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Señor Director
DIRECCIÓN DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

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Referencia : Expediente No. **13.025.488**
Asunto : Solitud de Patente para: **"MÉTODO PARA PRODUCIR EL FACTOR VON WILLEBRAND (VWF) DE ALTO PESO MOLECULAR RECOMBINANTE EN CULTIVO CELULAR"**
Solicitante : BAXTER HEALTHCARE S.A.; BAXTER INTERNATIONAL INC.
Fecha de solicitud : 7 de Febrero de 2013

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. **2452** notificado por fijación en lista el 4 de Marzo de 2014.

2. Mediante memorial del 29 de Mayo de 2014 se solicitó oportunamente plazo para responder la acción oficial arriba mencionado.

3. Mediante oficio No. **2452** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio se refieren a la presencia de materia que no es considerada una invención, a la claridad del pliego reivindicatorio, y a la novedad de las reivindicaciones 1 a 11 y 14 a 44 en vista del documento **D1: WO2009086309**.

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4. Respuesta a las objeciones

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desea presentar las siguientes modificaciones, aclaraciones y argumentos:

4.1 Modificación de una solicitud en trámite

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por 42 reivindicaciones.

El nuevo pliego reivindicatorio corresponde esencialmente al anterior, pero se ha definido la concentración de NH₄⁺ en el sobrenadante de cultivo celular de la reivindicación 1, y se han eliminado las reivindicaciones 44 y 45.

4.2 Con respecto a la materia no considerada como invención

Es preciso mencionar que, aunque las reivindicaciones 12 y 13 mencionan células, en ningún momento se está intentando obtener protección sobre dichas células. De hecho, dichas reivindicaciones únicamente especifican las células seleccionadas para el medio de cultivo de células basales provisto en el paso (a) del método de la reivindicación 1.

De esta forma, el objeto de protección de las reivindicaciones es el mismo de la reivindicación 1, es decir Un método para producir una composición del Factor Von Willebrand recombinante (rVWF), pero especificando una característica preferente, y no parte o la totalidad de un ser vivo.

Consecuentemente, las reivindicaciones 12 y 13 sí se refieren a una invención.

4.3 Argumentos a favor de la claridad del pliego reivindicatorio

En primer lugar, es preciso aclarar que la actividad del cofactor de ristocetina específico del rVWF mencionada en el paso (e) de la reivindicación 1 no corresponde a un resultado a alcanzar sino un parámetro del proceso que puede ser determinado mediante métodos descritos a lo largo de la descripción y comunes en el estado del arte. Adicionalmente, la determinación de la actividad del cofactor de ristocetina específico del rVWF es una práctica común y bien establecida en la técnica para caracterizar proteínas rVWF.

Adicionalmente, no es posible definir la invención de otra manera ya que es completamente inviable tratar de definir el sobrenadante del cultivo celular por sus componentes específicos. Ya que dicho sobrenadante no puede ser definido correctamente mediante sus características técnicas estructurales, es preciso recurrir a sus parámetros, como la actividad del cofactor de ristocetina específico del rVWF, para definirlo.

Con respecto a lo anterior, el numeral 2.6.5.9 de la “GUÍA PARA EXAMEN DE SOLICITUDES DE PATENTE DE INVENCION Y MODELO DE UTILIDAD” de su Despacho establece lo siguiente:

“Una reivindicación de producto, por ejemplo un compuesto químico, se puede caracterizar por su estructura y elementos, por su fórmula química, como un producto de un proceso, o excepcionalmente por sus parámetros.

Los parámetros son valores característicos de propiedades mensurables (por ejemplo el punto de fusión), o definidos como combinaciones matemáticas de varias variables.

El examinador no permitirá la caracterización de un compuesto químico solamente por sus parámetros, a menos que la invención no se pueda definir de otra manera. En cualquier caso, el parámetro tiene que poder ser determinado y medido sin

ambigüedad por métodos estándar conocidos en el campo en cuestión, o descritos claramente en la solicitud.

Lo mismo se aplica a una característica vinculada a un procedimiento, que es definido por parámetros”.

Resulta entonces que el parámetro en cuestión debe ser aceptado para definir la invención porque puede ser determinado y medido sin ambigüedad por métodos estándar conocidos en el campo en cuestión, y porque la invención no se puede definir de otra manera.

Lo anterior aplica también para las reivindicaciones 23 a 27.

Por otro lado, el contenido de NH_4^+ , mencionado en la nueva reivindicación 1 y en la reivindicación 32, no se refiere de ninguna forma a un resultado a alcanzar. De hecho, el contenido de NH_4^+ es mencionado siempre dentro del contexto de un paso activo que se debe llevar a cabo para realizar el método: **mantener** el contenido de NH_4^+ del sobrenadante de cultivo celular a una concentración por debajo de cierto nivel especificado en la reivindicación. Cualquier persona versada en la materia reconocerá evidentemente que mantener el contenido de NH_4^+ por debajo de cierto valor en un cultivo celular como el de la presente invención **es un paso activo** conocido en la técnica, y no un resultado a alcanzar. Anexos se presentan los artículos “IN SITU REMOVAL OF AMMONIUM IONS FROM HYBRIDOMA CELL CULTURE MEDIA: SELECTION OF ADSORBENT” y “Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine” que soportan lo anterior.

