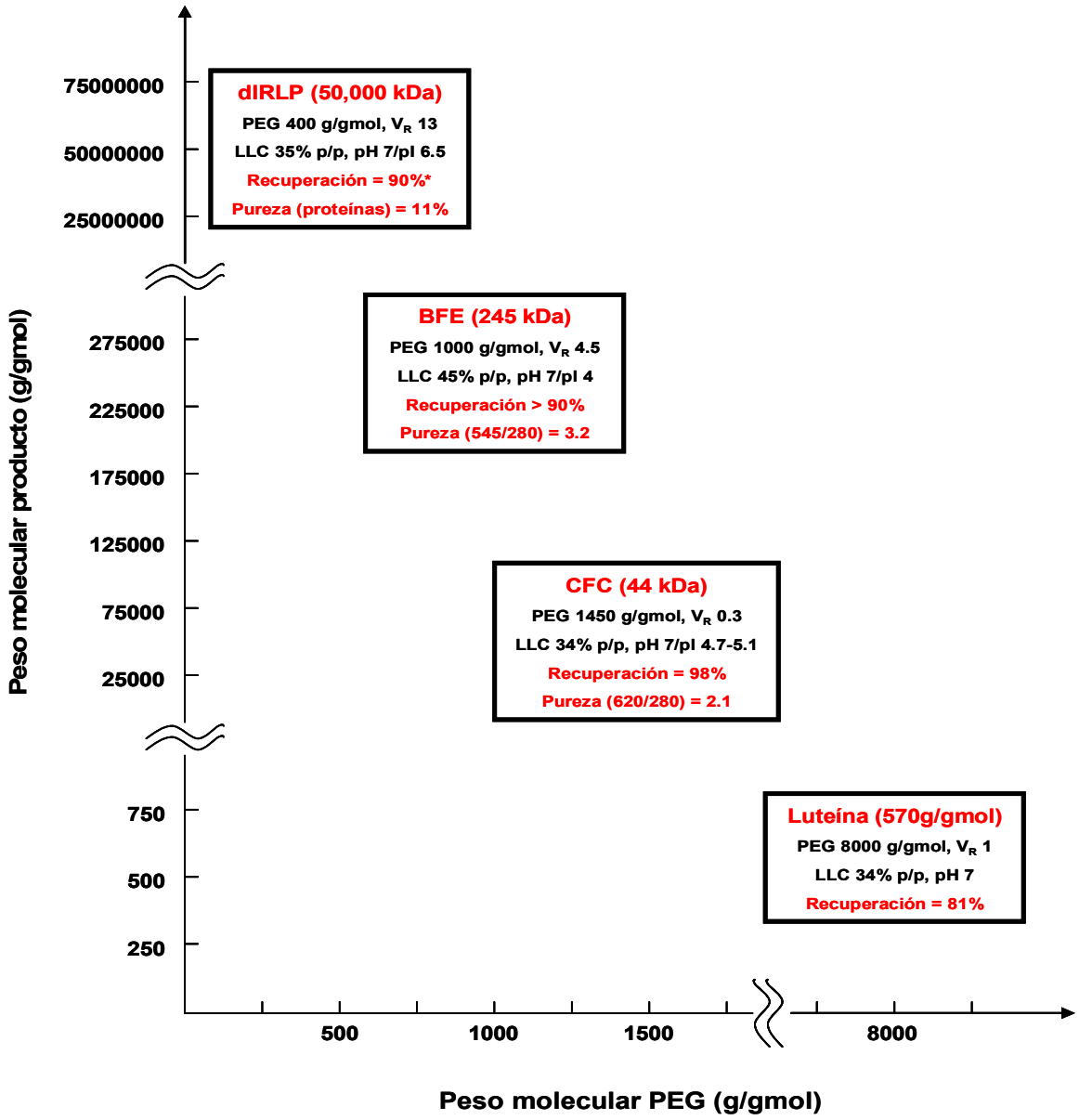

Figura 3.2 Mejores condiciones de sistema para la recuperación y purificación primaria de los modelos estudiados en fase superior. Mientras mayor el peso molecular de compuesto de interés, menor el peso de PEG que brindó mejores porcentajes de recuperación en fase superior.

Este fenómeno es debido al aumento del volumen excluido. A medida que las cadenas poliméricas incrementan su longitud, las fuerzas de interacción aumentan y el volumen libre entre ellas se reduce. Los compuestos como la B-ficoeritrina y las pseudo-partículas 2/6 de rotavirus no pueden alojarse en el reducido volumen disponible cuando se utiliza PEG de alto peso molecular. Por lo tanto no pueden ser recuperados en la fase superior del sistema a menos de que se utilice polímero de bajo peso molecular. Al aumentar estratégicamente el peso molecular de PEG de 400 a 3350 fue posible forzar a las pseudo-partículas virales a particionarse hacia la interfase del sistema.

En contraste, la luteína no se ve fuertemente afectada por el aumento de volumen excluido, ya que su bajo peso molecular le permite alojarse en el volumen libre de la fase sin experimentar impedimento estérico aun cuando se utilice polímero de alto peso molecular (8000 g/gmol). De igual forma es importante considerar el peso molecular de los contaminantes presentes. De esta manera es posible seleccionar una estrategia en la cual se elija un peso molecular de PEG tal que permita la separación selectiva de la proteína de interés y los contaminantes.

Hidrofobicidad

Otra característica fisicoquímica que tiene gran relevancia en el comportamiento de partición de los compuestos biológicos de interés en sistemas de dos fases acuosas es la hidrofobicidad de la molécula. El aumento en el peso molecular del polímero genera un incremento del volumen excluido en la fase superior del sistema. Adicionalmente, el aumento del peso molecular del polímero aumenta la hidrofobicidad de la fase superior. Esto es debido a dos razones. Primero, al aumentar el volumen excluido disminuye la cantidad de agua en la fase superior aumentando la hidrofobicidad. Segundo, la reducción estequiométrica de los grupos hidroxilo, situados en ambos extremos de la cadena polimérica, hace que el momento dipolar generado por la interacción hidrógeno – oxígeno se vuelva más débil en la fase superior, lo cual aumenta la hidrofobicidad de la misma. En la Tabla 3.2 se comparan los porcentajes de recuperación en fase superior e interfase para las dlRLP y luteína. La partición de dlRLP hacia la fase superior se ve disminuida por el aumento en la hidrofobicidad al usar PEG de alto peso molecular. En contraste, la

recuperación en fase superior de luteína, un compuesto hidrofóbico, se ve favorecida por el uso de polímero de alto peso molecular, ya que al generarse un ambiente más hidrofóbico su afinidad aumenta, lo cual se ve reflejado en un incremento del porcentaje de recuperación (Tabla 3.2).

Tabla 3.2 Influencia del peso molecular del polímero (PM PEG) sobre el porcentaje de recuperación de dlRLP y luteína en SDFA PEG – fosfato de potasio.

PEG (g/gmol)	Recuperación dlRLP (%)		Recuperación luteína (%)	
	Fase superior	Interfase	Fase superior	Interfase
400	60 ± 3.0	24 ± 1.2	NP	NP
600	55 ± 2.8	29 ± 1.5	NP	NP
1000	ND	84 ± 4.2	35 ± 2.0	65 ± 2.0
1450	ND	80 ± 4.0	62 ± 3.0	38 ± 3.0
3350	ND	96 ± 3.1	68 ± 2.6	32 ± 2.6
8000	ND	90 ± 4.5	75 ± 2.1	25 ± 2.1

Los sistemas se mantuvieron con LLC baja (20 – 35% p/p) y pH 7. ND: No detectado. NP: No probado.

Una manera adicional de aumentar la hidrofobicidad de la fase superior del sistema es incrementando la longitud de línea de corte (LLC). La longitud de línea de corte es un parámetro directamente relacionado con la concentración de polímero y sal en el sistema, asi como en cada una de sus fases. Un aumento en la LLC favorece una mayor concentración de polímero en la fase superior del sistema. Consecuentemente mientras mayor es la concentración de polímero en la fase superior del sistema, mayor es el volumen excluido de la misma. Por lo tanto, al incrementar la LLC aumenta la hidrofobicidad de la fase superior.

De los sistemas probados con luteína, los que mejor porcentaje de recuperación brindaron fueron aquellos construidos con elevado peso molecular de polímero (3350 y 8000 g/gmol) y alta LLC. En dichos sistemas la hidrofobicidad de la fase superior es considerable, por lo cual la luteína, un compuesto hidrofóbico, presenta afinidad por dicha fase.

Punto isoeléctrico

Este parámetro también es de importancia para predecir el comportamiento de partición de un compuesto en sistemas de dos fases acuosas, ya que esta íntimamente relacionado con la carga electroquímica superficial de la molécula. Cuando el pH del medio se encuentra por debajo del punto isoeléctrico (pI), la molécula (proteína) adquiere una carga electroquímica superficial positiva, ya que algunos residuos de los aminoácidos de la molécula se encuentran protonados. Una vez que el pH se eleva por encima del punto isoeléctrico la molécula adquiere una carga superficial negativa, debido a la desprotonación de los residuos.

El PEG cuenta con dos grupos hidroxilo en los extremos de la cadena polimérica, los cuales generan una ligera carga positiva. Debido a la elevada electronegatividad del oxígeno del grupo hidroxilo, se genera una distorsión en la nube electrónica, creando un momento dipolar positivo. De esta manera, los compuestos cargados negativamente (pH por encima del punto isoeléctrico) tienen cierta afinidad por la fase superior de los sistemas de fases acuosas PEG – solución salina debido a la atracción electroquímica.

Para BFE, CFC y dlRLP se probaron sistemas cuyo pH se encontraba por encima del su punto isoeléctrico. Por lo tanto BFE, CFC y dlRLP tenían carga superficial negativa. Esto permitió aumentar su afinidad por la fase superior del sistema. Debido a la naturaleza química de la sal formadora (fosfatos, sulfatos, etc) es imposible manipular con libertad el pH del sistema, ya que a ciertos valores de pH las sales tienden a precipitar, generando esto la perdida del sistema. Los sistemas polímero – fosfato son utilizados cuando se desean utilizar pH mayores o iguales a 7, mientras que los sistemas polímero – sulfato son utilizados a valores de pH inferiores a 7.

Por lo tanto es importante tratar de trabajar a valores de pH del sistema que estén por arriba del pI de la proteína de interés. Sin embargo, es necesario considerar que el aumentar innecesaria o excesivamente el pH promovería la migración de proteínas contaminantes hacia la fase superior del sistema, lo cual se vería reflejado tanto en la pureza del producto como en su porcentaje de recuperación.

3.3 Estrategia secuencial para el desarrollo de procesos de recuperación primaria de compuestos biológicos en sistemas de dos fases acuosas PEG – solución salina.

Considerando la información recabada de la investigación realizada se desarrolló una estrategia secuencial para el desarrollo de procesos de recuperación primaria de compuestos biológicos en sistemas de dos fases acuosas PEG – solución salina. La Figura 3.3 muestra el diagrama de decisión derivado de dicha estrategia secuencial. Siguiendo los pasos establecidos en el diagrama de decisión mostrado (Figura 3.3) es posible llevar a cabo el diseño predictivo de procesos de recuperación primaria de compuestos biológicos mediante sistemas de dos fases acuosas PEG – solución salina.

El compuesto de interés es seleccionado y caracterizado, de tal manera que sus propiedades fisicoquímicas sirvan como guía para el desarrollo del proceso de recuperación primaria. De igual manera es importante caracterizar los principales compuestos contaminantes presentes. Tomando en cuenta el punto isoeléctrico (pI) del compuesto de interés se elige si se utilizarán sistemas PEG – sulfato y PEG – fosfatos, dependiendo del rango de pH que permite manejar cada uno de estos. El uso de sistemas PEG – fosfato es recomendado sobre PEG – sulfato debido a que están mejor caracterizados. Si el compuesto no presenta anfoterismo (no cambia su carga electroquímica dependiendo de las condiciones del medio) se recomienda utilizar sistemas PEG – fosfato.

Posteriormente se considera el peso molecular y la hidrofobicidad del mismo para elegir los parámetros de sistema (peso molecular de polímero y longitud de línea de corte) bajo los cuales la partición del producto de interés hacia la fase superior se ve favorecida. Si las condiciones de recuperación y pureza mínima en la fase superior del sistema no son satisfechas se puede estudiar la influencia del pH, la relación de volúmenes (V_R) y el uso de sistemas de dos fases acuosas en serie, eligiendo para esto los sistemas con los que mejor resultados se hayan obtenido en términos de recuperación y pureza en la etapa experimental de peso molecular del polímero (PM PEG) y longitud de línea de corte (LLC).

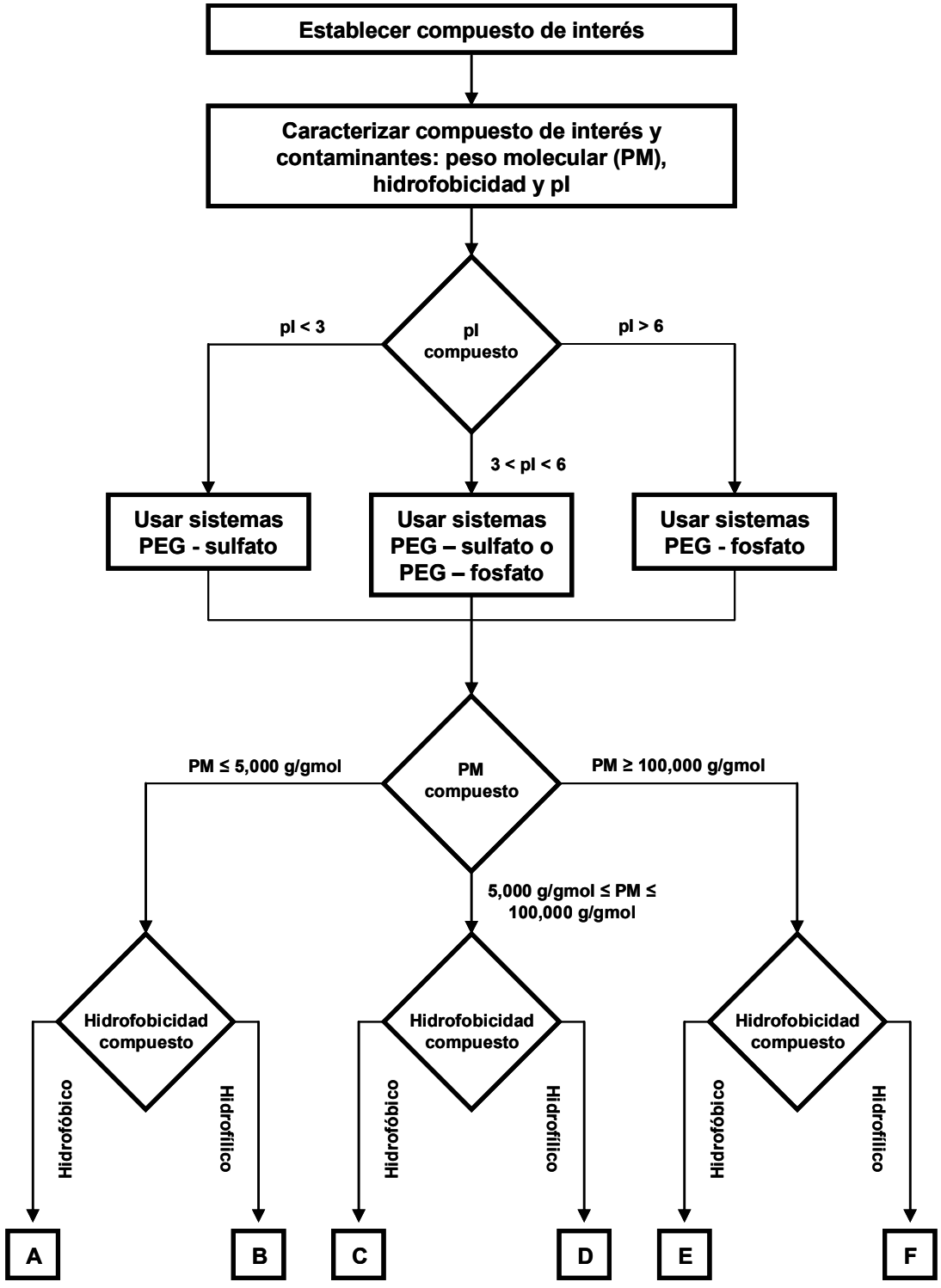

Figura 3.3 Diagrama de decisión para el desarrollo para procesos de recuperación y purificación primaria de compuestos biológicos mediante sistemas de dos fases acuosas PEG – solución salina.

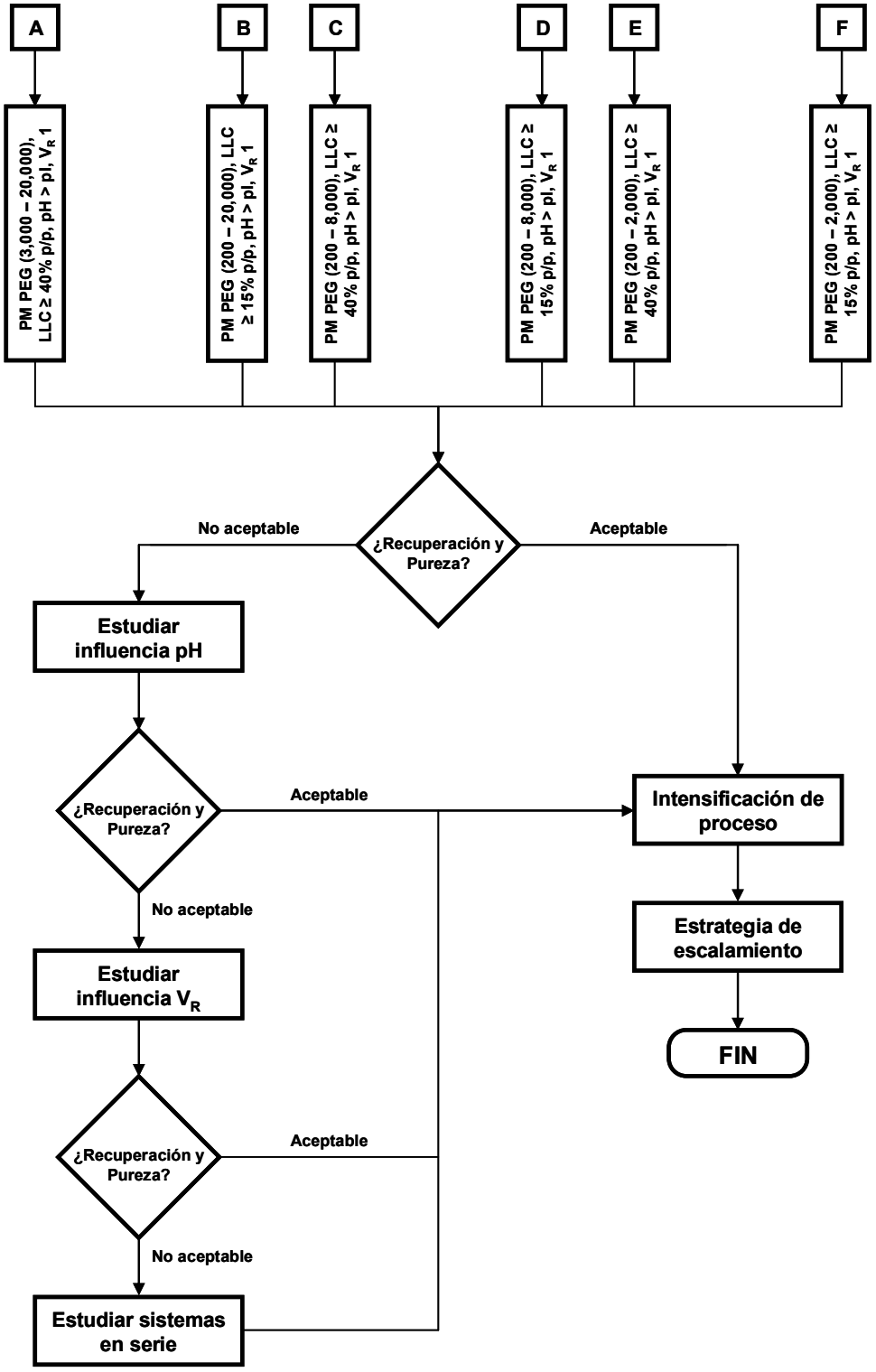

Figura 3.3 (Continuación) Diagrama de decisión para el desarrollo para procesos de recuperación y purificación primaria de compuestos biológicos mediante sistemas de dos fases acuosas PEG – solución salina.

Se recomienda evaluar una intensificación del proceso, con la finalidad de determinar si es posible procesar un mayor porcentaje de muestra (% p/p), de tal manera que tanto los costos de inversión como de operación se vean reducidos por cantidad de producto. Por último se debe establecer la estrategia de escalamiento del proceso diseñado a nivel laboratorio – piloto. A nivel industrial es imposible centrifugar los SDFA para acelerar el equilibrio de las fases, Por lo tanto es recomendable realizar estudios de cinética de separación de fases utilizando contenedores con diferentes geometrías (relaciones altura – diámetro, H/D). Ha sido reportado que mientras menor sea la relación H/D, menor será el tiempo necesario para que las fases del sistema lleguen al equilibrio (Solano-Castillo and Rito-Palomares, 2000).

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Bioprocess intensification: a potential aqueous two-phase process for the primary recovery of B-phycoerythrin from *Porphyridium cruentum*

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Abstract

A process for the primary recovery of B-phycoerythrin from *Porphyridium cruentum* exploiting aqueous two-phase systems (ATPS) was developed in order to reduce the number of unit operations and benefit from an increased yield of the protein product. The evaluation of system parameters such as poly(ethylene glycol) (PEG) molecular mass, concentration of PEG as well as salt, system pH and volume ratio was carried out to determine under which conditions the B-phycoerythrin and contaminants concentrate to opposite phases. PEG 1450-phosphate ATPS proved to be suitable for the recovery of B-phycoerythrin because the target protein concentrated to the top phase whilst the protein contaminants and cell debris concentrated in the bottom phase. An extraction ATPS stage comprising volume ratio (Vr) equal to 1.0, PEG 1450 24.9% (w/w), phosphate 12.6% (w/w) and system pH of 8.0 allowed B-phycoerythrin recovery with a purity of 2.9 (estimated as the relation of the 545–280 nm absorbances). The use of ATPS resulted in a primary recovery process that produced a protein purity of 2.9 ± 0.2 and an overall product yield of 77.0% (w/w). The results reported demonstrated the practical implementation of ATPS for the design of a primary recovery process as a first step for the commercial purification of B-phycoerythrin produced by *P. cruentum*.

Keywords: Bioprocess intensification; Aqueous two-phase systems; *Porphyridium cruentum*; B-phycoerythrin

1. Introduction

The increasing need to rapidly and economically bring new biotechnological products to market using scaleable and efficient technology has encouraged manufacturers to seek competitive advantages through bioprocess intensification. In this context, colouring compounds used in food, cosmetic, detergent and molecular genetic industries are products of great commercial significance [1,2]. The particular production of B-phycoerythrin (a red-coloured protein) by *Porphyridium cruentum* represents a very interesting case, because the industrial and commercial value of this product is considerable. The commercial value of highly purified B-phycoerythrin (purity greater than 4, defined as the relationship of 545–280 nm absorbances) for pharmaceutical or fluorescent uses can be more than US$ 50/mg [3,4]. B-phycoerythrin is the most valuable of the three main classes of phycoerythrins (B, R and C) from the photosynthetic systems of *P. cruentum*, due mainly to its wide range of potential commercial applications, such as natural dyes in foods, cosmetics and in the development of biosensors [2,5]. It is formed by three sub-units, α, β and γ (in a relative molar ratio of 6:6:1) of 18.0, 18.0 and 29 kDa molecular weight, respectively [6].

The recovery of B-phycoerythrin from *P. cruentum* has been attempted previously [5,7–10]. However, the resulting protocols have been characterised by an excessive number of unit operations (more than 10 steps), mainly in the primary recovery part of the downstream process. Consequently, affecting product yield and potential scaling up of these procedures at commercial scale. To overcome some of the disadvantages attributed to the established B-phycoerythrin purification protocols, the use of aqueous two-phase systems (ATPS) has been suggested as an attractive alternative. This technique has several advantages including bio-compatibility, ease of scale-up, low cost, etc. [11]. The use of ATPS for the recovery of protein products from fermentation broth has been addressed before [12–16]. However, no scientific reports, known to the authors, exist on the primary recovery and purification of B-phycoerythrin from *P. cruentum* cultures using ATPS.

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The research presented here aims to generate knowledge on the partition behaviour of B-phycoerythrin on ATPS to benefit the production of such colouring compounds. A practical approach which exploits the known effect of systems parameters such as the concentration of poly(ethylene glycol) (PEG) and salt (i.e. phosphate), phase volume ratio (Vr), molecular weight of PEG and system pH upon protein partition and purity was used to establish a greatly simplified primary recovery process for the purification of B-phycoerythrin from cell homogenate from *P. cruentum*. The way in which the developed process greatly simplifies the primary recovery of the protein product, potentially defines the first step for the development of a commercial process for the purification of B-phycoerythrin produced by *P. cruentum*.

2. Materials and methods

2.1. Characterisation of aqueous two-phase systems

The binodal curves were estimated by the cloud point method [17] using poly(ethylene glycol) (Sigma, St. Louis, MO, USA) of nominal molecular mass of 1000, 1450, 3350 and 8000 g/gmol (50% (w/w) stock solution) and di-potassium hydrogen orthophosphate/potassium di-hydrogen orthophosphate (Sigma) (30%, w/w). Fine adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide.

2.2. Culture medium and cultivation conditions

P. cruentum was cultivated in the culture medium described by Bermejo et al. [5]. The algae were grown in a batch culture (500 ml Erlenmeyer flasks) at 22–25 °C under natural light conditions, agitation and aeration was provided with an air flow rate of 3.2 cm^3/seg using a peristaltic pump (ELITE 799, Mexico). The cells were allowed to grow for 30 days and were harvested by centrifugation at 3500 rpm for 5 min (Eppendorf 5415C). After harvesting, cellular fragmentation was performed manually in a ceramic pot using glass beads and de-ionised water (4.3 cm^3/g wet biomass) for 15 min. Temperature was controlled with a dry ice bath. Complete cellular fragmentation was verified using an optical microscope (Olympus CK2). Cell debris removal was achieved by centrifugation at 3500 rpm for 5 min (Eppendorf 5415C) and the supernatant was processed with a solution of ammonium sulphate (0.47 g/cm^3 supernatant) containing 0.01% of sodium azide. This precipitation step was introduced just to concentrate the protein content in the cell homogenate in order to facilitate the estimation of protein concentration in the ATPS experiments. The precipitate was re-suspended in a potassium phosphate buffer (50 mM, pH 7.0) with sodium azide (5 mM), the resulting solution (referred to as crude extract) was introduced into the aqueous two-phase system previously selected as described further.

2.3. Influence of system parameters upon partition behaviour of B-phycoerythrin in PEG–salt systems

All experimental systems used to establish the operating conditions for the ATPS process were prepared for convenience on a fixed mass basis. Predetermined quantities of stock solutions of PEG and potassium phosphate were mixed with either, a single model system (containing purified B-phycoerythrin obtained from a commercial supplier, Sigma) or a complex model system (containing 20% (w/v) wet homogenate from *P. cruentum* fermentation; referred earlier as crude extract) to give a final weight of 1.0 g. The stock solution (PEG or salts) were mixed and phases dispersed by gentle mixing for 30 min at 25 °C. Adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide. Complete phase separation was achieved by low speed batch centrifugation at 1500 × g for 20 min at 25 °C. Visual estimates of the volumes of top and bottom phases and solids, were made in graduated tubes. The volumes of the phases were then used to estimate the volume ratio (volume of the top phase/volume of the bottom phase (Vr)). Samples were carefully extracted from the phases and diluted for biochemical analysis and subsequent estimation of B-phycoerythrin partition coefficient (K = concentration of solute in the top phase/concentration of solute in the bottom phase). The systems tie-line length (TLL), which represents the length of the line that connects the composition of the top and bottom phase of a defined ATPS was calculated as described by Albertsson [11]. The top phase recovery was estimated as the amount of protein presents in the upper phase (volume of the phase × protein concentration in the phase) and expressed relative to the original amount loaded into the system. Bottom phase and interface protein recovery was not estimated due problems associated to the very low amount of total protein presents in such phases. Results reported are the average of two independent experiments and errors were estimated to be a maximum of ±10% of the mean value.

2.4. Analytical procedures

Protein concentration in the samples was estimated by the method of Bradford [18]. The purity of B-phycoerythrin was determined as the relation of the 545–280 nm absorbance (i.e. purity of B-phycoerythrin = $Abs_{545\,nm}/Abs_{280\,nm}$). Bermejo et al. [5] reported the use of the 545–280 nm absorbance relation as an estimation of B-phycoerythrin purity, since the absorption spectrum of this protein exhibits a peak at 545 nm. Under this circumstances, a ratio greater than four corresponds to a highly purify B-phycoerythrin (defined as pure commercial B-phycoerythrin; Sigma).

3. Results and discussion

Predictive design of extraction processes exploiting aqueous two-phase systems depend upon the understanding of

the mechanism governing the behaviour of proteins in ATPS. However, the lack of accumulative knowledge of such phenomena demands a practical approach for the design of these type of processes. In this paper, for the design of an aqueous two-phase primary recovery process, the influence of systems parameters on the partition behaviour of B-phycoerythrin was studied using single model systems. Such systems were characterised by the presence of purified (commercially available) B-phycoerythrin only in the ATPS. These systems took no account of the influence upon the performance of ATPS of the whole range of proteins, contaminants and cell debris which may be present in the fermentation broth of the *P. cruentum*.

A practical approach which exploits the known effect of system parameters such as tie-line length, phase volume ratio, system pH and molecular weight of PEG on the protein partition behaviour can reduce the extent of the necessary empirical experiments to determine the process conditions for the development of an ATPS extraction process. For the fractionation of the cell homogenate from *P. cruentum*, the concentration of PEG and phosphate, system pH, the phase volume ratio (Vr) and the molecular weight of PEG were manipulated to maximise B-phycoerythrin recovery from the top PEG-rich phase. Initially, the effect of increasing TLL upon partition behaviour of B-phycoerythrin was evaluated. Changes in the TLL affect the free volume [19] available for a defined solute to accommodate in the phase an as a consequence in the partition behaviour of such solute in the ATPS.

The impact of increasing TLL upon B-phycoerythrin purity from model and complex ATPS, when PEG of four different molecular weight (i.e. 1000, 1450, 3350 and 8000 g/gmol) were used, is illustrated in Table 1. For all these systems, volume ratio and system pH were kept constant at 1.0 and 7.0, respectively. The partition experiments that used purified B-phycoerythrin in ATPS revealed that this protein exhibited a strong top-phase preference (data not shown), which imply that the majority of the target protein concentrated in the top phase. The top-phase preference of the B-phycoerythrin resulted in partition coefficients greater than 100.0 and with great variations (i.e. from 100 to 200) for all the systems studied. Such behaviour was explained by problems associated with the detection of the presence of B-phycoerythrin in the bottom phase, caused by the very low amount of the protein concentrated in this phase. As a consequence, it was very difficult to evaluate the impact of system parameters upon the partition behaviour of B-phycoerythrin, by monitoring the protein partition coefficient (K). As a result, it was decided to use the purity of B-phycoerythrin (expressed as the relation of the 545–280 nm absorbance) from the top PEG-rich phase as the response variable to evaluated the effect of system parameters on the behaviour of the protein in ATPS. The results of Table 1 showed that for both experimental systems: model (with purified B-phycoerythrin) and complex (crude extract from *P. cruentum*), increasing TLL caused the purity of B-phycoerythrin from the top PEG-rich phase of the different molecular weight of PEG (1000, 1450 3350 and 8000 g/gmol) used to remain relatively constant. Such behaviour may be explained by the minimum effect of the possible increase or decrease of the contaminant proteins in the top phase caused by the rise in the TLL. It has been reported that the free volume in the bottom phase decreases when the TLL is increased [19] and, as a result, the solutes in the lower phase may be

Table 1
Influence of increasing TLL upon the purity of B-phycoerythrin from PEG/phosphate ATPS

System	Molecular weight of PEG (g/gmol)	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Purity of B-phycoerythrin	
					Model system	Complex system
1	1000	15.6	12.6	28.3	4.2 ± 0.4	2.6 ± 0.1
2		17.6	13.6	36.1	4.6 ± 0.4	2.5 ± 0.1
3		19.8	14.8	38.0	4.7 ± 0.4	2.5 ± 0.1
4		22.2	16.0	49.4	5.1 ± 0.5	2.8 ± 0.3
5	1450	17.6	10.9	34.3	3.8 ± 0.3	1.8 ± 0.1
6		22.2	12.1	47.0	4.3 ± 0.4	2.3 ± 0.1
7		24.9	12.6	53.2	3.8 ± 0.3	2.6 ± 0.1
8		26.1	13.0	55.0	4.2 ± 0.4	2.6 ± 0.1
9	3350	16.9	10.1	33.6	3.0 ± 0.3	2.2 ± 0.1
10		18.7	11.2	39.6	2.5 ± 0.2	2.4 ± 0.1
11		21.0	12.9	45.0	3.0 ± 0.3	2.2 ± 0.1
12		22.1	14.0	48.1	3.6 ± 0.3	2.3 ± 0.1
13	8000	16.1	8.1	27.1	1.7 ± 0.1	1.0 ± 0.1
14		19.0	9.1	40.2	1.6 ± 0.1	1.1 ± 0.1
15		20.0	9.5	45.0	1.5 ± 0.1	1.0 ± 0.1
16		22.9	10.3	49.4	1.5 ± 0.1	1.0 ± 0.1

The tie-line lengths of the systems were estimated from the composition of PEG and phosphate as described in Section 2. The purity of B-phycoerythrin is expressed as the relation of the 545–280 nm absorbances. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively.

promoted to partition to the top phase. Consequently, the increase of contaminant proteins that concentrate in the top phase with increasing TLL is possible and as a result the purity of target product may be negatively affected. However, it seems that in this case the purity of B-phycoerythrin was not significantly affected by the increased concentration of the contaminant protein in the top phase, due probably to the increase in the concentration of B-phycoerythrin in the top phase with increasing TLL. The differences observed in the purity from top PEG-rich phases from the model and complex systems (see Table 1) is explained by the nature of the experimental vehicles (the purity from the starting material for the model system was 1.6, whilst that from the crude extract was approximately equal to 0.9). In the case of the model systems, the sole presence of the target protein, resulted in a high purity from the top phase. In contrast, for the complex system, the presence of contaminants from the homogenate from *P. cruentum* caused an effect in the partition behaviour and purity of B-phycoerythrin.

For all the system studied using cell homogenate from *P. cruentum* (or crude extract), the purity of B-phycoerythrin increased in ATPS compared with that from the crude extract (i.e. purity from the crude extract was approximately equal to 0.9). PEG 1000/phosphate ATPS characterised by TLLs of 49.4% (w/w) (PEG 22.2% (w/w) and phosphate 16.0% (w/w), Vr = 1.0 and system pH of 7.0) resulted in the maximal purity (2.8 ± 0.3) from these experiments. Once the impact of increasing TLL upon the purity of B-phycoerythrin from the top phase was evaluated, the effect of system pH on the purity of the protein was investigated using cell homogenate from *P. cruentum*.

Several authors [14–16,20,21] have discussed the influence of system pH on protein partition behaviour. In general, these reports concluded that increasing the pH (e.g. from 6.5 to 9.0) caused an increase in the protein concentration in the top phase and a decrease in the bottom phase. Such behaviour of proteins has been attributed to free-volume effects [16]. An alternative explanation may be associated to the speciation of the phosphate salts over the pH range and to conformational changes in the structural integrity of proteins [14]. Table 2 shows the influence of pH on the purity of B-phycoerythrin in PEG/phosphate ATPS, when four different molecular weights (i.e. 1000, 1450, 3350 and 8000 g/gmol) of PEG was used. ATPS from each molecular weight of PEG were selected base upon the best purity of the target protein from the results of Table 1. Purity of B-phycoerythrin decreased at the highest system pH evaluated (i.e. pH 9.0) regardless of molecular weight of PEG, except for the case of PEG 8000 in which a slight increase in the purity was observed (from 1.1 to 1.4; see Table 2). The decrease of purity can be associated with an increased in the contaminant proteins migration to the top phase with the increase in pH. Although, increasing the system pH from 7.0 to 9.0 resulted in changes in the purity of B-phycoerythrin from the ATPS studied, it is clear that no great differences to the previous protein purity obtained (see Table 1) was achieved. In the case of top phase recovery of B-phycoerythrin, it is evident that ATPS characterised by low molecular weight of PEG (i.e. 1000 and 1450 g/gmol) exhibited the highest recovery (>70%), compared with that from the ATPS of higher molecular weight of PEG (3350 and 8000 g/gmol; recovery less than 57%, see Table 2). Such behaviour in protein recovery can be associated to the decrease in purity (and top phase concentration of B-phycoerythrin) in these ATPS. Although, the small differences in purity and protein recovery obtained from the ATPS of PEG 1000 and 1450 g/gmol, ATPS with TLL of 49.4% (w/w) (PEG 1000 22.2% (w/w), phosphate 16.0% (w/w)), and 53.2% (w/w) (PEG 1450 24.9% (w/w), phosphate 12.6% (w/w)) with Vr = 1.0 at pH of 8.0 were selected as those that provided the best conditions to satisfy the needs of maximal protein purity (i.e. 2.8 ± 0.2) and top phase protein recovery (i.e. 73.0 ± 3.0 and 77.0 ± 3.0, respectively).

Table 2
Influence of changing system pH upon the purity and top phase recovery of B-phycoerythrin from PEG/phosphate ATPS

System	Molecular weight of PEG (g/gmol)	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	System pH	Purity of B-phycoerythrin	Top phase recovery of B-phycoerythrin (%)
a	1000	22.2	16.0	49.4	7.0	2.8 ± 0.3	73.0 ± 3.0
					8.0	2.8 ± 0.2	73.0 ± 3.0
					9.0	2.5 ± 0.2	72.0 ± 3.0
b	1450	24.9	12.6	53.2	7.0	2.6 ± 0.1	76.0 ± 3.0
					8.0	2.9 ± 0.2	77.0 ± 3.0
					9.0	2.2 ± 0.2	76.0 ± 3.0
c	3350	18.7	11.2	39.6	7.0	2.4 ± 0.1	50.0 ± 2.0
					8.0	1.6 ± 0.1	55.8 ± 2.0
					9.0	1.7 ± 0.1	55.8 ± 2.0
d	8000	19.0	9.1	40.2	7.0	1.1 ± 0.1	20.0 ± 2.0
					8.0	1.3 ± 0.1	56.0 ± 2.0
					9.0	1.4 ± 0.1	45.0 ± 2.0

System pH was adjusted as described in Section 2. The purity of B-phycoerythrin is expressed as the relation of the 545–280 nm absorbance. For all systems, volume ratio (estimated from non-biological experimental systems) was kept constant at 1.0. The top phase recovery is expressed relative to the original amount of B-phycoerythrin loaded into the system.

Table 3
Influence of changing system Vr upon the purity and top phase recovery of B-phycoerythrin PEG/phosphate ATPS

System	Volume ratio	Molecular weight of PEG (g/gmol)	PEG (%, w/w)	Phosphate (%, w/w)	Purity of B-phycoerythrin	Top phase recovery of B-phycoerythrin (%)
I	2.4	1000	29.5	9.0	2.7 ± 0.2	66.4 ± 3.0
II	1.8		24.0	12.0	2.5 ± 0.1	70.2 ± 3.0
III	0.9		18.0	15.5	2.6 ± 0.1	74.8 ± 3.0
IV	0.4		12.5	18.5	2.5 ± 0.1	61.3 ± 3.0
V	0.2		7.5	21.7	2.2 ± 0.1	32.0 ± 3.0
VI	2.4	1450	29.0	8.0	2.6 ± 0.1	64.3 ± 3.0
VII	1.8		23.5	11.0	2.2 ± 0.2	65.2 ± 3.0
VIII	0.9		17.7	14.0	1.8 ± 0.1	66.0 ± 3.0
IX	0.4		12.0	7.0	1.7 ± 0.1	55.0 ± 2.5
X	0.2		7.0	20.0	1.7 ± 0.1	31.5 ± 3.0

The volume ratio (Vr) in non-biological experimental systems along a single tie-line length (49.4 and 53.2% (w/w), for PEG 1000 and PEG 1450, respectively) was estimated after phase separation in graduate centrifuge tubes. The purity of B-phycoerythrin is expressed as the relation of the 545–280 nm absorbance. For all ATPS, system pH was kept constant at 8.0. The top phase recovery is expressed relative to the original amount of B-phycoerythrin loaded into the system.

In the selected ATPS (TLL of 49.4% (w/w), PEG 1000 22.2% (w/w), phosphate 16.0% (w/w), Vr = 1.0 at pH of 8.0 and TLL of 53.2% (w/w), PEG 1450 24.9% (w/w), phosphate 12.6% (w/w), Vr = 1.0 at pH of 8.0), a decrease in the Vr caused the purity of B-phycoerythrin to slightly decline (see Table 3). Hustedt et al. [22] proposed that the protein partition behaviour remains constant for systems along the same tie-line. Such proposal may be extended for the behaviour of B-phycoerythrin in ATPS along the same tie-line. Changes in the protein purity with Vr can be attributed to a concentration effect. A decrease in the Vr imply a reduction of the volume of the top phase. Consequently, the contaminant in this phase will concentrate further and as a result a decrease in the protein purity from that phase is possible. Although, a reduction in the volume of the top phase will also have a concentration effect on the target protein (B-phycoerythrin), it seems that such effect was either smaller than that of the contaminants or caused a possible precipitation of B-phycoerythrin, which in both cases resulted in a reduction on protein purity. From the results of this experiments, it was observed that a change in system volume ratio (different from Vr = 1.0) caused no benefits in the purity and top phase recovery of phycoerythrin, thus ATPS with Vr = 1.0 were preferred for the potential development of a primary recovery process.

In a further comparison of the effect of PEG molecular weight on the purity and top phase recovery of B-phycoerythrin from ATPS, the purity and recovery of B-phycoerythrin from ATPS decreases when high molecular weights of PEG were used (see Table 4). The effect of increasing molecular weight of PEG upon protein partition behaviour has been explained based upon the protein hydrophobicity [23,24] and phase excluded volume [12,25,26]. In the case of B-phycoerythrin, the decrease in protein purity when high molecular weights of PEG were used, may be explained by a migration of contaminant proteins from the bottom phase or interface to the top phase. An alternative explanation involves B-phycoerythrin migration from the top to the bottom phase or the interface. ATPS with low molecular weight of PEG (i.e. PEG 1000 and PEG 1450) exhibited the best protein purity and recovery. ATPS using PEG 1450-phosphate (instead of PEG 1000-phosphate) was selected for the extraction stage, since the cell debris concentrated in the bottom phase (data not shown). In contrast, in ATPS with PEG 1000 cell debris accumulated at the interface. Such a situation may cause contamination problems when the top PEG-rich phase is removed for further processing.

From the studies of the influence of system parameters upon the purity and top phase recovery of B-phycoerythrin from ATPS, process conditions (i.e. Vr = 1.0, PEG 1450 24.9% (w/w), phosphate 12.6% (w/w), TLL of 53.2% (w/w) and system pH of 8.0) were selected for the ATPS extraction stage. Such extraction conditions resulted in a protein product with a purity of 2.8 and top phase recovery of

Table 4
Influence of molecular weight of PEG upon the purity and recovery of B-phycoerythrin from PEG/phosphate ATPS

System	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Molecular weight of PEG (g/gmol)	Purity of B-phycoerythrin	Top phase recovery of B-phycoerythrin (%)
A	22.2	16.0	49.2	1000	2.8 ± 0.3	73.0 ± 3.0
B	24.9	12.6	53.2	1450	2.8 ± 0.1	77.0 ± 3.0
C	18.7	11.2	39.6	3350	1.6 ± 0.1	55.8 ± 2.0
D	19.0	9.1	40.2	8000	1.3 ± 0.1	56.0 ± 2.0

The purity of B-phycoerythrin is expressed as the relation of the 545–280 nm absorbance. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 8.0, respectively. The top phase recovery is expressed relative to the original amount of B-phycoerythrin loaded into the system.

77.0%. The general process proposed for the potential primary recovery of B-phycoerythrin produced by *P. cruentum* is characterised by three unit operation involving one ATPS extraction stage for the downstream processing to produce a B-phycoerythrin with a purity of 2.8, suitable for the purification steps. In contrast, the process reported by Bermejo et al. [5], involves a minimum of eight unit operation for the primary recovery stage to obtain a product with similar characteristics. Therefore, a direct comparison of the new proposed strategy with the existing process, highlights the superiority of the current approach. The research presented here, resulted in a primary recovery process that produced an overall B-phycoerythrin recovery of 77%. The recovery process proposed here increase the protein purity up to 2.8 in ATPS extraction stage, which raise the potential commercial application of this process as an alternative for the practical purification of B-phycoerythrin produced by *P. cruentum*.

4. Conclusions

This study reports for the first time the fractionation of cell homogenate of *P. cruentum* in aqueous two-phase systems for the development of a process for the potential primary recovery of B-phycoerythrin. It has been shown that tie-line length, volume ratio, molecular weight of PEG and system pH influence the purity of B-phycoerythrin from the top PEG-rich phase. PEG 8000-phosphate ATPS proved to be unsuitable for the primary recovery of B-phycoerythrin since top phase recovery and protein purity resulted in low values (i.e. $<$56% and 1.4, respectively). The operating conditions established for the PEG 1450-phosphate ATPS extraction resulted in a one-stage process for the potential recovery of B-phycoerythrin from *P. cruentum*, that concentrated the protein preferentially to the top phase and the contaminants to the opposite phase. Overall, the results reported here demonstrated the potential application of ATPS for the primary recovery of B-phycoerythrin as a first step for the development of a downstream process with commercial application.

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Simplified two-stage method to B-phycoerythrin recovery from *Porphyridium cruentum*☆

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Abstract

A simplified two-stage method for B-phycoerythrin (BPE) recovery from *Porphyridium cruentum* was developed. The proposed method involved cell disruption by sonication and primary recovery by aqueous two-phase partition. The evaluation of two different methods of cell disruption and the effect of increasing concentration of cell homogenate from *P. cruentum* culture upon aqueous two-phase systems (ATPS) performance was carried out to avoid the use of precipitation stages. Cell disruption by sonication proved to be superior over manual maceration since a five time increase in the concentration of B-phycoerythrin release was achieved. An increase in the concentration of crude extract from disrupted *P. cruentum* cells loaded to the ATPS (from 10 to 40%, w/w) proved to be suitable to increase the product purity and benefited the processing of highly concentrated disrupted extract. Kinetics studies of phase separation performed suggested the use of batch settlers with height/diameter (H/D) ratio less than one to reduce the necessary time for the phases to separate. The proposed ATPS stage comprising of 29% (w/w) polyethylene glycol (PEG) 1000 g/mol, 9% (w/w) potassium phosphate, tie-line length (TLL) of 45% (w/w), volume ratio (V_R) of 4.5, pH 7.0 and 40% (w/w) crude extract loaded in a batch settler with H/D ratio of 0.5 proved to be efficient for the recovery of 90% of B-phycoerythrin at the top PEG-rich phase. The purity of B-phycoerythrin increased up to 4.0 times after the two-stage method. The results reported here demonstrate the potential implementation of a strategy to B-phycoerythrin recovery with a purity of 3.2 (estimated by the absorbance relation of 545–280 nm) from *P. cruentum*.

Keywords: B-phycoerythrin; Recovery; Aqueous two-phase systems

1. Introduction

With the increasing commercial significance of colouring compounds from microbial origin used in food, cosmetic, detergent and molecular genetics industries, there is considerable interest in the development of efficient and scalable processes to bring such products to market. The reduced protocols reported for the primary recovery of intracellular coloured proteins involve release of the product by mechanical methods and their collection by centrifugation. The resulting homogenate is then fractionated to purify the colouring protein by an excessive number of stages. The entire protocols in most cases are complicated by the need for multiple chromatography steps to obtain a highly purified protein. The multi-step nature of the conventional methods can result in low process yield and high process cost [1]. Consequently, the potential scaling up of these procedures is perceived as economically unviable. To overcome some of the disadvantages attributed to the established purification protocols for coloured proteins, different approaches have been proposed. Such approaches involve the reduction of the chromatography steps [2] and the use of alternative recovery techniques such as aqueous two-phase systems (ATPS) [3]. One-step chromatography method involving precipitation with ammonium sulphate and ion-exchange chromatography has been recently proposed [2] for the particular recovery of R-phycoerythrin from *Polysiphonia urceolata*. In addition, the potential application of ATPS for the recovery of coloured proteins from microbial origin has also been addressed before [3]. In this context, B-phycoerythrin (BPE), a coloured phycobiliprotein found in nature (cyanobacteria, eukaryotic algae, etc.) [4,5], represents a very attractive study case. It has been reported that BPE can be used as a pigment in the food, cosmetic and pharmaceutical industry, and as a

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fluorescent marker [6–9]. The commercial value of highly purify BPE (defined as the absorbance ratio of $A_{545\,nm}$ to $A_{280\,nm}$ greater than 4.0), has been reported as high as $50 USD/mg [10]. This high value makes attractive the attempt to develop an efficient method for the recovery and purification of BPE.

The complexity of the processes for the recovery and purification of BPE has limited its potential practical implementation at commercial level. Such processes are usually characterized for having a high number of stages, low recovery and limitation to easy process scale-up. Previous attempt for the purification of BPE has been documented, for example the protocols reported by Bermejo et al. [8,11,12] involves ion-exchange high-pressure liquid chromatography or expanded bed absorption chromatography, which resulted in an effective way to purify BPE at laboratory scale. Although, some economic constrains may rise because at industrial scale, high pressure liquid chromatography involves high costs related to operation, these protocols represent attractive alternatives to consider for the purification stage of a potential downstream process to obtain pure BPE. In contrast, in the present research, the primary recovery of BPE is addressed using an alternative technique to produce material suitable for further purification.

Aqueous two-phase system is a technique that has proved to have great potential to recover and purify biological compounds [13–17]. A strategy for the primary recovery of BPE from *P. cruentum* using ATPS, ammonium sulphate precipitation and centrifugation was developed in our group [13]. However, the use of ammonium sulphate precipitation, centrifugation and pellet re-suspension were required in order to concentrate the BPE crude extract obtained by an unoptimized cell disruption stage using manual maceration. From this previous work, it was concluded that breakage of the cell wall of the algae *P. cruentum* was not a trivial task. Thus, the need for an alternative cell disruption method to eliminate unnecessary stages was evident. Furthermore, the potential use of ATPS to process high concentrate biomass extracts can also be exploited to optimize the proposed method.

It is clear that the development of a simplified method for the recovery of BPE from *P. cruentum* will benefit the performance of the previously established purification protocols. In this study, based upon a previous report from our group [13], experimental conditions were selected to address the potential process benefits using an alternative cell disruption strategy. Also, potential system intensification was attempted by evaluating the effect of increasing concentration of crude extract from disrupted *P. cruentum* cultures upon ATPS performance. Furthermore, in this work kinetics studies of phase separation under different extraction device geometries are presented, as a first step to establish initial operating conditions to BPE primary recovery from *P. cruentum*.

2. Materials and methods

2.1. P. cruentum culture and cell disruption

P. cruentum was cultivated in the culture medium previously described by Bermejo et al. [8]. The algae were grown in a batch culture (1000 ml Erlenmeyer flasks) at 22–25 °C under natural light conditions, agitation and aeration was provided with an air flow rate of 3.2 cm^3/seg using a peristaltic pump (ELITE 799, Rolf C Hagen Corp, Mansfield, MA, USA). The cells were allowed to grow for 30 days and were harvested by centrifugation (Eppendorf 5415C, Westbury, NY, USA) at 1,000 × *g* for 5 min. After harvesting, cell disruption was carried out using two different methods (i.e. manual maceration or sonication) in order to establish which of those two methods achieved a higher BPE concentration in the resulting crude extract. For manual maceration, the disruption was done using a ceramic pot under refrigeration using a dry ice bath. Wet biomass, glass beads and de-ionised water (4 cm^3/g wet biomass) were added to the ceramic pot and macerated for 10 min/g wet biomass. For sonication the disruption was done using a 50 cm^3 glass test tube and a sonicator (Branson 1510, Branson Ultrasonic Corp., Danbury, CT, USA). The glass tube was added with wet biomass and de-ionised water (4 cm^3/g wet biomass). The sonication time was 10 min/g wet biomass. During the sonication time, contents were mixed periodically to prevent the aggregation of the biomass at the bottom of the tube. In both disruption methods, cell fragmentation was verified using an optical microscope (Olympus CK2, Olympus, Melville, NY, USA). The homogenate obtained as the result of the cell disruption of the *P. cruentum* biomass was referred to as BPE crude extract (regardless the disruption method used), and included the cell debris generated.

2.2. Effect of concentration of BPE crude extract upon ATPS performance

ATPS were prepared for convenience on a fixed mass basis. Predetermined quantities of stock solutions of polyethylene glycol (PEG) and potassium phosphate (Sigma Chemicals, St. Louis, MO, USA) were mixed with different concentrations of BPE crude extract from 10 to 40%, (w/w); wet weight of disrupted biomass/total weight of the ATPS) to give a final weight of 10 g. The system parameters, selected from previous report [13], were: 29% (w/w) PEG 1000 g/mol, 9% (w/w) potassium phosphate, tie-line length (TLL) 45% (w/w), volume ratio (V_R) of 4.5 and pH 7.0. The stock solutions (PEG, potassium phosphate and BPE crude extract) were mixed and phases dispersed by gentle mixing for 10 min. Adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide when needed. Complete phase separation was achieved by low speed batch centrifugation at 1500 × *g* for 10 min. Visual estimates of the volumes of top and bottom phases were made in graduated tubes. The volumes of the phases were then used to estimate the experimental volume ratio (V_R, defined as the ratio between the volume of the top phase and the bottom phase). Samples were carefully extracted from the phases for biochemical analysis. The top and bottom phase recovery were estimated as the amount of BPE present in the phase (volume of the phase × BPE concentration in the phase) and expressed relative to the original amount loaded into the system. Interface BPE recovery was not experimentally determined due to the presence of cell debris in such phase. Results reported are the average of three inde-

pendent experiments and standard errors were estimated to be a maximum of ±5% of the mean value.

2.3. Influence of the system geometry upon ATPS performance and time of phase separation

In order to study the influence of the system geometry upon the recovery, purification factor of BPE and time of phase separation, batch settlers characterized by different geometries expressed as height/diameter (H/D) ratios from 0.5 to 2.5 were selected. For each set of experiments, two different ATPS comprising 29% (w/w) PEG 1000 g/mol, 9% (w/w) potassium phosphate were mixed with BPE crude extract to reach a final concentration of 40% (w/w) in the ATPS. The pH was adjusted to 7.0 when needed. After complete dissolution of the chemicals forming phases, the two phases of the ATPS identified in this work as "centrifuged" were separated by low-speed batch centrifugation at 1500 × *g* for 10 min at 25 °C. Samples were taken from these systems for biochemical analysis and further estimation of BPE recovery and purification factor (defined as the ratio of BPE purity from the phase divided by that from the crude extract). In contrast, once complete mixing of the chemicals forming phases was achieved phases from the "experimental" systems were allowed to separate under gravity (without further mixing) during which a record of the changing volume of the phases formed with elapsed time was kept. Volume of the phases was estimated using graduated settlers. Volume ratio (V_R) of the ATPS was estimated as a ratio of the volume of the top and bottom phases. The phase separation of PEG–potassium phosphate systems under gravity was expressed as the relative volume ratio (volume ratio of the "experimental" ATPS divided by that from the "centrifuged" system) relative to time. Results reported are the average of three independent experiments and the standard errors were estimated to be a maximum of ±5% of the mean value.

2.4. Analytical procedures

The total protein concentration in the samples was estimated by the method of Bradford [18]. The purity of BPE was determined as the relation of the 545–280 nm absorbance (purity of BPE = $Abs_{545\,nm}/Abs_{280\,nm}$). Bermejo et al. [8] reported the use of the 545–280 nm absorbance relation as an estimation of BPE purity, since the absorption spectrum of this protein exhibits a peak at 545 nm. Under this circumstances a ratio greater than 4.0 corresponds to a highly purify BPE (defined as pure commercial BPE; Sigma). The concentration of BPE and the other two coloured proteins produced by *P. cruentum* (Allophycocyanin (APC) and R-phycocyanin (RPC)) was estimated by measuring the absorbance at 565, 620 and 650 nm, and using the equation system reported previously [8,19].

3. Results and discussion

3.1. Cell disruption studies

P. cruentum (as well as other algae) has a cell wall and membrane structures, which confer it high resistance against a wide variety of cell disruption methods. Selection of an appropriate cell disruption method is required to break the cell wall and membrane structures of *P. cruentum* in order to achieve high release efficiency. The potential protocol for the recovery of BPE using ATPS initially designed [13] was characterized by the need of precipitation stages prior to ATPS. Such precipitation stages were introduced in order to avoid handling of a diluted BPE crude extract, obtained after cell disruption by manual maceration. A direct comparison between cell disruption by sonication and manual maceration was performed in order to increase the release of intracellular BPE. Fig. 1 illustrates the concentration of released BPE, allophycocyanin and R-phycocyanin when these two methods of cell disruption were used. It is clear that cell disruption by sonication proved to be superior for the release of intracellular coloured proteins from *P. cruentum* compared with cell disruption by manual maceration. In particular, the release of the product of interest (BPE) by sonication was 5.5 times higher than the one obtained using manual maceration (i.e. 0.22 mg/ml obtained by sonication compared with 0.04 mg/ml achieved by manual maceration). Coloured intracellular proteins are found inside the chloroplasts of the algae attached to the stromal side of the thylakoid membrane [20]. The constant and uniform forces generated by sonication were more effective than the irregular nature of that produced by manual maceration for detaching and releasing these pigments from the thylakoid membrane, and therefore the resulting concentration achieved was considerably higher.

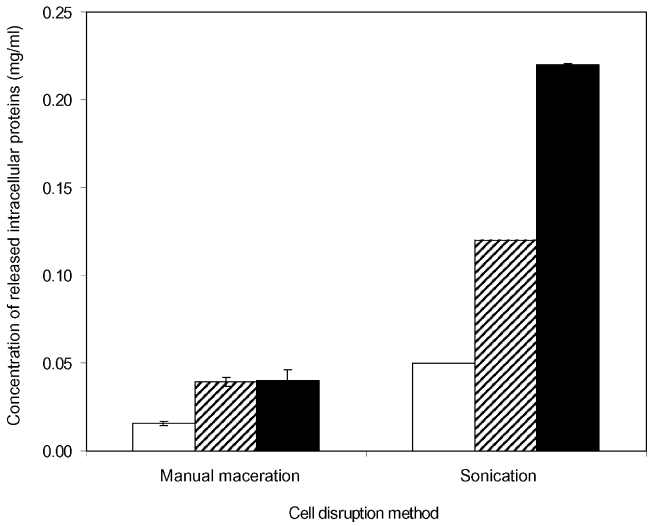

Fig. 1. Effect of methods of cell disruption upon the release of intracellular B-phycoerythrin (BPE), Allophycocyanin (APC) and R-phycocyanin (RPC) from *P. cruentum*. The concentrations of the three main *P. cruentum* intracellular coloured phycobiliproteins; RPC (□), APC (▨) and BPE (■) released are expressed relative to the method of cell disruption used. The concentrations were estimated as described in Section 2.

Interesting, the two selected cell disruption methods gave different patterns in the ratio of BPE to the other two coloured proteins (APC and RPC). Cell disruption by sonication produced a crude extract that exhibited a BPE to APC and RPC ratio greater than the one achieved by manual maceration. In red algae, as well as in cyanobacteria, the phycobiliproteins are assembled

into macromolecular water-soluble light harvesting complexes named phycobilisomes [21]. Phycobilisomes are formed by two structural subunits: a core complex and rod-like segments that are attached to the core complex. While the core complex is formed by phycobiliproteins that absorb in the red region (i.e. APC), the rod-like segments are formed by phycobiliproteins that absorb in the blue/green region of the spectrum (for example BPE and RPC) [5]. Rod-like segments of the phycobilisomes in *P. cruentum* are formed mainly by BPE (phycoerythrin reflects red light and is therefore responsible for the colour of most red algae). The rod-like segments are more exposed to the cytoplasm that the core complex which is attached to the thylakoid membrane. Therefore the cavitations generated by sonication are able to release BPE (that is located in the free end of the rod-like segments) more easily than the other two pigments (APC and RPC). This implies that the core complex is more likely to remain attached to the thylakoid membrane (or thylakoid membrane fragments) when sonication is used instead of manual maceration (which is a disruption method that generates a high mechanical impact over the membrane). As a result, the BPE to APC and RPC ratio achieved by sonication is greater than the one obtained by manual maceration. In the case of BPE purity obtained (purity of BPE = $Abs_{545\,nm}/Abs_{280\,nm}$), both disruption methods provided a crude extract with a purity of 0.8. This implies that even when the methods exhibited a different release patterns for the coloured proteins, the total amount of protein released in relation to the amount of BPE was similar. Using the concentrated BPE crude extract, obtained by sonication, allows eliminating concentration stages prior to ATPS, as the ammonium sulphate precipitation step utilized in the previous protocol reported [13]. Sonication was selected as the method for cell disruption to produce BPE crude extract for the subsequent experimental stages.

3.2. Effect of concentration of BPE crude extract upon ATPS performance

The potential to fractionate heavily loaded biological systems by ATPS has been proved [15,22]. Such biological systems include cells, cell debris, RNA, virus-like particles and proteins. Since an increment in the level of BPE crude extract concentration fractionated via ATPS may benefit the potential intensification of the proposed process, it was decided to examine the impact of highly concentrated BPE crude extract upon ATPS performance. The effect of the concentration of BPE crude extract upon ATPS performance is shown in Table 1. From our previous work [13] it was evident the strong top-phase preference exhibited by BPE in PEG-salt ATPS. Partition coefficient of BPE was difficult to evaluate due to the problems associated to the detection of BPE in the bottom phase. Thus, it was decided to use the top-phase BPE recovery and purity (or purification factor) as the response variables to evaluate the performance of ATPS. For the present work the systems conditions (e.g. molecular weight of PEG, TLL, and system pH) previously established [13] were selected. Large V_R (volume ratio; 4.5) was used to favour the recovery of BPE from the top phase by increasing the free volume available for the target protein to accumulate under increasing crude extract concentration conditions. From Table 1, it is clear that top-phase recovery over 90% was achieved, regardless the concentration of BPE crude extract loaded to the ATPS. Such robust behaviour can be explained in terms of the excluded volume of the top phase of the system [23]. Partition of BPE to the top-PEG-rich phase in ATPS is favoured by using PEG of low molecular weight [13]. Such behaviour has been attributed to the fact that the interaction net of short polymer chains generates a lower excluded volume than the one created by long chains. PEG of low molecular weight (for example, 1000 and 1450 g/mol) helps to overcome saturations problems at the top phase, and therefore BPE is able to migrate at the top phase. Additionally, the ATPS used were characterised by a volume ratio (V_R) greater than one (i.e. $V_R = 4.5$). As the volume of the top phase increases, the free volume available for the BPE to migrate at such phase also increases. An increase in the concentration of BPE crude extract in the system caused BPE purification factor (defined as the ratio between the BPE purity from the top phase and that from the crude extract) to rise (see Table 1). The BPE purity and the purification factor at the top phase increases from 1.3 to 3.2 and from 1.6 to 4.0, respectively (Table 1). When the concentration of BPE crude extract increments (from 10 to 40%, w/w), the amount of BPE presents in the system also increases. The increment in the amount of BPE (a protein with high affinity for the top phase) loaded to the systems favoured the increase of the amount of this target protein partitioned to the top phase. Once the particles with high affinity for the PEG-rich phase have partitioned, molecules with lower affinity can migrate to the upper phase. However, an expected decrease in the free volume available of the upper phase may compromise the further accumulation of the contaminant proteins in this phase [15,16].

Table 1
Influence of the concentration of *P. cruentum* crude extract upon B-phycoerythrin (BPE) recovery and purification factor

	Concentration of crude extract (%, w/w)	Top-phase BPE recovery (%)	Top-phase BPE purification factor
1	10	99.1 ± 3.15	1.6 ± 0.08
2	20	92.6 ± 0.29	2.0 ± 0.02
3	30	91.8 ± 0.22	2.1 ± 0.02
4	40	91.9 ± 0.17	4.0 ± 0.02

The top-phase B-phycoerythrin (BPE) recovery is expressed relative to the concentration of BPE from crude extract loaded into the systems. Concentration of the crude extract represents the total amount of disrupted BPE biomass loaded into the ATPS and it is expressed in wet weight of disrupted biomass relative to the total weight of the ATPS (%, w/w). The BPE purification factor is the ratio between the BPE purity obtained at the top phase of the system and the one from the BPE crude extract. The ATPS used comprised 29% (w/w) PEG 1000 g/gmol, 9% (w/w) potassium phosphate, TLL of 45% (w/w), volume ratio (V_R) of 4.5 and pH 7.0.

3.3. Influence of system geometry upon ATPS performance and time of phase separation

In order to facilitate the practical implementation of lab-scale ATPS protocols at commercial level, the evaluation of system

Table 2
Settlers used to evaluate the influence of the height/diameter (H/D) ratio on kinetics of the phase separation

Geometry	Settler device description	H/D ratio	Cross-section area (cm^2)
1	Glass vessel	0.5	29.0
2	Glass vessel	0.8	20.0
3	Glass vessel	1.2	18.0
4	Glass column	2.5	10.0

The H/D ratio values are the result of the height (H) of the ATPS in the settler divided by the diameter of the settler (D). The cross section-area (interface area) was estimated with the diameter of each settler used.

parameters that provide information relate to the kinetics of phase separation is needed. Exploitation of the potential use of settling equipment for the recovery of biological compounds using ATPS represents a major advantage of the technique for its industrial implementation. It is clear that passive settling to separate immiscible phases is simple in operation and incurs low cost. It has been reported that the settling velocity or phase separation time is strongly influenced by the geometry of the settler used [24,25]. The design or selection of an appropriated geometry of the batch settler is required to facilitate the implementation of ATPS at industrial scale. It is known that in batch settlers, the height (H) and the diameter (D) of the device influences the settling time due to the distance that the droplets must move and the wall effects caused, respectively. In this part of the study, a practical approach that involved the use of height/diameter (H/D) ratio of the settler as a dimensionless parameter was selected to evaluate the performance of ATPS in settlers of different geometries. Such parameter was selected to characterise the influence of the geometry of the settlers (from column to tank or vessel type; H/D ratios from 0.5 to 2.5; see Table 2) on the total time for the phases to separate.

Initially, the influence of the H/D ratio of the batch settlers upon the ATPS performance was evaluated. In this case, the BPE top-phase recovery and the BPE purification factor were not significantly affected by the change in system geometry (H/D ratio; data not shown). Such behaviour can be explained by the fact that changes in geometry (H/D ratio) do not affect the physicochemical properties or parameters of the ATPS (e.g. concentration of polymer and salt, ionic force, pH, V_R, etc.). In contrast, it has been reported that the system geometry have significant influence upon the time of phase separation [24,25]. However, the nature of such effect upon ATPS loaded with BPE crude extract has not been reported before. The influence of the H/D ratio upon the time of phase separation in ATPS loaded with 40% (w/w) of disrupted *P. cruentum* is illustrated in Fig. 2. The solid concentration (40% wet, w/w of the disrupted biomass) was selected based upon the results from the previous section of this work. An increment in the H/D ratio causes the time needed for the phase formation to rise. For the two lower H/D ratios used (i.e. H/D equal to 0.5 and 0.8) a relative V_R of 0.7, which represents 70% of the phase separation efficiency by sedimentation relative to that obtained by centrifugation (used only as a reference), was achieved after

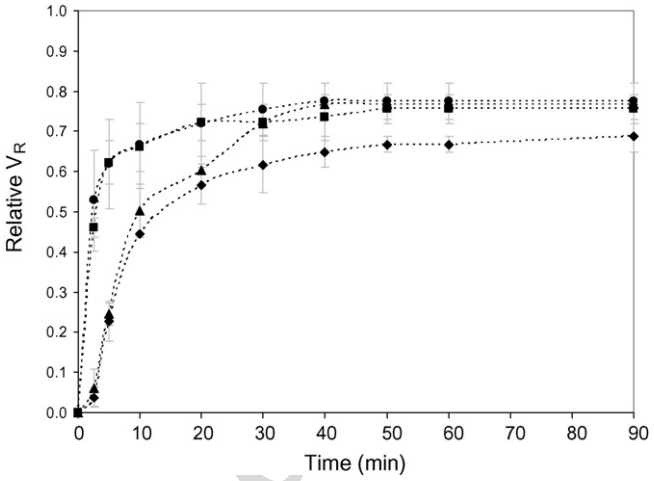

Fig. 2. Influence of the height/diameter ratio of the settler upon the kinetics of phase separation in batch ATPS. Four different height/diameter ratios were used: 0.5 (●), 0.8 (■), 1.2 (▲) and 2.5 (♦). The relative volume ratio (V_R) is defined as the ratio between V_R of the experimental system and the V_R of the centrifuged system as described in Section 2 and it is expressed relative to time. The ATPS used comprised 29% (w/w) PEG 1000 g/gmol, 9% (w/w) potassium phosphate, TLL 45% (w/w), V_R of 4.5, pH 7.0 and 40% (w/w) BPE crude extract load.

20 min. In contrast, for H/D 1.2 and 2.5 a relative V_R of 0.7 was achieved after 30 min and more than 90 min, respectively. This can be explained in terms of the interface area available (cross-section area) in the system (Table 2). Batch settlers with low H/D ratios are characterised by sufficient available interface area (compared with settlers with H/D higher than one) for solids distribution. Solids such as cell debris and other suspended particles present in the BPE crude extract can distribute across the interface (the middle part of settling device characterised by the presence of unseparated phases) and minimize solid accumulation. Thus, it is expected that phase separation under gravity will occur in a relatively short time. In contrast, in batch settlers with higher H/D ratios (e.g. H/D = 2.5) Table 2; the interface area is reduced, and therefore solids accumulate rapidly. Consequently, the time needed for phase separation by gravity is increased. The kinetics of phase separation followed up to 24 h, and the maximum relative V_R achieved was 0.82 for all H/D ratios (data not shown), which represents 82% phase separation efficiency achieved by sedimentation. Cell debris accumulated at the interface, extended to the bottom phase, thus a clear bottom phase was difficult to achieve in these ATPS, and caused an increment in the volume of such phase. Consequently, reaching a 100% phase separation efficiency of the systems separated by gravity was severely limited. The batch settlers with H/D ratio of 0.5 and 0.8 exhibited the best separation performance. These findings suggest the use of settlers with H/D ratios less that 1.0 to minimise the time for the phases to separate. This type of study provides an insight into the kinetics of phase separation necessary to design of appropriate equipment for the required pilot scale of the proposed ATPS process.

A direct comparison of the new proposed protocol for the recovery and purification of BPE from *P. cruentum* with the previous reported protocol involving BPE extract concentration and

cell debris separation stages [13] highlights the superiority of the new approach. Implementation of an alternative cell disruption method resulted in a reduction of the number of unit operations involved in the previous reported protocol by eliminating concentration stages resulting from poor extraction. Cell disruption achieved by sonication would of course be impractical at process scale and would need to be replaced by a mechanical cell disruption method (e.g. bead mill or homogenization). The outline of the new proposed method does not involve an additional cell debris removal stage due to the fact that ATPS was used to eliminate the cell debris. Thus, cell debris removal and BPE primary recovery stages were integrated. It is clear that, for BPE this two-stage method opens the way to potentially evaluate further bioprocess scale up and enhancement.

4. Conclusions

A two-stage method for the recovery of BPE from *P. cruentum* was developed that benefited from a reduced number of stages. It has been shown that cell disruption by sonication was preferred over manual maceration for the release of intracellular BPE, since a five times increase in BPE concentration, release was obtained with a similar degree of purity. An increase in BPE crude extract loaded to the ATPS (from 10 to 40%, w/w) proved to be suitable to increase the product purity and benefitted the processing of highly concentrated disrupted extract. In the case of kinetics of phase separation studies, it was concluded that H/D ratio has no effect upon the purity and the top-phase recovery of BPE when batch settlers were used. It was also concluded that the geometry of the separation device (expressed as H/D ratio) has an effect on the phase separation time. Batch settlers with H/D ratio less than one were particularly suitable to achieve a rapid phase separation. Overall the proposed ATPS process comprising of 29% (w/w) PEG 1000 g/gmol, 9% (w/w) potassium phosphate, TLL of 45% (w/w), V_R of 4.5, pH 7.0 and 40% (w/w) BPE crude extract loaded in a batch settler with H/D ratio of 0.5 proved to be efficient, recovering above 90% of BPE at the top phase (PEG-rich phase) with a purity of 3.2. It is clear that the results reported here, demonstrated the potential implementation of a method for BPE recovery from *P. cruentum* as a first step for the development of a process with commercial application.

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Potential Aqueous Two-Phase Processes for the Primary Recovery of Colored Protein from Microbial Origin

By *J. Benavides* and *M. Rito-Palomares**

The primary recovery of c-phycocyanin and b-phycoerythrin from *Spirulina maxima* and *Porphyridium cruentum*, respectively, using an established extraction strategy was selected as a practical model system to study the generic application of polyethylene glycol (PEG)-phosphate aqueous two-phase systems (ATPS). The generic practical implementation of ATPS extraction was evaluated for the recovery of colored proteins from microbial origin. A comparison of the influence of system parameters, such as PEG molecular mass, concentration of PEG as well as salt, system pH and volume ratio, on the partition behavior of c-phycocyanin and b-phycoerythrin was carried out to determine under which conditions target colored protein and contaminants concentrate to opposite phases. One-stage processes are proposed for the primary recovery of the colored proteins. PEG1450-phosphate ATPS extraction (volume ratio (V_R) equal to 0.3, tie-line length (TLL) of 34 % w/w and system pH 7.0) for the recovery of c-phycocyanin from *Spirulina maxima* resulted in a primary recovery process that produced a protein purity of 2.1 ± 0.2 (defined as the relationship of 620 nm to 280 nm absorbance) and a product yield of 98 % [w/w]. PEG1000-phosphate ATPS extraction (i.e., $V_R = 1.0$, PEG 1000, TLL 50 % w/w and system pH 7.0) was preferred for the recovery of b-phycoerythrin from *Porphyridium cruentum*, which resulted in a protein purity of 2.8 ± 0.2 (defined as the relationship of 545 nm to 280 nm absorbance) and a product yield of 82 % [w/w]. The purity of c-phycocyanin and b-phycoerythrin from the crude extract increased 3- and 4-fold, respectively, after ATPS. The results reported herein demonstrated the benefits of the practical generic application of ATPS for the primary recovery of colored proteins from microbial origin as a first step for the development of purification processes.

1 Introduction

The urgent need for manufacturers to rapidly and economically bring new biotechnological products to the market has oriented research to establish selective and scalable methods of primary product recovery that integrate effectively with upstream cell cultures. The resulting protocols will have to yield product in a state suitable for validation of polishing, formulation and delivery operations. Currently, biotechnological companies are focusing on the production of high-value products that will have impact in different industrial sectors, such as health, chemistry, food and others. In this context, coloring compounds used in food, cosmetic, detergent and molecular genetics industries are products of great commercial significance [1, 2]. The potential production of these substances by microorganisms presents a very interesting opportunity for biotechnological processes. Particularly, the production of colored proteins, named phycobiliproteins, from microbial origin represents a very interesting case because both the industrial application and commercial value of these products are considerable [1]. The phycobiliproteins are usually found in algae and cyanobacteria as a part of the photosynthetic system of these microorganisms [3–5]. Two microorganisms that have exhibited great potential for the production of these colorant compounds are *Spirulina maxima* and *Porphyridium cruentum*.

The commercial value of food grade c-phycocyanin (a blue-colored protein) produced by *Spirulina maxima* (purity of 0.7, defined as the relationship of 620 nm to 280 nm absorbance) is approximately US$ 0.13 per mg, while that of reactive grade c-phycocyanin (purity of 3.9) varies from US$ 1 to US$ 5 per mg [5]. In contrast, the commercial value of analytical grade c-phycocyanin (purity greater than 4.0) can be as high as US$ 15 per mg [6]. c-Phycocyanin (molecular weight of 44 kDa) is one of the two main biliproteins obtained from the photosynthetic systems of *Spirulina maxima*. It is formed by two sub-units, α and β, with a molecular weigh of 20.5 and 23.5 kDa, respectively, and its isoelectric point has been reported [7] to be around 5.8. In the case of *Porphyridium cruentum*, b-phycoerythrin (a red-colored protein) is the most valuable of the three main classes of phycoerythrins (B, R and C) on account of its photosynthetic system which offers a wide range of potential commercial applications, such as in natural dyes, food, cosmetics and in the development of biosensors [8, 9]. The commercial value of highly purified b-phycoerythrin (purity greater than 4, defined as the relationship of 545 to 280 absorbance) for pharmaceutical or fluorescent uses can be more than US$ 50 per mg [10, 11]. b-Phycoerythrin (molecular weight of 245 kDa) is formed by three subunits, α, β, and γ (in a relative molar ratio of 6:6:1) with a molecular weight of 18.0, 18.0, and 29 kDa, respectively [12].

The recovery of these colored proteins from microbial origin has been previously attempted [9, 13, 14]. However, the resulting protocols identified the need to improve the pri-

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mary recovery part of the downstream process to increase product yield that is affected by conventional procedures. Consideration of alternative strategies for the recovery of colored protein is needed to alleviate concerns regarding the potential scaling up of established protocols at the commercial scale. As an attractive alternative to address some of the disadvantages attributed to the established c-phycocyanin and b-phycoerythrin purification protocols, the use of aqueous two-phase systems (ATPS) has been suggested. ATPS is characterized by several advantages, not only concerning the recovery of protein products from fermentation broth [15–18], but also concerning bio-compatibility, ease of scale-up, low cost, etc. [19].

In this context, we recently reported [20] the establishment of a primary recovery process for the purification of b-phycoerythrin from *Porphyridium cruentum* in which the preliminary conditions were defined. For this second study, defined operating conditions from this previous report were selected to address the generic process applicability of the ATPS extraction strategy for the recovery of colored protein, using an additional model system (i.e., recovery of c-phycocyanin from *Spirulina maxima*). Also, multi-stage ATPS extraction was considered to potentially improve process yield and product purity. Furthermore, in this work the recovery of c-phycocyanin and b-phycoerythrin from *Spirulina maxima* and *Porphyridium cruentum*, respectively, is presented in the form of representative model systems to compare the performance of ATPS extractions for the primary recovery of colored proteins from microbial origin.

2 Materials and Methods

2.1 Culture Medium and Cultivation Conditions

Spirulina maxima and *Porphyridium cruentum* were cultivated in the culture medium described by Herrera *et al.* [13] and Bermejo *et al.* [9], respectively. The algae were grown in a batch culture (500 cm^3 Erlenmeyer flasks) at 22–25 °C, under natural light conditions. Agitation in *Spirulina maxima* culture was provided using a reciprocal shaker (Lab-Line at 80 min^{-1}), while agitation and aeration for the *Porphyridium cruentum* culture was provided with an air flow rate of 3.2 cm^3/seg using a peristaltic pump (ELITE 799, Mexico). The cells were allowed to grow for 12 and 30 days for *Spirulina maxima* and *Porphyridium cruentum*, respectively. Cells were harvested by centrifugation at 12,000 rpm for 10 min (Eppendorf 5415 C). Cell disruption was performed manually in a ceramic pot using glass beads and 0.1 mol/dm^3 $CaCl_2$ solution (10 cm^3/g wet biomass) for 10 min for *Spirulina maxima* and deionized water (4.3 cm^3/g wet biomass) for 15 min for *Porphyridium cruentum*. Temperature was controlled with a dry ice bath. Cell fragmentation was verified using an optical microscope (Olympus CK2). The removal of cell debris was achieved by centrifugation at 12,000 rpm for 10 min (Eppendorf 5415 C).

In the case of the *Spirulina maxima* homogenate, the supernatant (referred to as c-phycocyanin crude extract) was introduced into the aqueous two-phase systems previously selected. In contrast, the supernatant obtained from the *Porphyridium cruentum* homogenate was processed with a solution of ammonium sulfate (0.47 g/cm^3 supernatant). This precipitation step was introduced in order to concentrate the protein in the cell homogenate to facilitate the estimation of protein concentration in the ATPS experiments. The precipitate was resuspended in ammonium sulfate buffer (60 % v/v) with potassium phosphate (0.05 mol/dm^3, pH 7.0) and sodium azide (0.005 mol/dm^3), the resulting solution (referred to as b-phycoerythrin crude extract) was introduced into the aqueous two-phase system previously selected, as described below.

2.2 Design of the Potential Processes for the Colored Protein Recovery from Microbial Origin

The binodal curves for the ATPS were estimated by means of the cloud point method [21] using poly(ethylene glycol) (Sigma) with a nominal molecular mass of 1,000, 1,450, 3,350 and 8,000 g/gmol (50 % [w/w] stock solution) and di-potassium hydrogen orthophosphate/potassium di-hydrogen orthophosphate (Sigma) (30 %, w/w). Fine adjustment of the pH was carried out by the addition of orthophosphoric acid or sodium hydroxide. All experimental systems used to establish the operating conditions for the ATPS process were prepared for convenience on a fixed mass basis. Predetermined quantities of stock solutions of PEG and potassium phosphate were mixed with the experimental solution (containing 10 % [w/v] wet homogenate from *Spirulina maxima* fermentation, referred earlier as c-phycocyanin crude extract, or b-phycoerythrin concentrated solution referred earlier as b-phycoerythrin crude extract). The stock solutions (PEG and salts) were mixed and phases were dispersed by gentle mixing for 30 min at 25 °C.

Complete phase separation was achieved by low speed batch centrifugation at $1500 \times g$ for 20 min at 25 °C. Visual estimates of the volumes of top and bottom phases and solids were performed in graduated centrifuge tubes. The volumes of the phases were then used to estimate the volume ratio (volume of the top phase/volume of the bottom phase (V_R)). Samples were carefully extracted from the phases for biochemical analysis. The tie-line length (TLL) of the system, which represents the length of the line that connects the composition of the top and bottom phase of a defined ATPS, was estimated using the relation in which TLL is equal to $[(\Delta PEG)^2 + (\Delta salt)^2]^{1/2}$; where ΔPEG and $\Delta salt$ are the differences in the concentration between the top and bottom phase of PEG and salt obtained from the phase diagram as described by Albertsson [19]. Top phase recovery was estimated as the amount of protein present in the upper phase (volume of the phase × protein concentration in the phase) and was expressed relative to the original amount loaded into the systems.

The results reported are the average of three or two independent experiments (for c-phycocyanin and b-phycoerythrin, respectively) and the errors were estimated to be a maximum of ±10 % of the mean value. After comparing the influence of TLL, V_R, pH and molecular weight of PEG upon the partition of c-phycocyanin and b-phycoerythrin, the use of subsequent ATPS was studied. This was done in order to determine the influence of using subsequent ATPS upon the purity of the proteins of interest. The subsequent ATPS extraction stages were implemented in which the top phase from the previous extraction was transferred to a new ATPS. The operating conditions (PEG and phosphate concentration, system pH and V_R) of the subsequent process were kept constant and were similar to those defined for the first extraction.

2.3 Analytical Procedures

Protein concentration in the samples was estimated by the method of Bradford [22]. The purity of c-phycocyanin was determined as the relation of 620 to 280 nm absorbance (i.e., purity of c-phycocyanin = Abs 620 nm/Abs 280 nm), while the purity of b-phycoerythrin was determined as the relation of 545 to 280 nm absorbance (i.e., the purity of b-phycoerythrin = Abs 545 nm/Abs 280 nm).

3 Results and Discussion

The design of primary recovery aqueous two-phase extraction processes demands a practical approach to overcome the lack of accumulative knowledge regarding the mechanisms governing the behavior of proteins in ATPS [23]. In this paper, two experimental vehicles were selected (i.e., c-phycocyanin produced by *Spirulina maxima* and b-phycoerythrin produced by *Porphyridium cruentum*) as representative model systems to compare the performance of ATPS extractions for the primary recovery of two different colored proteins from microbial origin. Furthermore, such a process comparison was carried out to also prove the generic applicability of the ATPS recovery strategy. Based upon previous reports [20, 23] experimental ATPS characterized by the low molecular mass of PEG (i.e., 1,000 and 1,450 g/gmol) were selected to compare the effect of increasing TLL upon the partition behavior of the colored proteins and to increase the purity from the original crude extract. It has already been reported in [24] that changes in the TLL affect the free volume available for a defined solute to concentrate in the phase and as a consequence affect the partition behavior of the solute in the ATPS.

The influence of increasing TLL upon the purity of colored proteins (i.e., c-phycocyanin and b-phycoerythrin) from selected ATPS, when the PEG of two different molecular masses (i.e., 1,000 and 1,450 g/gmol) was employed is illustrated in Tab. 1. c-Phycocyanin and b-phycoerythrin exhibited a strong top phase preference (data not shown), which implies that the majority of the target protein was concentrated in the top phase. The top phase preference of the colored proteins resulted in partition coefficients greater than 100.0 and with great variations (i.e., from 100 to 200). Such behavior was associated with problems in the detection of colored proteins in the bottom phase, caused by the very low amount of these proteins concentrated in this phase. As a consequence, it was difficult to use the protein partition coefficient (K = concentration of solute in the top phase/concentration of solute in the bottom phase) to evaluate the impact of system parameters upon the partition behavior of c-phycocyanin and b-phycoerythrin. As a result, a decision was taken to use the purity of the colored proteins from the top PEG-rich phase as the response variable to evaluate the effect of system parameters on the behavior of the proteins in ATPS.

Tab. 1 demonstrates that increasing TLL caused the purity of c-phycocyanin from the top PEG-rich phase to decline, when PEGs of 1,000 and 1,450 g/gmol were used. The increase in the contaminant proteins in the top phase caused by the rise in TLL may explain the decline in the protein purity observed (see Tab. 1). The increase in TLL reduced the free volume in the bottom phase [24] promoting the solutes in the lower phase to partition to the top phase. Therefore, an increase in the amount of contaminant proteins that concentrate in the top phase with increasing TLL is possible and as a result the purity of c-phycocyanin is negatively affected. However, in the case of b-phycoerythrin (from the crude extract from *P. cruentum*) an increasing TLL caused

Table 1. Comparison of the influence of Tie Line Length upon c-phycocyanin and b-phycoerythrin purity.

System	Molecular mass of PEG [g/gmol]	TLL [% w/w]	Purity of c-phycocyanin	Purity of b-phycoerythrin	Purification factor for c-phycocyanin	Purification factor for b-phycoerythrin
1	1000	38	1.2 ± 0.12	2.5 ± 0.1	1.7	3.6
2		50	0.9 ± 0.09	2.8 ± 0.3	1.3	4.0
3	1450	34	1.5 ± 0.15	1.8 ± 0.1	2.1	2.6
4		55	1.0 ± 0.10	2.6 ± 0.1	1.4	3.7

The tie-line lengths (TLL) of the systems were estimated from the composition of PEG and phosphate as described in Materials and Methods.
The purity of c-phycocyanin and b-phycoerythrin was defined as the relationship of 620 nm to 280 nm absorbance and 545 nm to 280 nm absorbance, respectively. Purification factors represent the increase in purity of the target protein relative to the purity of the initial crude extract (i.e., 0.7 for both colored proteins).
For all systems, the volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively.

the purity of the protein from the top PEG-rich phase to increase. It seems that in the case of this particular colored protein its purity was benefited by the increase in the concentration of the chemical forming phases. The opposite behavior observed between c-phycocyanin and b-phycoerythrin when TLL was increased may be explained as a result of the differences in the molecular size of the two colored proteins. The molecular weight of b-phycoerythrin (245 KDa) is considerably greater than that of c-phycocyanin (44 KDa) leaving reduced space in the top phase to accommodate migrating contaminants or force them to migrate from the bottom phase by the increased TLL.

For all the systems studied using cell homogenate, the purity of the colored proteins increased in the ATPS (see purification factors for both colored proteins in Tab. 1) compared with that from the crude extract (i.e., the purity of the crude extract from *S. maxima* and *P. cruentum* was approximately 0.7). PEG 1450-phosphate ATPS (characterized by a TLL of 34 % [w/w], a $V_R = 1.0$ and a system pH of 7.0) resulted in a maximum purity for c-phycocyanin (i.e., 1.5 ± 0.15), which represent an increase in purity of 2.1 times with respect to the purity of the initial crude extract. In the case of b-phycoerythrin, a maximum purity of 2.8 ± 0.3 was obtained from PEG 1000-phosphate ATPS (with a TLL of 50 % [w/w], a $V_R = 1.0$ and a system pH = 7.0). In this case, the purity of b-phycoerythrin from the crude extract (i.e., 0.7) increased fourfold after ATPS (see Tab. 1). Once the impact of TLL upon the purity of the colored proteins from the top phase was evaluated, the effect of the system pH on the purity of the proteins was investigated using cell homogenate (crude extract) from *S. maxima* and *P. cruentum*.

The effect of the system pH on protein partition behavior in ATPS has been previously discussed [15, 16]. These reports concluded that an increase in the system pH caused the protein concentration in the top phase to rise and in the bottom phase to decrease. Such behavior of proteins has been attributed to free-volume effects [24], speciation of the phosphate salts over the pH range and to conformational changes in the structural integrity of proteins [25]. Fig. 1 shows a comparison of the effect of the pH on the purity of the colored proteins in PEG-phosphate ATPS, when two different molecular masses of PEG (i.e., 1,000 and 1,450 g/gmol) were employed. A comparison of the effect of an increasing system pH (from 7.0 to 8.0) upon the purity of c-phycocyanin and b-phycoerythrin revealed a similar opposite trend (as that observed with increased TLL) between the two colored proteins, regardless of the molecular mass of PEG (see Figs. 1a and 1b). The purity of c-phycocyanin decreased when the system pH was increased, while that from b-phycoerythrin remained constant (see Fig. 1a) or slightly increased (see Fig. 1b). The decrease in purity may be associated with an increase in the migration of contaminant proteins to the top phase with the increase in the pH. When the system pH is increased, some contaminant proteins may acquire a negative charge and since PEG is positively charged, it is possible that such contaminant proteins could migrate to the top phase decreasing the purity of the c-phycocyanin. Although, increasing the system pH resulted in changes in the purity of both c-phycocyanin and b-phycoerythrin from the ATPS studied, it is clear that no great differences in the previous protein purity obtained (see Tab. 1) were achieved. Thus, it was decided to keep the system pH at 7.0 for the ATPS selected for further studies.

An important system parameter for the characterization of an ATPS extraction stage is the volume ratio (V_R) defined as the ratio of the volume of the phases of the systems. It has been proposed that protein partition behavior remains constant for the systems along the same tie-line [20] and that variations in the partition behavior observed with changes in

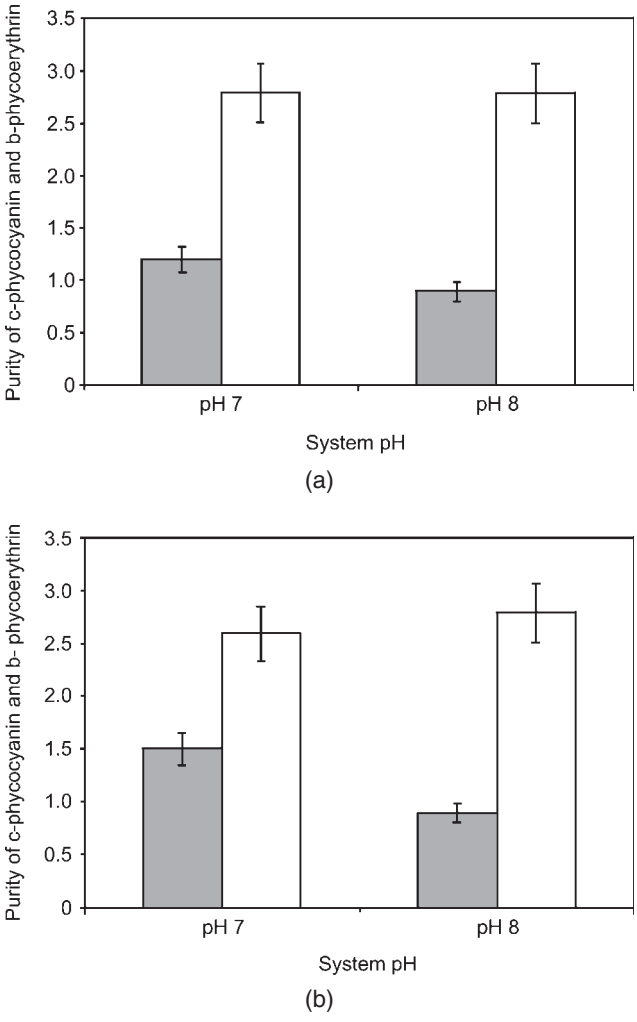

Figure 1. Comparison of the influence of changing the system pH upon the purity of c-phycocyanin and b-phycoerythrin from PEG 1000-phosphate ATPS (a) and PEG 1450-phosphate ATPS (b).
The purity of b-phycoerythrin (white bars) and c-phycocyanin (gray bars) are expressed relative to the system pH.
The system pH was adjusted as described in *Materials and Methods*. The characteristics for the ATPS used are represented in Tab. 1 (i.e., systems 1–4). The crude extracts of c-phycocyanin and b-phycoerythrin were used as experimental vehicles. The purity of c-phycocyanin and b-phycoerythrin is defined as the relation of 620 nm to 280 nm absorbance and 545 nm to 280 nm absorbance, respectively.

V_R can be attributed to a concentration effect [23]. Such a proposal may be extended for the behavior of the colored proteins in the ATPS. Fig. 2 illustrates the comparison of the effect of the system V_R upon the purity of c-phycocyanin and b-phycoerythrin. It is clear that c-phycocyanin and b-phycoerythrin exhibited an opposite behavior when the system V_R was increased. A rise in the V_R implies an increment in the volume of the top phase. Consequently, the volume available to accommodate solutes in that phase is augmented. It is possible that in the case of c-phycocyanin, this protein of low molecular weight (44 KDa) is displaced when it competes with contaminant proteins (from the crude extract) for the additional available volume generated by the increase in the system V_R. Therefore, the reduction in the purity of c-phycocyanin with increased V_R (see Fig. 2) can be explained by the increment in the contaminant concentration in the top phase. In contrast, the increase in the system V_R caused the purity of b-phycoerythrin to rise.

The molecular weight of b-phycoerythrin (245 KDa) is greater than that of c-phycocyanin and it is expected to have a different partition behavior under an increased system V_R. Although it is possible that a competition between the contaminant proteins and b-phycoerythrin for the additional volume available (when V_R increases) in the top phase occurred, it seems that the high molecular weight colored protein occupied the majority of such volume [20]. From the results of these experiments it is clear that a system V_R smaller than one favored the purity of c-phycocyanin from the top phase (i.e., $V_R = 0.3$, resulted in a protein purity of 2.1 ± 0.2), while a system V_R equal to one resulted in a maximum b-phycoerythrin purity (i.e., 2.8 ± 0.2) from the ATPS studied (see Fig. 2).

In a further comparison of the effect of PEG molecular mass on the purity of the colored proteins from ATPS, the purity of both proteins (i.e., c-phycocyanin and b-phycoerythrin) decreased when high molecular masses of PEG were applied (see Fig. 3). The effect of increasing the molecular mass of PEG upon protein partition behavior has been explained based upon the protein hydrophobicity [26, 27] and the phase excluded volume [16, 28, 29]. The increase in the molecular mass of PEG enhances the potential interactions between the long chains of the polymer. Consequently, the free volume in the upper phase decreases and promotes the migration of molecules (such as c-phycocyanin and b-phycoerythrin) from the top to the bottom phase or interface. As a result, the purity of both colored proteins is negatively affected. An alternative explanation involves the decrease in the amount of water in the upper phase when the molecular mass of PEG is increased.

Since the colored proteins are highly hydrophilic, it is anticipated that the affinity for the upper phase of the target proteins decreased and subsequently promoted the migration of the molecules to the bottom phase or interface. It is clear that the negative effect of the increasing molecular mass of PEG on protein purity observed in Fig. 3 was greater for b-phycoerythrin than for c-phycocyanin. This situation may be explained based upon the high molecular weight of this particular protein compared to that of the c-phycoerythrin. In this case it is anticipated that a reduction in the free volume affected the high molecular weight proteins more severely. It is clear that the low molecular mass of PEG (i.e., 1,000 and 1,450 g/gmol) should be used for the extraction of colored proteins in ATPS.

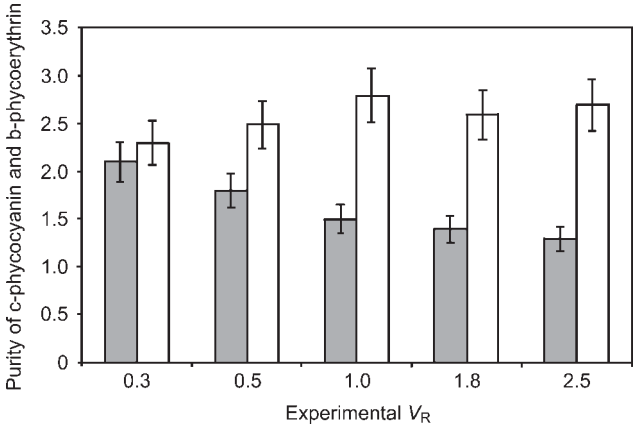

Figure 2. Comparison of the influence of changing the system V_R upon the purity of c-phycocyanin and b-phycoerythrin from PEG-phosphate ATPS. The purity of b-phycoerythrin (white bars) and c-phycocyanin (gray bars) from PEG 1000 and 1450 ATPS, respectively, are expressed relative to the system V_R. The volume ratio (V_R) in non-biological experimental systems along a single tie-line length (50 and 34 % w/w for PEG 1000 and PEG 1450, respectively) was estimated after phase separation in graduate centrifuge tubes. The crude extracts of c-phycocyanin and b-phycoerythrin were used as experimental vehicles. The purity of c-phycocyanin and b-phycoerythrin is defined as the relation of 620 nm to 280 nm absorbance and 545 nm to 280 nm absorbance, respectively.

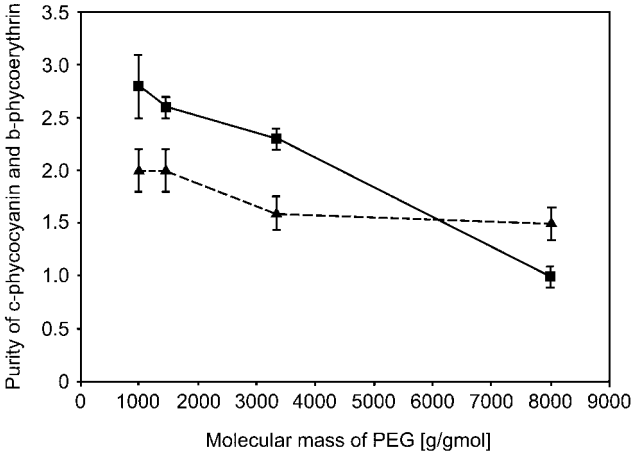

Figure 3. Comparison of the influence of molecular mass of PEG upon the purity of c-phycocyanin and b-phycoerythrin from PEG-phosphate ATPS. The purity of b-phycoerythrin (squares) and c-phycocyanin (triangles) are represented relative to the molecular mass of PEG. The system volume ratio (V_R) for b-phycoerythrin and c-phycocyanin was kept constant at 1.0 and 0.3, respectively. Crude extracts of c-phycocyanin and b-phycoerythrin were used as experimental vehicles. The purity of c-phycocyanin and b-phycoerythrin is defined as the relation of 620 nm to 280 nm absorbance and 545 nm to 280 nm absorbance, respectively.

From the studies on the influence of system parameters upon the purity of colored proteins, optimum process conditions for c-phycocyanin (i.e., $V_R = 0.3$, PEG 1450, TLL 34 % w/w and system pH 7.0) and b-phycoerythrin (i.e., $V_R = 1.0$, PEG 1000, TLL 50 % w/w and system pH 7.0) were selected for the ATPS extraction stages. Such extraction conditions resulted in a protein purity of 2.1 and 2.8 for c-phycocyanin and b-phycoerythrin, respectively. In order to further increase the protein purity, subsequent ATPS extraction stages were applied. At these extraction stages, the top PEG rich-phase (where the c-phycocyanin or the b-phycoerythrin was present) from the previous ATPS was further processed by the addition of fresh phosphate to create a new ATPS extraction stage. The process conditions for the subsequent ATPS extraction stages were kept constant and equal to those used for the first ATPS extraction.

Fig. 4 illustrates the effect of the application of multiple ATPS extraction stages on the purity of colored proteins. In addition, protein recovery, purity and purification factors for the colored proteins after each process stage are presented in Tab. 2. The protein recovery values take into account the losses incurred during the handling of the different streams of the processes. It is evident that the use of consecutive ATPS caused the c-phycocyanin purity (from the top phase) to rise (from 2.1 to 2.4). It seems that further removal (in the bottom salt-rich phase) of additional protein contaminants was achieved in the subsequent extraction stages. However, it is clear that no significant increment in protein purity was obtained when a third ATPS extraction stage was used.

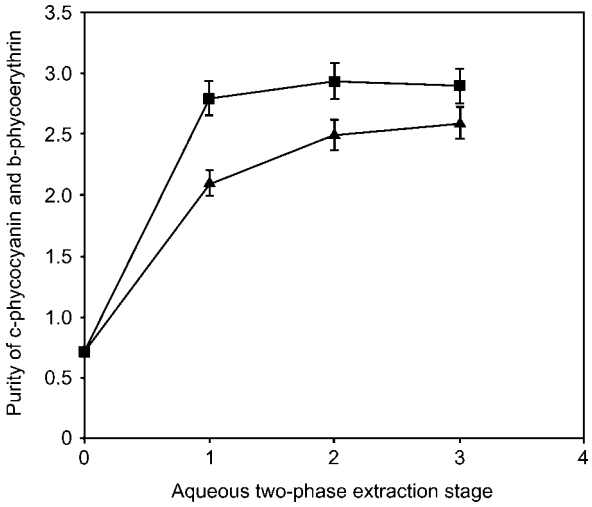

Figure 4. Effect of the implementation of multiple ATPS extraction stages on the purity of c-phycocyanin and b-phycoerythrin.
The purity of b-phycoerythrin (squares) and c-phycocyanin (triangles) are represented relative to the number of ATPS extraction stages. Crude extracts of c-phycocyanin and b-phycoerythrin were used as experimental vehicles.
The purity of c-phycocyanin and b-phycoerythrin is defined as the relation of absorbance 620 nm to 280 nm, and 545 nm to 280 nm, respectively. Extraction conditions for c-phycocyanin (i.e., $V_R = 0.3$, PEG 1450, TLL 34 % w/w and system pH 7.0) and b-phycoerythrin (i.e., $V_R = 1.0$, PEG 1000, TLL 50 % w/w and system pH 7.0) were kept constant for all the ATPS extraction stages.

Table 2. Protein yield, purity and purification factor from each process step for the recovery of colored proteins from microbial origin.

	Protein yield from each individual step [%]	Cumulative yield [%]	Purity of the colored protein	Purification factor
Crude extract				
c-phycocyanin	Not evaluated	100	0.7 ± 0.07	1.0
b-phycoerythrin	Not evaluated	100	0.7 ± 0.07	1.0
First ATPS extraction				
c-phycocyanin	98.0	98.0	2.1 ± 0.2	3.0
b-phycoerythrin	82.0	82.0	2.8 ± 0.2	4.0
Second ATPS extraction				
c-phycocyanin	87.0	85.0	2.4 ± 0.2	3.4
b-phycoerythrin	77.0	63.0	2.9 ± 0.2	4.1
Third ATPS extraction				
c-phycocyanin	87.0	74.0	2.4 ± 0.2	3.4
b-phycoerythrin	77.0	49.0	2.9 ± 0.2	4.1

The yield from each step represents the practical recovery of the colored protein and is expressed relative to the original amount of the protein loaded to the process step.
The purity of c-phycocyanin and b-phycoerythrin was defined as the relationship of 620 nm to 280 nm absorbance and 545 nm to 280 nm absorbance, respectively.
Purification factors represent the increase in purity of the target protein relative to the purity of the initial crude extract (i.e., 0.7 for both colored proteins).

The purity of c-phycocyanin from the top phase of the third extraction stage remained almost constant in comparison with that from the second ATPS extraction stage. Product yield decreased from 98 % to 85 % and 74 % after the second and the third ATPS extraction stage, respectively. Therefore, a one-stage ATPS extraction process was proposed to fractionate the fermentation broth of *Spirulina maxima* to recover c-phycocyanin. Such a proposed process resulted in an overall product yield of 98 %, a purity of 2.1 and a threefold increase in purity with respect to the purity of the crude extract (see Tab. 2).

In the case of b-phycoerythrin, additional ATPS extraction stages produced no significant increase in protein purity. It is clear that no further removal of protein contaminants was achieved when consecutive ATPS extraction stages were implemented. Apparently, the process conditions selected for the first extraction stage concentrated the majority of the target protein and allowed for the maximum removal of contaminants. Product yield decreased from 82 % to 63 % and 49 % after the second and the third ATPS extraction stages, respectively, without improvement in protein purity. In this case, one-stage ATPS extraction resulted in a potential primary recovery process to fractionate *P. cruentum* homogenate and produce b-phycoerythrin with a purity of 2.8 suitable for further purification. The purity of b-phycoerythrin from the crude extract increased 4-fold after ATPS (see Tab. 2). The proposed processes exhibited a reduced number of unit operations for the primary recovery stage that can be integrated to the protocols previously reported [9, 13]. Therefore, the strategy proposed here for the primary recovery of colored proteins, highlights the superiority of the current approach.

4 Conclusions

This study reports the comparison of the fractionation of cell homogenate of *Spirulina maxima* and *Porphyridium cruentum* in aqueous two-phase systems for the development of extraction processes for the potential primary recovery of colored proteins (i.e., c-phycocyanin and b-phycoerythrin). It has been shown that tie-line length, volume ratio, molecular mass of PEG and system pH influence the purity of c-phycocyanin and b-phycoerythrin from the top PEG-rich phase. ATPS with a PEG of high molecular mass (i.e., 8,000 g/gmol) proved to be unsuitable for the primary recovery of the colored proteins since the lowest values of purity were obtained in theses systems. The operating conditions established for the PEG 1450 and PEG 1000-phosphate ATPS extraction resulted in one-stage processes for the potential recovery of c-phycocyanin from *Spirulina maxima* and b-phycoerythrin from *P. cruentum,* respectively. The chosen operating conditions preferentially concentrated the target protein to the top phase and the contaminants to the opposite phase. The results reported here demonstrate the benefits of the practical application of ATPS for the primary recovery of colored proteins from microbial origin as a first step for the development of commercial purification processes.

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Rotavirus-like particles primary recovery from insect cells in aqueous two-phase systems☆

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Abstract

Virus-like particles have a wide range of applications, including vaccination, gene therapy, and even as nanomaterials. Their successful utilization depends on the availability of selective and scalable methods of product recovery and purification that integrate effectively with upstream operations. In this work, a strategy based on aqueous two phase system (ATPS) was developed for the recovery of double-layered rotavirus-like particles (dlRLP) produced by the insect cell-baculovirus expression system. Polyethylene glycol (PEG) molecular mass, PEG and salt concentrations, and volume ratio (Vr, volume of top phase/volume of bottom phase) were evaluated in order to determine the conditions where dlRLP and contaminants concentrated to opposite phases. Two-stage ATPS consisting of PEG 400-phosphate with a Vr of 13.0 and a tie-line length (TLL) of 35% (w/w) at pH 7.0 provided the best conditions for processing highly concentrated crude extract from disrupted cells (dlRLP concentration of 5 μg/mL). In such conditions intracellular dlRLP accumulated in the top phase (recovery of 90%), whereas cell debris remained in the interface. Furthermore, dlRLP from culture supernatants accumulated preferentially in the interface (recovery of 82%) using ATPS with a Vr of 1.0, pH of 7.0, PEG 3350 (10.1%, w/w) and phosphate (10.9%, w/w). The purity of dlRLP from culture supernatant increased up to 55 times after ATPS. The use of ATPS resulted in a recovery process that produced dlRLP with a purity between 6 and 11% and an overall product yield of 85% (w/w), considering purification from intracellular and extracellular dlRLP. Overall, the strategy proposed in this study is simpler than traditional methods for recovering dlRLP, and represents a scalable and economically viable alternative for production processes of vaccines against rotavirus infection with significant scope for generic commercial application.

Keywords: Aqueous two-phase systems; Rotavirus like-particles; Insect cells

1. Introduction

Virus-like particles are composed of the main structural proteins of a virus, but lack its genetic material. They are produced by the recombinant expression of the structural proteins that, in the absence of non-structural proteins and genetic material, assemble into structures identical to the native virus [1,2]. Virus-like particles (VLP) have a wide range of applications, including vaccination, gene therapy, biosensors, and nanomaterials [3,4]. A relevant model for study is the rotavirus. Annually, more than 500,000 children die as victims of acute gastroenteritis caused by rotavirus [5]. VLP are attractive candidates for a prophylactic vaccine against rotavirus infection, because many drawbacks of traditional viral vaccines are absent in VLP, such as infection by inefficiently inactivated viruses or by reversion of attenuated strains. Double-layered rotavirus-like particles (dlRLP) consist of two concentric protein layers. The inner most layer is comprised by 60 dimers (120 molecules) of VP2 and the second shell is formed by 260 trimers of VP6. Such dlRLP induce a good immune response when administered intranasally [4].

The structure of dlRLP makes their production and purification a unique challenge [2,6,7]. However, in order for dlRLP to

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be a viable alternative for immunization, economical production of complete particles of good quality at large scale needs to be achieved. An expression system that has been proven to be especially suitable for the production of VLP is the insect cell-baculovirus expression vector system (IC-BEVS) [8]. Although the production of dlRLP has been studied before, the existing reports addressed mainly the upstream part of the process [1,7,9,10]. In general, purification of VLP is a complex task. A purification process must preserve the best possible yield and purity of intact VLP obtained as final products, minimizes processing time and decrease the consumption of resources (reagents) in order to make the process economically viable. Difficult to scale and time-consuming operations, such as ultracentrifugation, are currently used even at industrial scale. Such protocols are characterized by low product recovery and high cost associated to the scaling up of the process [11,12]. Consequently, process problems associated with these protocols limit their commercial application. The lack of reports addressing the downstream stages of the process for the recovery of dlRLP is evident.

The development of efficient and scalable biotechnological processes is needed for the commercial implementation of recovery processes. An alternative practical approach, exploiting the use of aqueous two-phase systems [13] to develop a process for the recovery of dlRLP is examined in this work. Aqueous two-phase systems (ATPS), constituted by a mixture of polymers (e.g. polyethylene, PEG) and salts (e.g. phosphate or sulphate), result in two-phases for the extraction of biomolecules. This technology has several potential advantages, including bio-compatibility, ease of scale-up and low cost [14]. Andrews et al. [15] have reported the use of ATPS for the recovery of hepatitis B VLP from yeast cells. They evaluated qualitatively the potential of ATPS to separate VLPs from cell debris and contaminants. However, no quantitative information was provided regarding concentration, purity or yield of VLPs obtained after ATPS. Such findings raised the generic potential application of ATPS for the recovery of VLPs. In the present study, a practical approach, which exploits the known effect of system parameters such as PEG and salt concentration and the nominal molecular mass of PEG upon product partition, was used. Purified dlRLP were initially used as a model system for evaluating their partition behaviour. Subsequently, the real complex system, consisting of cellular homogenate and supernatant from insect cell cultures was studied to obtain different conditions where the target product and the contaminants (e.g. cell debris) partitioned preferentially to opposite phases. The results of this work provide a strategy that greatly improves the traditional way in which dlRLP are recovered, with significant scope for generic commercial application.

2. Materials and methods

2.1. Production of dlRLP

dlRLP were produced in High Five insect cells (Invitrogen, Carlsbad, CA, USA), as described in Mena et al. [2]. Briefly, cells were cultivated in suspension in SF900-II medium (Invitrogen, Carlsbad, CA, USA) in shaker flasks. Cultures were simultaneously infected at a cell concentration of 0.5×10^6 cell/mL with a baculovirus coding for the fusion protein EGFPVP2 (kindly provided by Professor Jean Cohen, INRA, France) [16] and another coding for VP6 from strain SA11 (kindly provided by Dr. Susana López, IBT-UNAM, México) at a multiplicity of infection of five plaque forming units/mL of each virus. Viral titters were determined as described by Mena et al. [17]. Cells were harvested by centrifugation at 48 h post-infection at 10,000 rpm for 10 min (Beckman, UK). The supernatant was identified in this study as extra-cellular dlRLP source (cell debris-free extract). After cell harvesting, the biomass obtained from 200 mL of culture was re-suspended in 6 mL of Tris EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, 2% of sodium deoxicholate). Cell rupture was achieved by sonication for 2 min (periods of 10 s, separated by pauses of 5 s). The resulting homogenate was identified in this study as intracellular dlRLP source (crude extract). Material for the model systems was obtained by purifying dlRLP by the traditional method of ultracentrifugation [1]. Briefly, dlRLP from culture supernatants were concentrated by a 35% sucrose cushion by ultracentrifugation. dlRLP were obtained by ultracentrifugation in an isopicnic CsCl gradient at $148{,}000 \times g$ for 18 h. The band corresponding to dlRLP was recovered and ultrafiltrated through a 300 kDa MW cut-off membrane.

2.2. Analytical procedures

The concentration and purity of dlRLP was determined by gel exclusion HPLC, as described in Mena et al. [2]. The chromatographic system consisted in a controller, pumps, and a photodiodes array and a fluorescence detectors connected in series (Waters, Massachusetts, USA). The mode of operation of the chromatography was isocratic, with a Tris-EDTA pH 8.0 buffer mobile phase at a flow rate of 0.9 mL/min using an Ultrahydrogel 2000 size-exclusion column, or an Ultrahydrogel 2000 and an Ultrahydrogel 500 columns in series (Waters, Massachusetts, USA). Prior to injection into the HPLC equipment, samples were ultrafiltered (10 and 300 kDa MW cut-off, Nanosep, Pall Life Sciences, Ann Arbor, MI, USA) and centrifuged at $10{,}000 \times g$ for 15 min. EGFP fluorescence was determined with the fluorescence detector, and concentration of EGFPVP2 was determined by comparison with a standard curve of pure EGFP concentration versus fluorescence (excitation and emission wavelengths of 484 and 510 nm, respectively). Charpilienne et al. [16] have determined that the molar extinction coefficient of EGFPVP2 is that of EGFP. Thus, dlRLP concentration was determined from its content of EGFP. Namely, the molecular masses of the dlRLP and EGFP are 49.5×10^6 and 27×10^3, respectively. Therefore, 1 g of EGFP is contained in 15.27 g of dlRLP. The purity of dlRLP relative to contaminant proteins was estimated by absorbance at 280 nm using the photodiodes array detector (Waters, Massachusetts, USA). Transmission electron microscopy was performed as described by Mena et al. [2]. Briefly, dlRLP negatively stained with uranile acetate after fixation in a 200 mesh grid coated with Formvar-carbon (Structure Probe Inc., West Chester, PA, USA) were observed with an electron microscope Jeol 1200EXII (Jeol, Peabody, MA, USA).

2.3. Characterisation of aqueous two-phase systems

The binodal curves were estimated by the cloud point method [18] using poly(ethylene glycol) (PEG, Sigma Chemicals, St. Louis, MO, USA) of nominal molecular mass of 400, 600, 1000, 1450, 3350 and 8000 g/mol (50% (w/w) stock solution) and ammonium sulphate or di-potassium hydrogen orthophosphate/potassium di-hydrogen orthophosphate (Sigma) (30%, w/w). Fine adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide.

2.4. Influence of system parameters upon partition behaviour of dlRLP from insect cells in PEG-salt systems

All experimental systems used to establish the operating conditions for the ATPS process were prepared for convenience on a fixed mass basis. Predetermined quantities of stock solutions of PEG, ammonium sulphate or potassium phosphate were mixed with either a single model system (containing purified dlRLP) or a complex system (containing 10% (w/v) supernatant or cell homogenate from insect cell cultures; referred above as extracellular dlRLP or crude extract (intracellular dlRLP), respectively), to give a final weight of 1.0 and 10 g for model and complex systems, respectively. The stock solutions (PEG or salts) were mixed and phases dispersed by gentle mixing for 30 min at 25 °C. Adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide. Complete phase separation was achieved by low speed batch centrifugation at 1500 × *g* for 20 min at 25 °C. Estimates of the volumes of top and bottom phases and solids, utilizing a calibrated syringe with a total volume of 100 μL or with a micropipette in the case of model systems, or 15 mL graduated centrifuge tubes in complex systems. The volumes of the phases were used to estimate the volume ratio (volume of the top phase/volume of the bottom phase, Vr). Samples were carefully extracted from the phases (top and bottom phase) and analyzed. In the particular case of sample from interface, top and bottom phases were withdraw with care to avoid interface perturbation. The remaining material identified as interface was then re-suspended in Tris-EDTA buffer and consequently diluted for biochemical analysis. The systems tie-line length (TLL), which represents the length of the line that connects the composition of the top and bottom phase of a defined ATPS, was estimated using the relation in which TLL is equal to $[(\Delta PEG)^2 + (\Delta salt)^2]^{1/2}$; where ΔPEG and Δsalt are the differences in concentration between top and bottom phase of PEG and salt, obtained from the interception of the tie-line of the ATPS with a defined Vr with the phase diagram as described by Albertsson [19]. The top phase and interface dlRLP recovery was estimated as the amount of dlRLP present in the upper phase or interface and expressed relative to the original amount loaded into the system. Bottom phase recovery was not estimated because the amount of dlRLP present in such phase was undetectable. Results reported are the average of three independent experiments and errors were estimated to be a maximum of ±5% of the mean value.

3. Results and discussion

3.1. Partition behaviour of purified dlRLP from insect cells in PEG-salt aqueous two-phase systems

The unknown mechanism governing the behaviour of dlRLP in ATPS limited the predictive design of extraction processes using ATPS. In this study, the influence of system parameters on the partition behaviour of dlRLP was studied using single model systems before designing the aqueous two-phase process. Such systems were characterised by the presence of purified dlRLP (in a concentration of 1000 ng/mL in the systems). These systems do not account for the influence of the whole range of proteins, contaminants, and cell debris which may be present in the crude extract of insect cells upon the performance of ATPS.

It has been established [13] that, the extent of the empirical experiments necessary to determine the process conditions of an ATPS extraction can be reduced by using a practical approach which exploits the known effect of system parameters such as tie-line length (TLL), phase volume ratio (Vr), system pH and molecular mass of PEG on the protein partition behaviour. In the present work, it was decided to use the practical approach exploited for proteins to examine the partition behaviour of purified dlRLP. Initially, the effect of increasing TLL upon partition behaviour of dlRLP was evaluated. Changes in the TLL affect the free volume available for a defined solute to accommodate in the phase and, as a consequence, the partition behaviour of such solute in the ATPS [20].

Table 1 illustrates the impact of increasing TLL upon recovery of dlRLP from model ATPS, when PEG of six different molecular mass (from 400 to 8000 g/mol) were used. For all these systems, volume ratio and pH were kept constant at 1.0 and 7.0, respectively. In the partition experiments that used purified dlRLP, these could not be detected in the bottom phase. It is possible that the majority of the dlRLP concentrated in the top phase or interface, or that dlRLP were destabilized in the bottom phase due to its high ionic strength. To test this second hypothesis, dlRLP were submitted to 500 mM of ammonium phosphate for 30 min, treated as the samples from ATPS, and analyzed by HPLC. As can be seen in Fig. 1, such a treatment completely eliminated fluorescence from the chromatograms. Exposure of dlRLP for 30 min to 500 mM of ammonuim phosphate completely abated fluorescence. The decrease in fluorescence can only be a consequence of the loss of structure of EGFP, since quenching can be discarded as salts were eliminated prior to injection to the HPLC system. As the EGFP portion of EGFPVP2 is inside dlRLP, it is most likely that the lack of fluorescence was preceded by a destabilization of the particle. A similar effect can be expected from the exposure of dlRLP to the bottom phase of ATPS, which has an ammonium phosphate concentration superior to 1.0 M.

The absence of a detectable amount of dlRLP in the bottom phase impeded, as for the case of soluble material, the estimation of the partition coefficient (K = concentration of dlRLP in the top phase/concentration of dlRLP in the bottom phase) in all the systems studied. Alternative, partition ratio of the systems could be estimated and used to evaluate the impact of system

Table 1
Influence of increasing tie-line length (TLL) upon the recovery of double-layer rotavirus-like particles (dlRLP) from PEG/phosphate ATPS

System	Molecular mass of PEG (g/mol)	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Top phase recovery of dlRLP (%)	Interface recovery of dlRLP (%)
1	400	19.4	16.0	35	60 ± 3	24 ± 1.2
2		25.1	17.9	52	57 ± 2.9	26 ± 1.2
3	600	14.0	15.5	22	55 ± 2.8	29 ± 1.5
4		18.3	17.4	42	49 ± 2.5	32 ± 1.6
5	1000	18.2	15.0	35	ND	84 ± 4.2
6		18.9	16.0	40	ND	88 ± 4.4
7		22.2	19.0	48	ND	83 ± 4.2
8		24.1	20.1	57	ND	95 ± 4.1
9	1450	15.1	13.0	27	ND	80 ± 4.0
10		17.5	14.3	37	ND	74 ± 3.7
11		21.9	18.0	53	ND	97 ± 3.0
12		23.0	19.8	59	ND	77 ± 3.9
13	3350	10.1	10.9	17	ND	98 ± 2.1
14		11.0	11.4	23	ND	85 ± 4.3
15		12.2	11.8	27	ND	96 ± 3.1
16		13.7	12.3	31	ND	96 ± 3.1
17	8000	12.2	9.7	22	ND	90 ± 4.5
18		13.2	10.3	26	ND	80 ± 4.0

The tie line lengths (TLL) of the systems were estimated from the composition of PEG and phosphate as described in Section 2. The top phase and interface recovery is expressed relative to the original amount of purified dlRLP loaded into the systems. Concentration of dlRLP in the ATPS was 1000 ng/mL. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively. ND, not detected.

parameters upon the partition behaviour of purified dlRLP by monitoring the partition ratio. However, to address the primary recovery of the product, it was decided to use the dlRLP practical phase recovery (expressed as the amount of dlRLP in the phase relative to the total amount loaded into the ATPS) from the top PEG-rich phase or interface as the response variables to evaluate the effect of system parameters on the behaviour of the dlRLP in ATPS. In some cases, not all the purified dlRLP introduced into the ATPS could be detected in any of the phases or the interface.

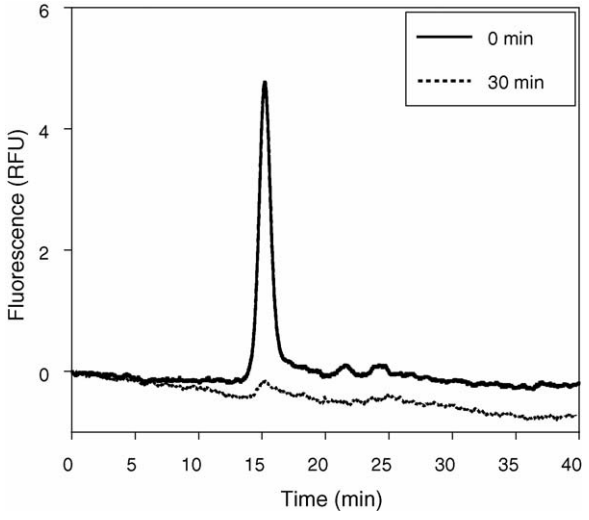

Fig. 1. Effect of high salt concentration on dlRLP stability. Seven hundred nanograms of dlRLP were subjected to 500 mM of ammonium phosphate for 30 min, treated as dlRLP recovered from ATPS, and analyzed by HPLC. Zero minute refers to the sample without treatment and 30 min to the treated sample.

Again such discrepancy may be caused by particle instability and loss of structural integrity (as measured by loss of fluorescence) of EGFP in ATPS (see Fig. 1). The results of Table 1 showed that, increasing TLL caused no significant effect upon the recovery of dlRLP from the top PEG-rich phase or the interface for model systems (with purified dlRLP) of each molecular mass of PEG used. In systems with low molecular mass of PEG (i.e. 400 and 600 g/mol) approximately 50–60% of the purified dlRLP can be recovered from the top phase. However, an increase in the molecular mass of PEG of the systems caused a change in the accumulation of dlRLP from the top phase to the interface. The effect of increasing molecular mass of PEG upon solute partition behaviour has been explained on the basis of protein hydrophobicity [21,22] and phase excluded volume [23,24]. In the case of dlRLP, the decrease in free volume available in the top phase for solute dissolution with the increase in the molecular mass of PEG, may explain the potential migration of dlRLP from the top phase to the interface. ATPS with low molecular mass of PEG (i.e. PEG 400 and PEG 600) exhibited the best conditions for dlRLP top phase recovery. ATPS using PEG of higher molecular mass (greater than 600 g/mol) can be considered for interface dlRLP recovery. In this case the majority of dlRLP (more than 95%; see Table 1) can be potentially recovered from the interface. Once the impact of increasing TLL upon the recovery of dlRLP was evaluated, the effect of system pH upon dlRLP partition behaviour was investigated using a model system.

The influence of system pH on protein partition behaviour has been discussed before [25,26]. In general, these reports concluded that increasing the pH (e.g. from 6.5 to 9.0) caused changes in the partition behaviour of proteins attributed to free-

Table 2
Influence of double-layer rotavirus-like particles (dlRLP) concentration upon product recovery from PEG/phosphate ATPS

System	Molecular mass of PEG (g/mol)	PEG (%, w/w)	Phosphate (%, w/w)	dlRLP concentration (ng/mL)	Top phase recovery of dlRLP (%)	Interface recovery of dlRLP (%)
a	400	19.4	16.0	1000	60 ± 3	24 ± 1.2
				5000	32 ± 2	13 ± 1.0
b	3350	10.1	10.9	1000	ND	98 ± 2.1
				5000	ND	32 ± 1.6

The top phase and interface recovery is expressed relative to the original amount of purified dlRLP loaded into the systems. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively. ND, not detected.

volume effects [26], to speciation of the phosphate salts over the pH range, and to conformational changes in the structural integrity of proteins [27]. However, for all the ATPS studied, no significant influence of the system pH (from 7.0 to 9.0) upon the recovery of dlRLP was observed (data not shown). Since potential recovery of dlRLP from the interface in selected ATPS (e.g. PEG 3350) exceeded 90% of the total amount loaded into the systems (see Table 1), further evaluation of additional systems parameters (i.e. volume ratio) was not pursued at this stage.

In an attempt to improve the recovery of dlRLP using an ATPS process (by increasing the amount of dlRLP that can be processed by the systems), the effect of an increase in the dlRLP concentration on the performance of ATPS was evaluated. It was decided to examine the impact of increasing dlRLP concentration (from 1000 to 5000 ng/mL) on product recovery by using selected ATPS with PEG with molecular mass of 400 and 3350 g/gmol. The ATPS used in this part of the study (i.e. systems "a" and "b" in Table 2; equivalent to systems 1 and 13 in Table 1) were selected on the basis of product recovery (from the top phase and interface) from the previous experiments (see Table 1). Systems 1 and 13 exhibited the best top phase and interface dlRLP recovery, respectively, from the ATPS studied. Table 2 illustrates the effect of dlRLP concentration in the systems on product recovery. It is clear that interface and top phase dlRLP recovery decreased for both ATPS when the concentration of product of interest (dlRLP) increased. It is probable that the dlRLP migrated to the bottom phase once the free-space of top phase and interface is saturated. However, dlRLP were not detected in the lower phase, possibly because of a negative effect of the high salt concentration environment on dlRLP structural integrity. It is clear that an attempt to improve the recovery systems (using purified dlRLP) by increasing the amount of biological material that can be processed (simulated by an increase in dlRLP concentration in the ATPS), resulted in a decrease of the percentage of product recovered either from the top phase or interface (Table 2).

In general, from the ATPS studied with purified dlRLP, the systems with Vr = 1.0, PEG 400 19.4% (w/w), phosphate 16.0% (w/w) at pH 7.0 provided the required conditions to concentrate dlRLP from insect cells in the top phase (i.e. top phase recovery of 60%). In contrast, systems with Vr = 1.0, PEG 3350 10.1% (w/w), phosphate 10.9% (w/w) at pH 7.0 facilitated the accumulation of dlRLP in the interface (i.e. interface recovery of 98%). It is clear that such findings will facilitate the potential generic application of ATPS process to recover dlRLP from complex systems (i.e. crude extract and cell debris free-supernatant).

3.2. *Recovery of intracellular dlRLP from insect cells in PEG-salt aqueous two-phase systems*

Once the feasibility of dlRLP recovery in ATPS was established using model systems, the potential recovery of dlRLP from complex systems in ATPS was investigated. Initially, the processing of the homogenate of insect cell culture (crude extract) for the recovery of intracellular dlRLP in ATPS was attempted. This complex system was characterized by the presence of intracellular dlRLP, contaminant proteins and particularly cell debris derived from the cell disruption stage. In order to evaluate the potential concentration of cell debris and dlRLP in opposite phases, cell debris partition behaviour in selected ATPS was studied by visual observation. Table 3 illustrates the partition behaviour of cell debris from insect cell culture in PEG/phosphate ATPS. According to the results with model sys-

Table 3
Partition behaviour of cell debris from insect cell culture in PEG/phosphate ATPS

System	Molecular mass of PEG (g/mol)	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Cell debris phase preference
I	400	19.4	16.0	35	I
II	400	25.1	17.9	52	I
III	1000	18.2	15.0	35	I
IV	1000	24.1	20.1	57	I
V	3350	10.1	10.9	17	B–I
VI	3350	13.7	12.3	32	I

The tie line lengths (TLL) of the systems were estimated from the composition of PEG and phosphate as described in Section 2. Cell debris preference was estimated by visual observation. I and B denote interface and bottom phase preference for cell debris, respectively. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively.

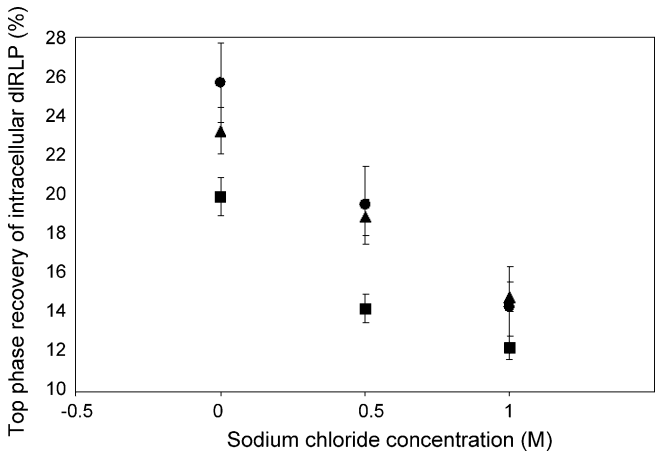

Fig. 2. Influence of sodium chloride concentration upon the top phase recovery of intracellular double-layer rotavirus-like particles (dlRLP) from PEG/salt ATPS. The top phase recovery is expressed relative to the original amount of intracellular dlRLP from crude extract loaded into the systems and it is reported relative to the total concentration of sodium chloride in the ATPS. The selected systems were; 19.4% (w/w) PEG 400 and 16.0% (w/w) phosphate (triangle symbols), 14.0% PEG 600 and 15.5% (w/w) phosphate (square symbols) and 20.0% (w/w) PEG 600 and 13.5% (w/w) ammonium sulphate (circle symbols). For all systems, pH was kept constant at 7.0.

tems (see Tables 1 and 2), ATPS of low and high molecular mass of PEG (i.e. 400 and 3350 g/mol) were selected, together with a system of PEG molecular mass of 1000 g/mol. Cell debris from the crude extract of insect cells exhibited an interface preference. In system of high molecular mass of PEG and short TLL (i.e. system V in Table 3) cell debris exhibited both, interface and bottom phase preferences. Although a clear explanation for the behaviour of the cell debris from insect cell homogenate is not currently available, the results obtained facilitate the selection of operating conditions to favour cell debris separation from the intracellular dlRLP. Such situation can avoid the addition of a cell debris removal process step. It is clear that in order to fractionate insect cell homogenate to recover intracellular dlRLP in ATPS, low molecular mass of PEG (i.e. 400 and 600 g/mol) need to be used. In these systems the majority of purified dlRLP were concentrated on the top phase. Consequently, by using such systems cell debris and intracellular dlRLP can be accumulated at the interface and top phase, respectively.

Processing of intracellular dlRLP in ATPS was attempted using systems of low molecular mass of PEG. In was decided to evaluate the influence of molecular mass of PEG (i.e. 400 and 600 g/mol) and the type of salt forming phase (i.e. phosphate and sulphate) on the recovery of intracellular dlRLP. Furthermore, as suggested by Andrews et al. [15] for the purification in ATPS of VLP of hepatitis B virus, the effect of molar concentration of sodium chloride in the ATPS upon the recovery of dlRLP was also studied. Such strategy was followed in an attempt to increase the top phase dlRLP recovery. Fig. 2 illustrates the influence of sodium chloride concentration upon the top phase recovery of intracellular dlRLP from PEG 400 and 600/salt ATPS when two salts forming phase (i.e. phosphate and sulphate) were used. It is clear that an addition of sodium chloride to the systems resulted in a negative effect on the top phase recovery of the dlRLP, regardless of the use of phosphate or sulphate salts (see Fig. 2). Such behaviour may be explained by the migration of dlRLP to the interface caused by the saturation of the free-volume of the top phase by sodium chloride addition. The observed turbidity of the top PEG-rich phase when sodium chloride was added may suggest phase saturation. An additional explanation may involve the possible degradation of dlRLP due to the excessive ionic strength generated by addition of sodium chloride [28]. Therefore, regardless of the mechanism behind the decrease of recovery of dlRLP, sodium chloride in the concentrations tested in this work should not be added to ATPS for the purification of dlRLP. The differences observed in the recovery of dlRLP from the top PEG-rich phases from the model and complex systems (see Table 1 and Fig. 2) are explained by the nature of the experimental vehicle (purified dlRLP and insect cell homogenate). In the case of model systems, the sole presence of the target product resulted in a top phase dlRLP recovery of 49–60% (Table 1). In contrast, for the complex systems, the presence of contaminants (particularly cell debris) from the insect cell homogenate affected the partition behaviour and top phase recovery of dlRLP (20–26%; Fig. 2). Apparently, the presence of a variety of contaminants from the insect cell homogenate contributed to the top PEG-rich phase saturation. Consequently, the free-volume available for dlRLP allocation was greatly reduced and the top phase dlRLP recovery was negatively affected. Such situation can be addressed by decreasing the amount of contaminants in the homogenate or by increasing the volume of the top phase of the ATPS. A decrease in the amount of contaminants from the homogenate can be achieved by a dilution strategy. However, a dilution strategy implies processing greater volumes which will necessarily affect the potential implementation of the resulting process.

In an attempt to increase the top phase recovery of dlRLP, ATPS with a larger top PEG-rich phase (Vr greater than 1) were selected. In these systems it was expected that the free-space available for dlRLP and contaminants accumulation was favoured and as a result top phase dlRLP recovery can be increased. Table 4 illustrates the effect of system Vr and dlRLP concentration upon product recovery in PEG/salt systems. It is clear that an increase in the system Vr benefited the top PEG-rich dlRLP recovery from the ATPS of 400 and 600 molecular mass of PEG. Furthermore, an increment in the concentration of the crude extract (as represented by the increase in the dlRLP concentration from 2000 to 5000 ng/mL) did not negatively affect the top phase product recovery in ATPS characterized by Vr greater than 1. The increase in the top phase product recovery observed with increasing Vr in ATPS was similar regardless the salt forming phase (i.e. phosphate or sulphate) of the ATPS used. However, a slight advantage was obtained when ATPS of low molecular mass of PEG (i.e. 400 g/mol) was used. In general, from the ATPS evaluated, the system comprising Vr = 13.0, PEG 400/phosphate, TLL 35% (w/w) at pH 7.0 provided the best conditions to process high concentrated crude extract (dlRLP concentration of 5000 ng/mL) and accumulate intracellular dlRLP in the top phase (i.e. top phase dlRLP recovery of 47%) and cell debris in the interface. In order to further increase the process recovery, a subsequent ATPS extraction stage was used. In

Table 4
Influence of system volume ratio (Vr) and crude extract concentration upon the recovery of intracellular double-layer rotavirus-like particles (dlRLP) from PEG/salt ATPS

System	Molecular mass of PEG (g/mol)	TLL (%, w/w)	System volume ratio (Vr)	dlRLP concentration (ng/mL)	Top phase recovery of dlRLP (%)
i	400	35	1.0	2000	23 ± 2.0
			11.0	2000	50 ± 3.4
			13.0	5000	47 ± 1.0
ii	600	27	1.0	2000	26 ± 2.0
			5.0	2000	31 ± 1.2
			6.0	5000	42 ± 3.0

The tie line lengths (TLL) of the systems were estimated from the composition of PEG and salt as described in Section 2. Systems i and ii comprise PEG400/phosphate and PEG600/sulphate ATPS, respectively. System volume ratio (Vr) estimated from non-biological experimental systems after phase separation in graduated tubes. The top phase and interface recovery is expressed relative to the original amount of intracellular dlRLP from crude extract loaded into the systems. For all systems, pH was kept constant at 7.0.

this additional extraction stage, the interface (where the unrecovered dlRLP together with cell debris was present) from the previous ATPS, was further processed by the addition of fresh PEG and phosphate. The process conditions for the subsequent ATPS extraction stage were kept constant and equal to those used for the first ATPS extraction. The second extraction had a high efficiency of dlRLP recovery, most likely due to the lower amount of dlRLP and contaminants present in the crude extract. Such result agrees with the effect of dlRLP load in model systems (see Table 2). The use of a consecutive aqueous two-phase systems resulted in a two-stage ATPS process with a 90% overall dlRLP top phase recovery (i.e. 47 and 43% from the first and second ATPS extraction stage, respectively and relative to the original amount of dlRLP loaded to the first extraction stage). It is clear that such strategy proved to be effective to increase the dlRLP recovery (from the top PEG-rich phase) by further removing (in the interface and bottom phase) the majority of cell debris and protein contaminants.

3.3. Recovery of extracellular dlRLP in PEG-salt aqueous two-phase systems

In this study, extracellular dlRLP were defined as those obtained from the insect cell culture supernatant. It has been determined that approximately 60% of the total dlRLP from the insect cell culture are contained in the supernatant and the remaining 40% are intracellular [2]. Therefore, it is relevant (in order to increase process yield) to also pursue the potential recovery of dlRLP from the supernatant. In this particular case, the problem of cell debris accumulation at the interface is not longer present. Thus, it is beneficial to process a cell debris-free extract in ATPS for the recovery of dlRLP. Consequently, ATPS in which dlRLP can be accumulated at the interface are recommended for the recovery of extracellular dlRLP. From the studies of the influence of system parameters upon dlRLP recovery in ATPS using model systems (see Table 1), ATPS of low and high molecular mass of PEG (i.e. 400, 1000 and 3350 g/mol) were selected to process the supernatant from insect cell culture. Such systems were evaluated to establish the conditions for an ATPS extraction stage of extracellular dlRLP. Table 5 illustrates the effect of molecular mass of PEG upon the recovery of extracellular dlRLP from PEG/phosphate ATPS. Approximately 50% of the dlRLP were accumulated at the interface of ATPS of low molecular mass (i.e. 600 and 1000 g/mol). Nonetheless, it is evident that the use of ATPS (comprised of PEG 10.1% (w/w), phosphate 10.9% (w/w), Vr of 1.0 at pH of 7.0) with PEG of high molecular mass (i.e. 3350 g/gmol) was more favourable for the accumulation of dlRLP at the interface (i.e. interface recovery of dlRLP of 82%; see Table 5).

The study presented here resulted in a process strategy that produced intracellular and extracellular dlRLP recovery of 90 and 82%, respectively (Table 6). By considering that 40% of the dlRLP are intracellular and 60% of dlRLP are extracellular [2], the overall recovery of dlRLP from insect cell culture in ATPS process was approximately 85%. The purity of the resulting product increased from 0.2% in the culture supernatant [2] to 6–11% (see Table 6), which represented an increase of 30–55 times. The outline of the new proposed process is summarized in Fig. 3. In the current study centrifugation was used to obtain cell debris-free supernatant containing the extracellular dlRLP and the biomass for the cell disruption stage. Cell disruption was archived by sonication. This would, of course, be impractical at

Table 5
Influence of molecular mass of PEG upon the recovery of extra-cellular double-layer rotavirus-like particles (dlRLP) from PEG/phosphate ATPS

System	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Molecular mass of PEG (g/mol)	Interface recovery of dlRLP (%)
A	14.0	15.5	22	600	44 ± 1.1
B	24.1	20.1	57	1000	48 ± 6.0
C	10.1	10.9	17	3350	82 ± 2.3

The interface recovery is expressed relative to the original amount of extra-cellular dlRLP from cell debris-free supernatant loaded into the systems. For all systems, volume ratio (estimated from non-biological experimental systems) and the system pH were kept constant at 1.0 and 7.0, respectively.

Table 6
Potential aqueous two-phase systems for the primary recovery of double-layer rotavirus-like particles (dlRLP) from insect cells

Type of dlRLP (source)	Molecular mass of PEG (g/mol)	TLL (%, w/w)	System volume ratio (Vr)	Top phase recovery of dlRLP (%)	Interface recovery of dlRLP (%)	Purity of dlRLP (%)
Intracellular (crude extract)	400	35	13.0	90 ± 5.0	ND	11.0
Extra-cellular (cell debris-free extract)	3350	17	1.0	ND	82 ± 2.3	6.4

The top phase recovery of intracellular dlRLP represents the sum of the top phase product recovery from the first and second ATPS extractions and is expressed relative to the original amount of intracellular dlRLP from crude extract loaded to the first extraction system. Interface dlRLP recovery is expressed relative the original amount of extra-cellular dlRLP from cell debris-free supernatant, loaded into the system. Purity of dlRLP was estimated as described in Section 2. ND, not detected.

process scale but can be easily substituted by a mechanical cell disruption (e.g. bead mill or homogenization). ATPS extraction was then applied for the processing of both intracellular and extracellular dlRLP at conditions defined in Table 6. An ultrafiltration stage can then be implemented for polymer removal to obtain a potential process recovery of 85% (w/w). This novel process greatly reduces the processing time and the consumption of reagents and simplifies the traditional way in which dlRLP expressed in insect cell-baculovirus system can be recovered, with significant scope for generic commercial application. It is clear that, for dlRLP, this bioengineering strategy opens the way to further bioprocess improvement. Particularly, products, such as VLP, in which their potential production using conventional processes is not economically feasible.

3.4. Direct comparison of the primary recovery of dlRLP with PEG-salt aqueous two-phase systems and zonal centrifugation

To evaluate the primary recovery of dlRLP with other purification methods, dlRLP from culture supernatants were purified by CsCl gradients or recovered by ATPS, and compared. dlRLP obtained by both methods were observed by electron microscopy. Representative micrographs are shown in Fig. 4. It can be seen that, in both cases, dlRLP were intact and had a similar morphology to that previously reported [2]. Analysis by gel-permeation HPLC (Fig. 5) showed that particles recovered using both methodologies had the same retention time, meaning that they had a similar size distribution (Fig. 5, peak a). The chromatograms in Fig. 5 also show that the purity of dlRLP recovered by ATPS was higher to that obtained after zonal centrifugation. The purity of particles obtained by cesium chloride gradients, as determined from integrating the peaks, was of 2.3%, with a yield of 1.8%. Such values contrast with the performance of ATPS, with yields of 85% and a purity of 6–11%. It should be noted that in the case of dlRLP purified by CsCl gradients, a large peak (b) was detected by fluorescence. It has been previously shown that such a peak corresponds to EGFP, which is the product of the cleavage of the fusion protein GFP-VP2. The appearance of non-fused EGFP indicates that dlRLP were destabilized and EGFP-VP2 was afterwards cleaved [2]. The large area of the EGFP peak in the sample purified by CsCl gradients underlines the extreme conditions to which particles are exposed during zonal centrifugation. In contrast, degradation of dlRLP recovered by ATPS was much lower (Fig. 5B). It can be concluded that the primary recovery of dlRLP by ATPS results in higher yields, low dlRLP destabilization, and a higher ratio between assembled and disassembled proteins.

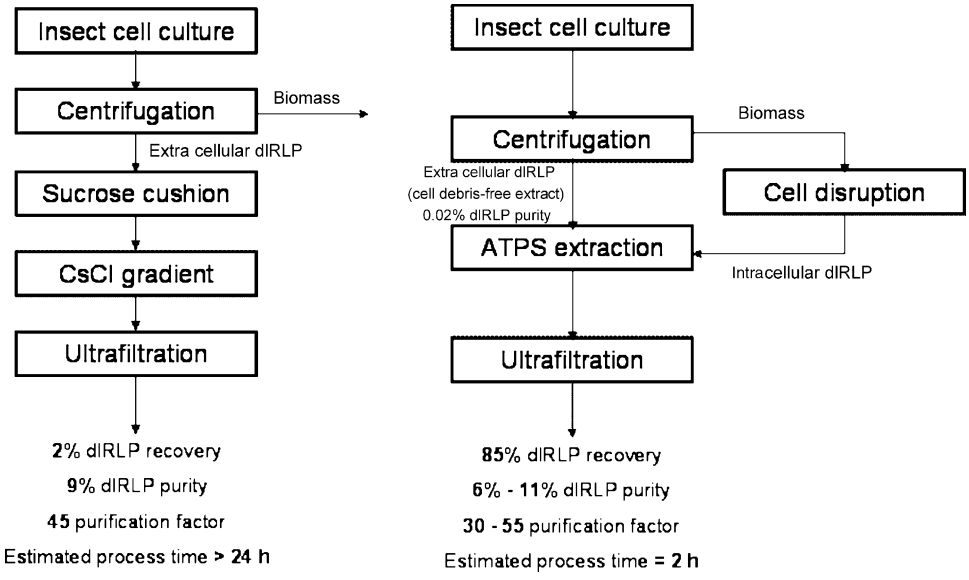

Fig. 3. Simplified representation of the current protocol for the primary recovery of double-layer rotavirus-like particles dlRLP produced by insect cells and the new proposed strategy using aqueous two-phase systems

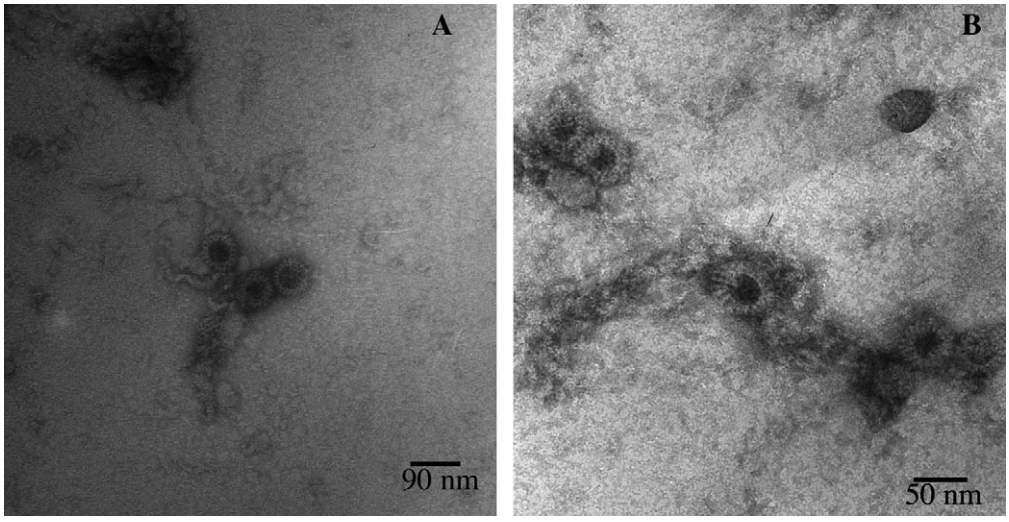

Fig. 4. Electron microscopy of dlRLP recovered by cesium chloride gradients (A) or ATPS (B). Magnification of (A) 85,000× and (B) 140,000×.

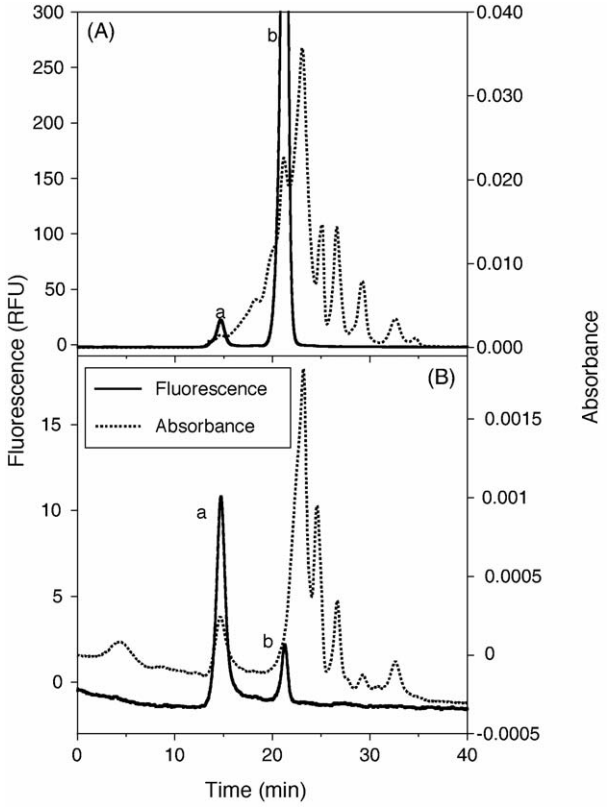

Fig. 5. Gel-permeation chromatograms obtained from dlRLP recovered by (A) cesium chloride gradients or (B) ATPS. An Ultrahydrogel 500 and a Ultrahydrogel 2000 columns were connected in series. Peak (a) has been identified as dlRLP, and peak (b) as EGFP.

4. Conclusions

This study reports the fractionation of cell homogenate and supernatant of insect cell-baculovirus expression system in aqueous two-phase systems for the development of a process for the primary recovery and potential purification of dlRLP. It was shown that molecular mass of PEG influenced the partition behaviour of purified dlRLP when model systems were used. dlRLP accumulated at the top PEG-rich phase when ATPS of PEG molecular mass of 400 and 600 g/mol were used and at the interface in ATPS comprising molecular mass of PEG1000 g/mol and higher. Increasing dlRLP concentration (>1000 ng/mL) in the ATPS resulted in reduced product yield. Cell debris from the insect cell homogenate accumulated at the interface which compromises the potential recovery of intracellular dlRLP from that phase. Addition of sodium chloride to the ATPS proved to be unsuitable to increase the recovery of dlRLP since a reduction of product yield from the top phase was observed. The operating conditions established for the PEG400 and PEG3350-phosphate ATPS extraction resulted in a process for the potential recovery of dlRLP from insect cell culture. These conditions accumulated the intracellular dlRLP preferentially to the top phase and the extracellular dlRLP to the interface. Overall, the results reported here demonstrate the potential application of ATPS for the recovery of structural VP2/VP6 proteins of dlRLP as a first step in a process simple to scale-up.

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Recovery in aqueous two-phase systems of lutein produced by the green microalga *Chlorella protothecoides*

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Abstract

In this study the potential use of aqueous two-phase system (ATPS) to establish a viable process for the recovery of lutein from the green microalga *Chlorella protothecoides* is evaluated. The partitioning behaviour of lutein, a representative model of natural compounds of commercial interest, was investigated in a polyethylene glycol (PEG)-phosphate system. An evaluation of system parameters including PEG molecular mass, the concentrations of PEG, phosphate and product concentration was conducted, to estimate conditions under which lutein partitions preferentially to the top phase whilst cell debris partition to the opposite phase. The necessary addition of ethanol to the ATPS for the dissolution of lutein affected the phase formation and such effect was evaluated using the change in the volume ratio produced. ATPS extraction comprising Vr = 1.0, PEG 8000 22.9% (w/w) and phosphate 10.3% (w/w), pH 7.0 provided the conditions for the concentration of lutein into the upper phase and the cell debris preferentially to the bottom phase. The use of ATPS resulted in a primary recovery process to obtain lutein with an overall product yield of 81.0 ± 2.8%. The findings reported here demonstrate the potential of ATPS for the further development of a prototype process to recover lutein from *C. protothecoides* as a first step for the generic application of this technique.

Keywords: Aqueous two-phase systems; *Chlorella protothecoides*; Lutein

1. Introduction

With the increasing trend of the market to obtain natural products with bioactive nutraceutical properties, there is considerable interest in the development of efficient and scalable processes to obtain such products. The urgent need to define credible production systems has restricted basic research to establish selective and scalable methods of product recovery that integrate effectively with upstream operations to rapidly yield product in a suitable state for the validation of polishing, formulation and delivery operations. Biotechnological processes represent an attractive alternative to synthetic procedures to produce natural products [1–3]. In this context, the recovery of colour and functional compounds from microbial origin represents a very interesting case. Particularly, in this research lutein produced by *Chlorella protothecoides* was selected as a representative model of this group of natural products of commercial interest. Lutein is one of the most important carotenoids in human serum and foods [4]. Lutein, an intracellular product of *C. protothecoides*, has been widely used for the pigmentation of animal tissues and products, as well as for coloration of foods, drugs and cosmetics. Although the production of lutein has been addressed before, the existing reports addressed mainly the upstream part of the process and the purification by solvent extraction and chromatography [4–7]. However, the recent health benefits associated to carotenes that include the prevention of certain types of cancer and age related macular degeneration [1,8], make the production of lutein from microorganisms an attractive case of study. Nevertheless, the existing protocols [5,6], are characterized by potential high costs associated to the scaling up of the process. Recently, a high-speed counter-current chromatography method for the isolation and purification of lutein has been reported [6]. However, only a small sample size (e.g. 200 mg) could be treated in one run, and the consumption of organic solvents was great. Furthermore, high-speed counter-current chromatography is not easily accessible. Additionally, most processes for commercial recovery of carotenes include a saponification step to increase their solubility in water, and

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large amounts of organic solvents [5], which generates industrial effluents with a harmful environmental impact.

The development of efficient and scalable biotechnological processes is needed for the commercial implementation of prototype processes. An alternative practical approach, exploiting the use of aqueous two-phase systems (ATPS) [9] to address the limitation of the existing protocols for the potential recovery of lutein produced by *C. protothecoides*, is examined in this work. ATPS, assembled from a mixture of polymers (e.g. polyethylene; PEG) and salts (e.g. phosphate or sulphate), result in two-phases for the extraction of bio-molecules. This technology has several potential advantages, including bio-compatibility, ease of scale-up and low cost [3]. In the present research, a practical approach, which exploits the known effect of system parameters such as PEG and phosphate concentration and the nominal molecular mass of PEG upon product partition, was used. This approach was followed to evaluate the feasibility of using ATPS for the recovery of lutein from *C. protothecoides* as the first step in the development of a prototype biotechnological process. Initially, the effect of the necessary addition of ethanol to the ATPS for the dissolution of lutein on the phase formation was evaluated using the change in the volume ratio produced. The research was then conducted using a model system (characterized by the use of a lutein-rich paste) and a complex system (characterized by the use of homogenate from the fermentation of *C. protothecoides*), to obtain different conditions where the target product and the cell debris contaminants partitioned preferentially to opposite phases. These conditions were then used to further investigate the potential process intensification, evaluating the effect of increasing lutein concentration on the system performance.

2. Materials and methods

2.1. Characterization of aqueous two-phase experiments

The binodal curve used in this study were estimated by the cloud point method [10] using poly(ethylene glycol) (PEG, Sigma, St. Louis, MO, USA) of nominal molecular mass of 1000, 1450, 3350 and 8000 g/mol (50% (w/w) stock solution) and di-potassium hydrogen orthophosphate/potassium di-hydrogen orthophosphate (Sigma; 30% (w/w) stock solution). Fine adjustment of pH was made by addition of orthophosphoric acid or sodium hydroxide.

2.2. Culture medium and cultivation conditions

C. protothecoides was cultivated in the culture medium described by Shi et al. [7]. The algae were grown under heterotrophic conditions in a batch culture initially in 500 ml Erlenmeyer flasks containing 250 ml medium at 28 ± 1 °C under continuous shaking (180 rpm) in the dark. Further heterotrophic cultivation was performed in a 7 l fermentor (Bio-Flow III, New Braunswick) containing 2.5 l medium. The cultivation conditions were controlled as follows: pH: 6.6 ± 0.1; temperature: 28 ± 1 °C; agitation: 480 rpm; dissolved oxygen concentration: 50% saturation. After harvesting, biomass was recovered by centrifugation (3500 rpm for 5 min; Eppendorf 5415C) and lutein extraction was performed by addition of ethanol (30% wet weight biomass/volume). Cell debris removal was achieved by centrifugation at 3500 rpm for 5 min (Eppendorf 5415C). Cell debris were resuspended in ethanol (30% wet (w/v)) and the resulting solution used to evaluate their partition behaviour in ATPS. The supernatant derived from lutein extraction (referred to as crude extract) was introduced into the aqueous two-phase system previously selected as described below.

2.3. Aqueous two-phase experiments

Aqueous two-phase systems were prepared for convenience on a fixed mass basis using a top-loading balance. Predetermined quantities (see Table 1) of stock solutions of PEG and potassium phosphate were mixed with either a single model (containing a stock solution of purified lutein in ethanol) or a complex model (containing either cell debris suspension or cell debris-free extract from *C. protothecoides* fermentation; referred above as crude extract) systems. Subsequently, di-ionied water was added to give a final weight of 5 g. The stock solution of purified lutein was obtained by extracting 50 mg of lutein-rich paste (Lutein 5% TG Roche; Mannheim, Germany) in ethanol for 15 h at 30 °C under continuous shaking (200 rpm). The final concentration of ethanol in the ATPS was estimated to be around 6% (w/w) (weight of ethanol referred to the total weight of the system). The stock solutions (PEG and phosphate) were mixed and phases dispersed by gentle mixing for 60 min at 25 °C. Complete phase separation was achieved by low speed batch centrifugation at 2000 × *g* for 10 min at 25 °C. Visual estimates of the volumes of top and bottom phases were made in graduated centrifuge tubes. The volumes of the phases were then used to estimate the volume ratio (Vr = volume of the top phase/volume of the bottom phase). Samples were carefully extracted from the phases and diluted for analysis and subsequent estimation of lutein partition coefficient (K = concentration of solute in the top phase/concentration of solute in the bottom phase). The system tie-line length (TLL), which represents the length of the line that connects the composition of the top and bottom phases of a defined ATPS, was estimated as described by Albertsson [11]. Results reported are the average of three independent experiments and errors were estimated to be ±10% of the mean value.

2.4. Analytical methods

Total lutein concentration in the commercial paste was quantified using the average extinction coefficient for lutein of $E^{1\%}_{1\,cm} = 2550$ in ethanol and a molecular mass of 569 g/mol. All ATPS fractions were collected and their

Table 1
Systems selected for the evaluation of the partition behaviour of lutein and the effect of ethanol on phase formation

System	Molecular mass of PEG (g/mol)	PEG (%, w/w)	Phosphate (%, w/w)	TLL (%, w/w)	Volume ratio	Volume ratio (6% (w/w) ethanol)
1	1000	15.6	12.6	28.3	0.95 ± 0.09	2.4 ± 0.2
2		17.6	13.6	36.1	1.03 ± 0.1	2.1 ± 0.2
3		19.8	14.8	38.0	1.09 ± 0.1	2.4 ± 0.2
4		22.2	16.0	49.4	1.08 ± 0.1	2.1 ± 0.2
5	1450	17.6	10.9	34.3	1.03 ± 0.1	3.3 ± 0.3
6		22.0	12.1	47.0	1.1 ± 0.1	3.0 ± 0.3
7		24.9	12.6	53.2	1.1 ± 0.1	3.0 ± 0.3
8		26.1	13.0	55.0	1.07 ± 0.1	3.2 ± 0.3
9	3350	16.9	10.1	33.6	0.9 ± 0.1	2.7 ± 0.2
10		18.7	11.2	39.6	1.0 ± 0.1	2.2 ± 0.2
11		21.0	12.9	45.0	1.1 ± 0.1	2.1 ± 0.2
12		22.1	14.0	48.1	1.0 ± 0.1	2.2 ± 0.2
13	8000	16.1	8.1	27.1	1.0 ± 0.1	3.7 ± 0.3
14		19.0	9.1	40.2	0.9 ± 0.1	3.3 ± 0.3
15		20.0	9.5	45.0	1.08 ± 0.1	3.0 ± 0.3
16		22.9	10.3	49.4	1.08 ± 0.1	3.2 ± 0.3

Systems were selected to evaluate the impact of increasing tie-line length (TLL) and molecular mass of PEG upon the partition behaviour of lutein. The volume ratio was estimated (from blank systems and systems containing ethanol) as described in Section 2.

absorbance readings recorded at 445 nm using a Beckman DU® 650 spectrophotometer (Fullerton, CA). A system blank, without lutein, was prepared for each treatment combination and used as analytical blank for the corresponding phase (top or bottom). Total lutein concentrations were expressed as lutein equivalents using the average $E^{1\%}_{1\,cm}$ of 2550, and a molecular mass of 569 g/mol.

3. Results and discussion

3.1. The effect of ethanol on volume ratio of aqueous two-phase systems

The predictive design of aqueous two-phase processes demands the full understanding of the mechanisms governing the behaviour of molecules in ATPS. However, the lack of knowledge of such mechanism, requires that for each extraction process, once general conditions have been selected on the basis of experience or process limitations (e.g. polymer and salt type) more specific partition conditions (polymer and salt concentration, volume ratio, etc.) need to be empirically established. In the present research before designing the aqueous two-phase process for the recovery of lutein produced by the green microalga *C. protothecoides*, the influence of ethanol addition on the characteristic of ATPS was studied. In these experiments, ethanol was the solvent selected to favour the dissolution of lutein in the ATPS due to its wide use in food and pharmaceutical applications and relative low cost in contrast with other alcohols and organic solvents. In this particular case, the change in the volume ratio (compared with that from a system without ethanol) was used to evaluate the effect of ethanol in the ATPS. The effect of organic solvents on the partitioning of molecules in ATPS have been reported [12,13], revealing that the phase-forming properties can be influenced by the addition of an organic solvent, such as alcohol. These findings were recently applied to enhance the partition efficiency of geniposide in a polymer salt system [13]. However, it is important to consider that, in this paper, the use of ethanol was required to promote the dissolution of lutein to a concentration similar to that found in a fermentation from *C. protothecoides* [7].

Table 1 illustrates the effect of ethanol addition on the volume ratio (Vr) of the ATPS. It is clear that the addition of ethanol caused the Vr to rise for all systems. Regardless of the molecular mass of the PEG, the changes in Vr were more significant in the ATPS close to the binodal (short TLLs). Such situation can be associated to the nature of such systems. Albertsson [11] reported that ATPS located close to the binodal curve exhibited certain sensitivity to changes in system compositions. Subtle changes in the composition of ATPS caused by different factors (as in this case by the addition of ethanol), resulted in great changes in the phase composition and, as a result, in the final characteristics (Vr in this particular case) of the ATPS. In addition, the increase in the Vr of the ATPS loaded with ethanol may be explained by the particular effect of the solvent in the phases of the systems. It has been reported [13] that when complex systems are used, the presence of the solutes from biological suspensions has an impact on the final characteristic of the ATPS (e.g., volume ratio, position of the binodal curve). In the particular case of the volume ratio, it is suggested [14] that the accumulation of the solute to either phase caused the volume of that phase to increase. Thus, the volume ratio is increased by top-phase solvent accumulation or decreased by bottom phase solvent accumulation. It seems that for the ATPS studied the addition of ethanol affected the top phase and as a result the volume ratio of the systems. Thus, the partition behaviour and recovery of the target product loaded to the ATPS with ethanol will be influenced positively if lutein

exhibits top phase preference (where ethanol accumulates preferentially). Once the effect of the addition of ethanol on the ATPS was identified, the influence of system parameters on lutein partitioning behaviour was evaluated.

3.2. Influence of systems parameters on partition behaviour of lutein in PEG-salt aqueous two-phase systems

In order to select the ATPS in which the target product (lutein) and cell debris concentrate in opposite phases (preferentially the target product in the top phase to facilitate further processing), the partitioning behaviour was studied using systems comprising a stock solution of lutein from a paste rich in the product of interest. These model systems did not account for the influence, upon the performance of ATPS, of contaminants such as proteins, lipids, pigments, etc. that may be present in the extraction broth from a *C. protothecoides* fermentation or any other complex source. For the examination of the partition behaviour of lutein, a practical approach that exploits the known effect of system parameters such as tie-line length (TLL) and molecular mass of PEG on biomolecule partition coefficient was used. Thus, sixteen ATPS were selected (see Table 1) based upon experience [3,15]. These systems were characterized by increasing TLL using four different molecular mass of PEG (i.e. 1000, 1450, 3350 and 8000 g/mol) and keeping Vr = 1.0 and pH = 7.0 constant. The partition experiments that used purified lutein in ATPS revealed that this product exhibited a strong top phase preference (data not shown), which imply that the majority of the lutein concentrated in the top phase. The top-phase preference of the lutein resulted in partition coefficients greater than 50 for all the systems studied. Such behaviour was explained by problems associated with the detection of the presence of lutein in the bottom phase, caused by the very low amount of the product concentrated in this phase. As a consequence, it was very difficult to evaluate the impact of system parameters upon the partition behaviour of lutein, by monitoring the biomolecule partition coefficient (K). As a result, it was decided to use the recovery of lutein (expressed relative to the initial amount of lutein loaded to the ATPS) from the top PEG-rich phase as the response variable to evaluated the effect of system parameters on the behaviour of the product in ATPS. For the selection of the operating conditions to concentrate lutein in one phase, the concentration of PEG, phosphate and the molecular mass of PEG were manipulated to maximize recovery in the top phase.

Table 2 illustrates the impact of increasing TLL (for four different molecular mass of PEG) upon cell debris partition coefficient and lutein top phase recovery. As in the case of ethanol addition, the presence of cell debris affected the final Vr of the ATPS. The volume ratio increase by top phase cell debris accumulation or decrease by bottom phase cell debris accumulation. In the current study, ATPS both close and distant to the binodal curve (short and long TLL) for each molecular mass of PEG (1000, 1450, 3350, 8000 g/mol) were selected to examine the behaviour of the volume ratio of the biological systems. In general, the determination of the volume ratio for loaded ATPS requires a clear definition of the top, bottom and any other phase which develop. The latter clearly depends on the nature and complexity of the biological suspension. To simplify the estimation of the volume ratio in the ATPS loaded with cell debris, when the formation of an interface was observed, this was considered as a part of the top or bottom phase according to the initial volume ratio of the non-biological ATPS. Cell debris from *C. protothecoides* exhibited preferentially a bottom phase preference. In these systems a decrease in the volume

Table 2
Influence of increasing tie-line length (TLL) and molecular mass of PEG on cell debris partition behaviour and top phase recovery of lutein in the ATPS

System	Molecular mass of PEG (g/mol)	TLL (%, w/w)	Volume ratio (6% (w/w) ethanol)	Volume ratio (ATPS loaded with biomass)	Cell debris phase preference	Top phase recovery Y_{TOP} (%)
1	1000	28.3	2.4 ± 0.2	2.6 ± 0.2	T	35 ± 2.0
2		36.1	2.1 ± 0.2	2.0 ± 0.2	B	54 ± 4.1
3		38.0	2.4 ± 0.2	2.1 ± 0.2	B	71 ± 3.2
4		49.4	2.2 ± 0.2	2.0 ± 0.2	B	72 ± 3.0
5	1450	34.3	3.3 ± 0.3	2.9 ± 0.2	B	62 ± 3.0
6		47.0	3.0 ± 0.3	2.7 ± 0.2	B	72 ± 2.8
7		53.2	3.0 ± 0.3	3.3 ± 0.3	T	72 ± 3.8
8		55.0	3.2 ± 0.3	3.5 ± 0.3	T	77 ± 2.5
9	3350	33.6	2.7 ± 0.2	2.4 ± 0.2	B	68 ± 2.6
10		39.6	2.3 ± 0.2	2.1 ± 0.2	B	68 ± 3.4
11		45.0	2.1 ± 0.2	2.0 ± 0.2	B	70 ± 2.4
12		48.1	2.2 ± 0.2	1.9 ± 0.1	B	76 ± 2.6
13	8000	27.1	3.7 ± 0.3	4.0 ± 0.1	T	75 ± 2.1
14		40.2	3.3 ± 0.3	3.0 ± 0.3	B	78 ± 2.6
15		45.0	3.0 ± 0.3	2.7 ± 0.2	B	78 ± 2.1
16		49.4	3.2 ± 0.3	2.9 ± 0.2	B	81 ± 2.8

Composition of the systems (1–16) is defined in Table 1. Vr, estimated from blank or loaded with biomass systems containing ethanol was determined after phase separation in graduated tubes. T and B denote top and bottom phase preference for cell debris. The recovery of lutein from the top phase (Y_{TOP}) is expressed relative to the initial amount of lutein content in the stock solution loaded to the ATPS.

ratio was observed compared to that from the cell debris-free ATPS, this implied that cell debris was accumulated in the bottom phase. Top phase cell debris accumulation was observed mainly in ATPS of low molecular mass of PEG (1000 and 1450 g/mol). Although a clear explanation for such behaviour is not currently available, the results obtained facilitate the selection of operating conditions to favour cell debris accumulation into a define phase.

In the case of lutein partition behaviour, it seems that for all the ATPS studied, the product partitioned preferentially to the top phase (lutein was not detected at the bottom phase) regardless the TLL of the system. It seems that increasing TLL caused the top phase recovery of lutein in ATPS to rise for each molecular mass of PEG used (e.g. PEG 1000, 1450, 3350 and 8000 g/mol). Such situation can be associated to the fact that the free volume of the top phase of ATPS rises when the TLL is increased [15]. Thus, the space available to allocate the solute is significantly affected by an increase in the TLL of the system. In the case of PEG 1000 ATPS, increasing TLL caused a significant increment in the top phase recovery of lutein (from 35 ± 2.0 to 72 ± 3.0). Systems comprising PEG 1450, 3350 and 8000 exhibited a similar trend but with less emphasis 62 ± 3.0 to 77 ± 2.5, 68 ± 2.6 to 76 ± 2.6 and 75 ± 2.1 to 81 ± 2.8, respectively (see Table 2). Such partition behaviour may be explained by changes in the free volume [16] and density of the phases [10]. It has been reported that the free volume in the bottom phase decreases when the TLL is increased [16]. As a result, the solutes in the lower phase may be promoted to partition to the top phase. Consequently, top phase recovery of lutein rise when TLL was increased.

For all the ATPS studied, more than 50% of lutein can be potentially recovered from the upper phase. However, system 16 in Table 2 exhibited the best recovery of lutein from the top phase (i.e. $81 \pm 2.8\%$). It is important to mention that the moderate values of top phase product recovery of systems of low molecular mass of PEG (1000 and 1450 g/mol) and short TLL (less than 35% (w/w)) were possibly affected by product accumulation at the interface confirmed by the mass balance (see systems 1 and 5 in Table 2). In PEG 3350 and 8000-salt systems of long TLL, lutein exhibited a more favourable partitioning to the top phase and cell debris to the bottom phase in comparison with that of PEG 1000, and 1450-salt systems. For an extraction system, a PEG 3350 and PEG 8000-phosphate system with long TLL (systems 12 and 16 in Table 2) were selected as the most favourable to maximize the partitioning of the product of interest and cell debris contaminants to opposite phases. It is clear that such result will facilitate the potential application of ATPS processes in the recovery of lutein from *C. prototheocoides*.

3.3. Effect of lutein concentration on PEG-salt aqueous two-phase systems performance

In an attempt to intensify the potential recovery process, the effect of increasing lutein concentration on the performance of ATPS was evaluated. It was decided to examine the impact of an increment on lutein concentration (from 28 to 120 ppm) on the product top phase recovery using selected ATPS characterized by PEG 3350 and 8000 g/mol molecular mass of PEG. Concentration range was selected to simulate final levels (28–120 ppm) in the system that would be obtained by extracting the product from fermentation origin [7]. The ATPS used in this part of the research (i.e. systems 12 and 16 in Tables 1 and 2) were selected on the basis of top phase product recovery from the previous section and bottom phase accumulation of cell debris. Systems 12 and 16 exhibited the best top phase recovery from ATPS of high molecular mass of PEG (3350 and 8000 g/mol). Fig. 1 illustrates the effect of lutein concentration in the system on top phase product recovery. It is clear that top phase recovery of lutein decreased for both ATPS when the concentration of the product of interest (lutein) increased. Such behaviour can be explained on the basis of losses of soluble product caused by lutein accumulation at the interface. In the case of losses of soluble product in the top phase caused by the increase in lutein concentration, PEG 8000-salt ATPS exhibited better performance compared with that of the PEG 3350 system. It is clear that and attempt to intensify recovery process by increasing the amount of biological material that can be processed (simulated by an increase in lutein concentration in the system), resulted in a negative influence to the top phase lutein recovery (Fig. 1).

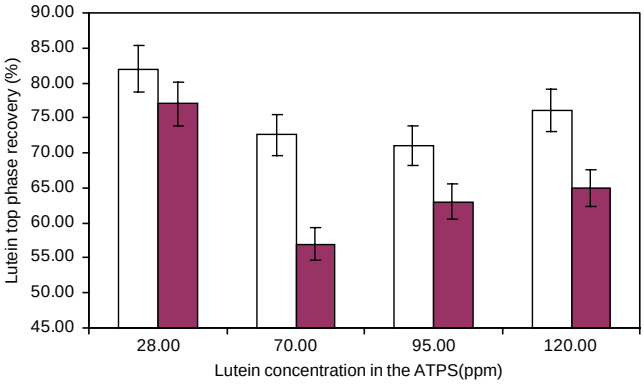

Fig. 1. Influence of increasing lutein concentration on product top phase recovery in PEG 3350 and PEG 8000—phosphate aqueous two-phase systems. The recovery of lutein from the top phase (Y_{TOP}) in PEG 3350 (■) and PEG 8000 (□) ATPS is expressed relative to the initial amount of lutein loaded to the ATPS. PEG 3350 and PEG 8000 ATPS were systems 12 and 16 identified in Table 1, respectively. For all systems, pH was kept constant at 7.0. The ATPS were constructed as described in Section 2. Results reported are the average of two independent experiments and errors were estimated to be ±5% of the mean value.

In general, from the ATPS studied, the system comprising Vr = 1.0, PEG 8000 22.9% (w/w), phosphate 10.3% (w/w) at pH = 7.0 provided the required conditions to concentrate lutein from *C. prototheocoides* in the top phase (i.e. top phase recovery of 81%) and cell debris in the bottom phase. It is clear that such findings will facilitate the potential generic application of the ATPS process to recover

lutein from microbial origin. In the case of top phase product recovery, although a yield of 81% for the ATPS primary step may be acceptable, potential strategies to improve the un-optimised performance of the ATPS process under higher product concentrations could be explored. They may include modifications in the level of ethanol in the ATPS, in order to maximize product solubility prior to subsequent top phase processing. However, at this stage the partitioning behaviour and the mechanism governing the partition of lutein in ATPS cannot be fully explained and further characterization to untangle such phenomena will facilitate the development of a commercial recovery process.

4. Conclusion

This is the first study to report the phenomenon of partitioning of lutein produced by *C. protothecoides* in aqueous two-phase systems. It was shown that tie-line length and molecular mass of PEG influenced the lutein concentration in the top phase. The addition of ethanol to favour the product dissolution increased the volume ratio of the systems. Increasing lutein concentrations (>28 ppm) in the ATPS resulted in reduced product yield. PEG 8000-phosphate ATPS proved to be suitable for the potential recovery of lutein (81%). The operating conditions selected for this particular ATPS system resulted in the partition of lutein preferentially to the top phase and cell debris to the bottom phase. Although the performance of the ATPS selected can be improved by further investigation, the findings reported here demonstrate the potential application of ATPS processes for the recovery of intracellular lutein produced by *C. protothecoides* as a first step for the development of a biotechnological process with commercial application.

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Capítulo 4. Conclusiones y Recomendaciones

Los procesos de recuperación y purificación primaria de compuestos biológicos generalmente cuentan con un gran número de etapas, lo cual se traduce en una pérdida significativa del producto de interés. Adicionalmente algunas de las técnicas convencionales no son fácilmente escalables, lo cual genera elevados costos de inversión, operación y mantenimiento a nivel industrial. Es necesario el diseño de procesos de recuperación primaria, con reducido número de etapas, para la recuperación primaria de compuestos biológicos de interés comercial. Por lo tanto se estableció la aplicación genérica de sistemas de dos fases acuosas polietilénglicol (PEG) – solución salina para el desarrollo de bioprocesos que permitan la recuperación primaria de compuestos biológicos.

Los parámetros del sistema (longitud de línea de corte, LLC; relación de volúmenes, V_R; pH y peso molecular del polímero, PM PEG; porcentaje de muestra en el sistema, etc) tienen influencia significativa sobre el comportamiento de partición de los modelos experimentales seleccionados: B-ficoeritrina (BFE) producida por *Porphyridium cruentum*, C-ficocianina (CFC) producida por *Spirulina maxima*, pseudo-partículas 2/6 de rotavirus (dlRLP) producidas por células de insecto High Five^{TM} y luteína producida por *Chlorella protothecoides*.

Los resultados experimentales indican que los parámetros de sistema que favorecen la partición de BFE a la fase superior del sistema son: PEG 1000 g/gmol, LLC 45% p/p, V_R 4.5 y 40% p/p de extracto crudo de BFE. Bajo estas condiciones es posible recuperar el 92% de BFE alimentada al sistema con una pureza (Abs545nm/Abs280nm) de 3.2. Para CFC los parámetros que favorecen su partición hacia la fase superior del sistema son: PEG 1450 g/gmol, LLC 34% p/p, V_R 0.3 y pH 7; logrando recuperar bajo estas condiciones el 98% del producto con una pureza (Abs620nm/Abs280nm) de 2.1.

En el caso de las pseudo-partículas 2/6 de rotavirus (dlRLP) ambas fracciones, intracelular y extracelular, son recuperadas con estrategias independientes. La fracción intracelular es recuperada utilizando un proceso con dos etapas de extracción en serie (PEG – fosfato de potasio, PEG 400 g/gmol, V_R 13.0, pH 7 y LLC 35% p/p), logrando

recuperar el 90% de las dlRLP intracelulares en la fase superior del sistema, mientras que los restos celulares se acumularon en la interfase. Debido a la ausencia de restos celulares la fracción extracelular es recuperada en la interfase del SDFA, con una estrategia que involucra una sola extracción (PEG – fosfato de potasio, PEG 3350 g/gmol, V_R 1.0, pH 7 y LLC 17% p/p), logrando recuperar el 82% de las dlRLP extracelulares. En lo que respecta a la recuperación de luteína, los parámetros de sistema que favorecen su partición hacia la fase superior son PEG 8000 g/gmol, LLC 34% p/p, V_R 1 y pH 7, logrando recuperar, bajo dichas condiciones el 81% del producto.

El comportamiento de partición de los compuestos de interés (bajo diferentes parámetros de sistema) esta íntimamente relacionado con sus propiedades fisicoquímicas (peso molecular, hidrofobicidad y punto isoeléctrico). Tomando como base la información obtenida del comportamiento de partición de los productos biológicos seleccionados se establecieron reglas heurísticas que correlacionan las características fisicoquímicas de los compuestos y los parámetros de SDFA. Estas reglas permiten el desarrollo predictivo de procesos de recuperación primaria para compuestos biológicos utilizando sistemas de dos fases acuosas PEG – solución salina, utilizando como información de partida las características fisicoquímicas del compuesto de interés y los contaminantes presentes.

En lo referente al peso molecular del compuesto de interés, mientras mayor sea este menor debe ser el peso molecular del PEG utilizado, si es que se desea llevar a cabo la recuperación en la fase superior del sistema. Esto es debido, principalmente al fenómeno de volumen excluido. Compuestos de bajo peso molecular (< 5000 g/gmol) pueden ser recuperados en la fase superior aun cuando se utiliza PEG de alto peso molecular, como en el caso de la luteína. La hidrofobicidad del compuesto de interés mostró tener influencia significativa sobre el comportamiento de partición. Mientras más hidrofóbico es el compuesto que se intenta recuperar, el peso del PEG debe ser mayor, para de esa manera lograr que una mayor cantidad de agua sea excluida de la fase superior. De igual manera, el uso de longitudes de línea de corte elevadas (> 40% p/p) genera un aumento en la hidrofobicidad de la fase polimérica, lo cual se ve reflejado en un aumento en el porcentaje de recuperación de compuestos hidrofóbicos. También es importante considerar la carga electroquímica superficial de la molécula a

recuperar. En caso de que la molécula presente anfoterismo (cambio de carga electroquímica superficial en función del pH) es recomendable trabajar a valores de pH por arriba del punto isoeléctrico, de tal manera que la carga de compuesto sea negativa y tenga mayor afinidad por el PEG (el cual esta ligeramente cargado positivamente).

No tan solo es necesario considerar las características fisicoquímicas del compuesto de interés para llevar a cabo el diseño del proceso de recuperación primaria. Es recomendable considerar las características de los contaminantes presentes. De esta manera es posible seleccionar los parámetros de sistema en los que es más posible que la partición del compuesto de interés y los contaminantes se realicen en fases opuestas del sistema.

Recomendaciones

Las reglas heurísticas y la estrategia secuencial reportadas en este documento son una primera aproximación para el desarrollo predictivo de procesos de recuperación primaria de compuestos biológicos utilizando SDFA PEG – solución salina. Sin embargo, existen áreas de oportunidad que en el trabajo realizado.

Los modelos experimentales estudiados (B-ficoeritrina (BFE) producida por *Porphyridium cruentum*, C-ficocianina (CFC) producida por *Spirulina maxima*, pseudo-partículas 2/6 de rotavirus (dlRLP) producidas por células de insecto High Five^{TM} y luteína producida por *Chlorella protothecoides*) permitieron generar reglas heurísticas para el desarrollo predictivo de procesos de recuperación primaria. Es recomendable estudiar otros sistemas experimentales (proteínas hidrofóbicas de elevado peso molecular, compuestos hidrofílicos de bajo peso molecular) que permitan comprobar la robustez de la estrategia propuesta.

A pesar de que se investigó la influencia de algunos parámetros de sistema (peso molecular del polímero, longitud de línea de corte, relación de volúmenes, pH, porcentaje de muestra, etc) es recomendable hacer estudios de la influencia de otros factores que pueden afectar el comportamiento de partición de compuestos biológicos en SDFA. Parámetros tales como la adición de sales neutras (como por ejemplo NaCl) y

la temperatura pueden afectar el equilibrio de las fases del sistema, y por lo tanto el comportamiento de partición que exhibe cierto compuesto en el sistema.

Otro factor que es conveniente considerar es el uso de derivados de polietilénglicol (PEG) para la formación de los sistemas. Estos derivados son modificaciones químicas de la molécula de PEG, a la cual diferentes grupos funcionales son agregados con el objetivo de incrementar la afinidad del producto de interés por la fase polimérica. A pesar que el uso de estos polímeros modificados es un área de oportunidad, la falta información y caracterización de dichos sistemas dificulta su estudio. Es necesario caracterizar el equilibrio líquido-líquido de dichos sistemas, para posteriormente estudiar la influencia de los parámetros de proceso sobre el comportamiento de partición de los compuestos de interés.

La presente investigación se enfoca en sistemas polímero (PEG) – solución salina. Si bien estos sistemas de dos fases acuosas son los más caracterizados y utilizados (particularmente los sistemas PEG – fosfato de potasio), es recomendable llevar a cabo investigaciones con sistemas polímero-polímero. A pesar de que estos sistemas no se encuentran tan bien caracterizados, tecnologías emergentes (como por ejemplo la cromatografía líquida a contracorriente), utilizan equilibrios polímero-polímero para llevar a cabo el fraccionamiento de compuestos biológicos. Esto hace atractivo el desarrollo predictivo de procesos de recuperación primaria de productos biotecnológicos utilizando sistemas polímero-polímero.

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ANEXO

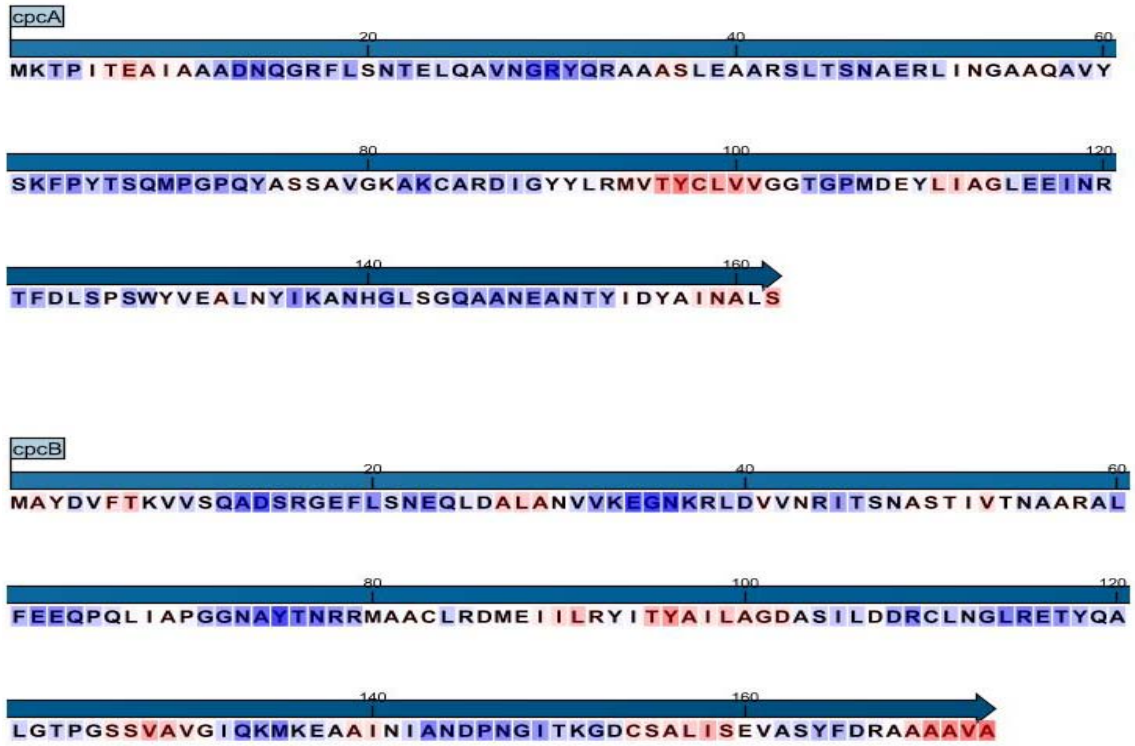

Figura A.1 Representación de las regiones hidrofílicas e hidrofóbicas de las subunidades α (cpcA) y β (cpcB) de C-ficocianina mediante la escala Kyte – Doolittle. El programa computacional CLC Protein Workbench (Versión 2.0.2, Demo) fue utilizado para determinar la hidrofobicidad de las subunidades de la proteína. A lo largo de las secuencias de aminoácidos se muestran en color azul las regiones hidrofílicas, mientras que en rojo se muestran las regiones hidrofóbicas. De esta manera es posible determinar (de manera cualitativa) la hidrofobicidad relativa de la proteína mediante la proporción de regiones hidrofílicas e hidrofóbicas.

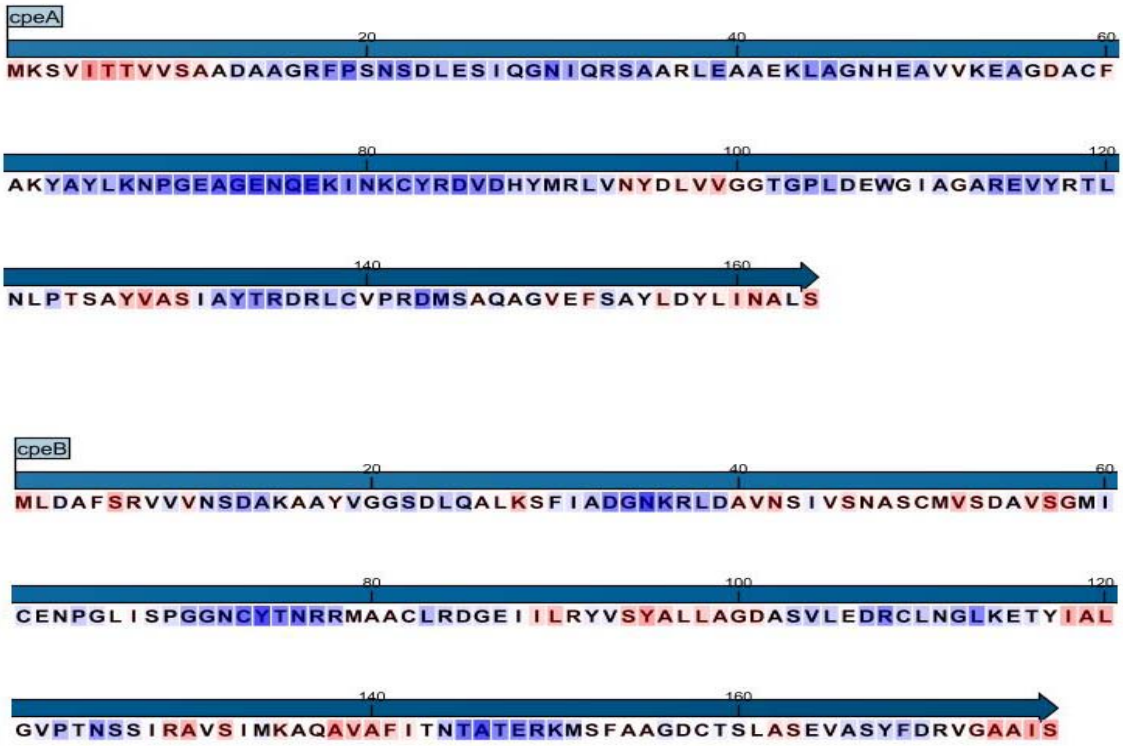

Figura A.2 Representación de las regiones hidrofílicas e hidrofóbicas de las subunidades α (cpeA) y β (cpeB) de B-ficoeritrina mediante la escala Kyte – Doolittle. El programa computacional CLC Protein Workbench (Versión 2.0.2, Demo) fue utilizado para determinar la hidrofobicidad de las subunidades de la proteína. A lo largo de las secuencias de aminoácidos se muestran en color azul las regiones hidrofílicas, mientras que en rojo se muestran las regiones hidrofóbicas. De esta manera es posible determinar (de manera cualitativa) la hidrofobicidad relativa de la proteína mediante la proporción de regiones hidrofílicas e hidrofóbicas.

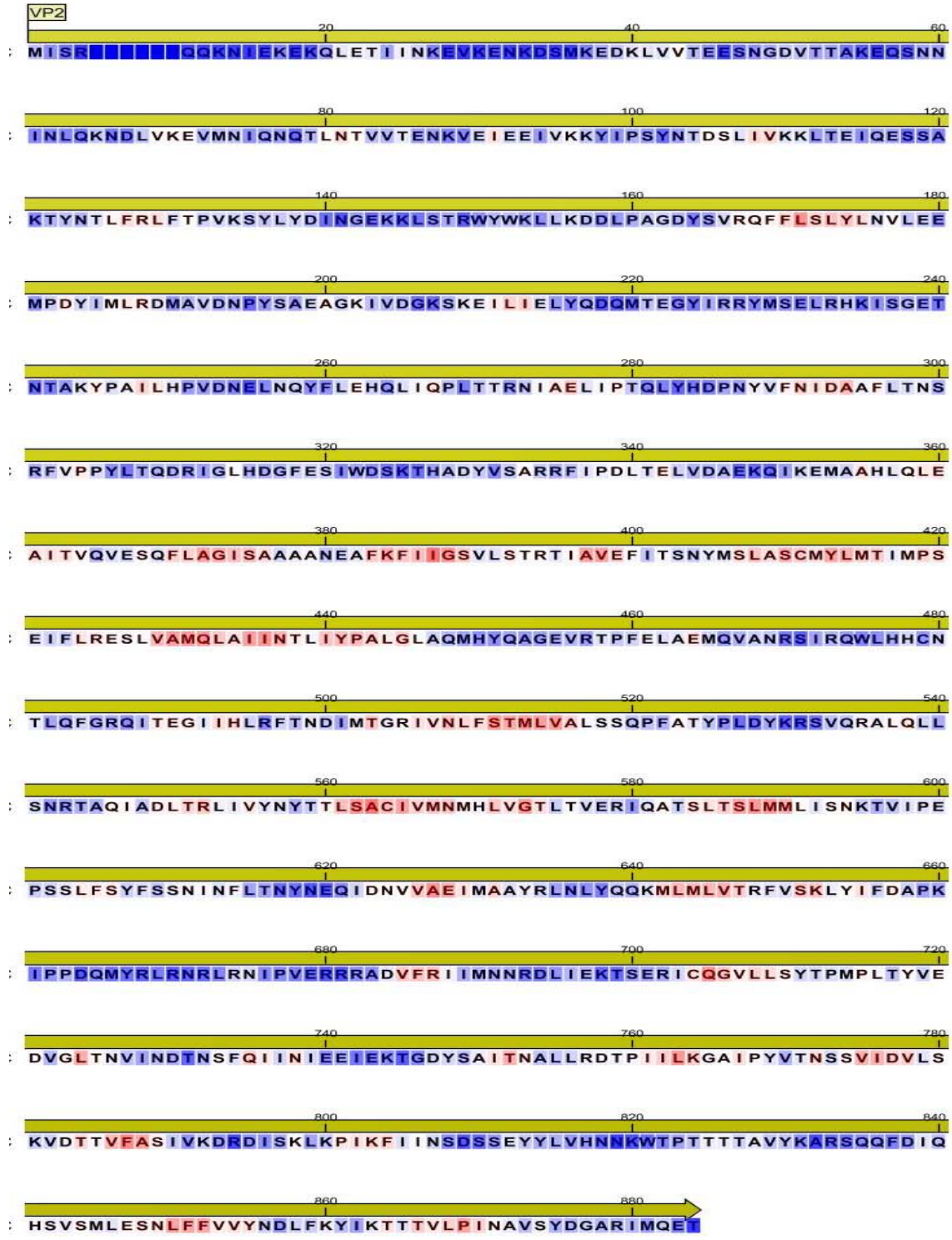

Figura A.3 Representación de las regiones hidrofílicas e hidrofóbicas de la proteína VP2 de rotavirus mediante la escala Kyte – Doolittle. El programa computacional CLC Protein Workbench (Versión 2.0.2, Demo) fue utilizado para determinar la hidrofobicidad de las subunidades de la proteína. A lo largo de las secuencias de aminoácidos se muestran en color azul las regiones hidrofílicas, mientras que en rojo se muestran las regiones hidrofóbicas. De esta manera es posible determinar (de manera cualitativa) la hidrofobicidad relativa de la proteína mediante la proporción de regiones hidrofílicas e hidrofóbicas.

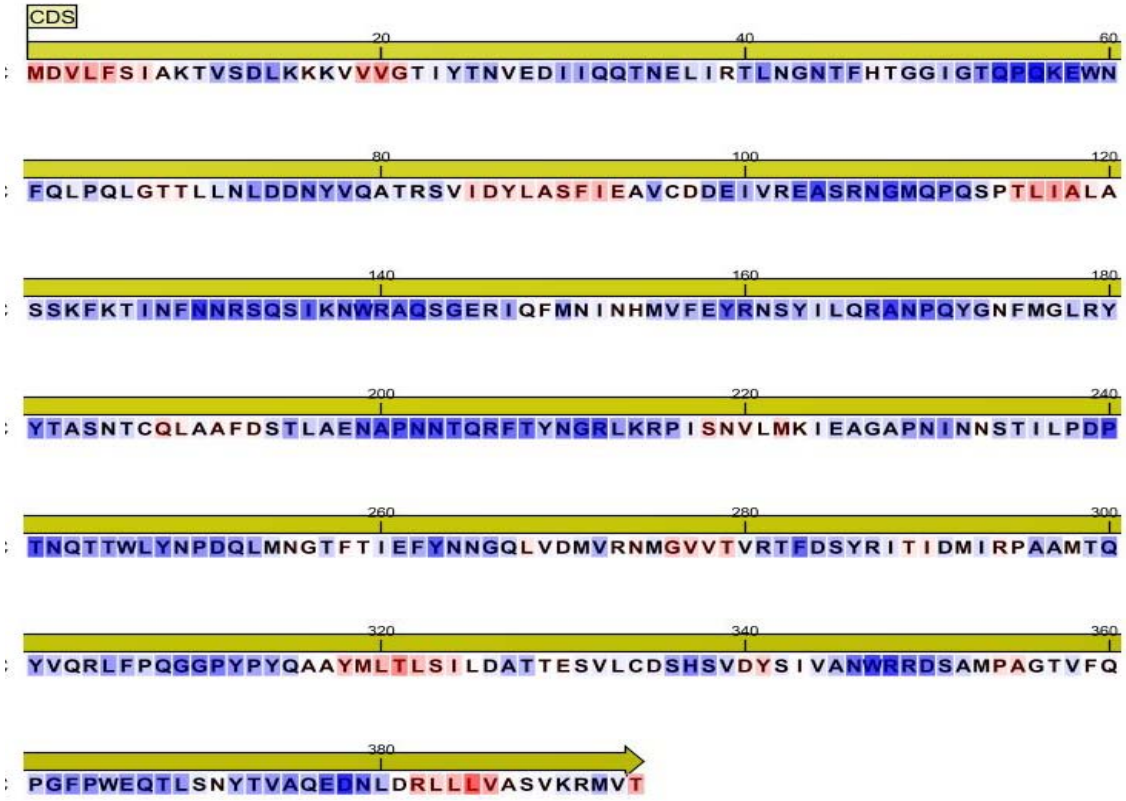

Figura A.4 Representación de las regiones hidrofílicas e hidrofóbicas de la proteína VP6 de rotavirus mediante la escala Kyte – Doolittle. El programa computacional CLC Protein Workbench (Versión 2.0.2, Demo) fue utilizado para determinar la hidrofobicidad de las subunidades de la proteína. A lo largo de las secuencias de aminoácidos se muestran en color azul las regiones hidrofílicas, mientras que en rojo se muestran las regiones hidrofóbicas. De esta manera es posible determinar (de manera cualitativa) la hidrofobicidad relativa de la proteína mediante la proporción de regiones hidrofílicas e hidrofóbicas.