Con respecto a la reivindicación 2, es preciso mencionar que ésta se encuentra debidamente soportada en la descripción (ver por ejemplo página 15 renglón 23 a página 16 renglón 1, página 35 renglón 2 a 12 y página 156 renglones 10 a 13). Adicionalmente, las técnicas para suplementar el medio de cultivo de células basales son ampliamente conocidas en la técnica, por lo que la reivindicación 2 resultaría perfectamente clara para cualquier persona normalmente versada en la materia.

Ahora con respecto a la reivindicación 3, es preciso mencionar que ésta se encuentra debidamente soportada en la descripción (ver por ejemplo página 15 renglón 23 a página 16 renglón 1, página 51 renglón 23 a página 52 renglón 19 y página 54 renglón 16 a página 55 renglón 11). Adicionalmente, los hidrolizados de plantas son ampliamente conocidos en el arte, por lo que esta reivindicación resulta perfectamente clara para cualquier persona normalmente versada en la materia.

Similarmente, las reivindicaciones se encuentran debidamente soportadas en la descripción (ver por ejemplo página 4 renglón 12 a página 5 renglón 16 y página 51 renglones 9 a 22). Los fragmentos mencionados de la descripción muestran claramente que los medios mencionados en las reivindicaciones 5 y 6 son ampliamente conocidos en el estado de la técnica.

Contrario a lo afirmado por el Señor Examinador, la reivindicación 33 no hace referencia a un resultado a alcanzar, sino a la selección de la proteína. Es preciso mencionar que la co-expresión de proteínas es un concepto ampliamente conocido en el campo técnico, y no puede ser interpretado de ninguna forma como un resultado a alcanzar. Adicionalmente, los métodos de co-expresión, que son ampliamente conocidos en el estado de la técnica, se encuentran descritos en, por ejemplo, el ejemplo 2 de la descripción.

Por último, las reivindicaciones 43 y 44 han sido eliminadas, por lo que las objeciones referentes a éstas no prosperan en el nuevo pliego.

En consecuencia de lo anterior, se considera que las objeciones formuladas en cuanto a la falta de claridad de las reivindicaciones han sido superadas.

4.4 Argumentos a favor de la novedad de la presente solicitud

Queremos llamar la atención del Despacho, que el Tribunal de Justicia de la Comunidad Andina se ha pronunciado en diversas oportunidades en relación a la forma

de evaluar la novedad. En la interpretación 36-IP-2010 el Tribunal ha dicho que *"El Tribunal ha tomado como referencia criterios de las Cámaras de Recursos de la Oficina Europea de Patentes (OEP), mediante los cuales "la falta de novedad de una invención se configurará si su objeto deriva clara, precisa y directamente del estado de la técnica, y si en éste son conocidas todas las características de aquélla"*. En el presente caso se observa que el objeto reivindicado no se deriva clara, precisa y directamente del estado de la técnica dado que:

- Del estado de la técnica no puede desprenderse el paso de mantener el contenido de NH_4^+ del sobrenadante de cultivo celular a una concentración por debajo de 10 mM;
- Del estado de la técnica no puede desprenderse que el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 30 mU/ μg del rVWF.

Dado que no es posible derivar clara, precisa y directamente de la anterioridad la presente invención con todas sus características técnicas, entonces la presente solicitud goza de novedad.

De acuerdo a todos los argumentos presentados, la presente solicitud cumple a cabalidad con los requerimientos exigidos en la Decisión 486 y las objeciones formuladas por el Examinador se ven superadas.

4. Puesto que se ha dado respuesta a todas las objeciones formuladas por el Examinador, se solicita atentamente proceder al otorgamiento de la solicitud de patente. En este sentido, respetuosamente recordamos al Examinador que la patente no podrá ser negada con base en objeciones diferentes a las expuestas en la acción oficial No. 2452, toda vez que al no haber sido trasladadas a mi representada, dicha decisión violaría tanto el tenor literal del artículo 45 de la Decisión 486 como el derecho constitucional al debido proceso. En este evento, y con base en el inciso 2 del artículo 45, respetuosamente solicitamos al Examinador expedir una nueva acción oficial que permita a mi representada

contra argumentar las mismas o modificar la solicitud con el fin de cumplir los requisitos de patentabilidad.

5. Anexos

- Nuevo pliego reivindicatorio conformado por 42 reivindicaciones
- Artículo "IN SITU REMOVAL OF AMMONIUM IONS FROM HYBRIDOMA CELL CULTURE MEDIA: SELECTION OF ADSORBENT"
- Artículo "Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine"

Señor Director,


LUZ CLEMENCIA DE PÁEZ
C.C. 35.456.344 de Usaquén
T.P.A. No. 23.555

COLO-13936-0841-051-6
SML\SML\ID-268409\WPC13936
No. M-2014-268409\SML

REIVINDICACIONES

1. Un método para producir una composición del Factor Von Willebrand recombinante (rVWF), caracterizado porque el método comprende las etapas de:
 - (a) proporcionar un medio de cultivo de células basales;
 - (b) suplementar el medio de cultivo de células basales con cobre para proporcionar una concentración de cobre final de al menos 2.4 µg/L;
 - (c) proporcionar una o más células que comprenden un ácido nucleico que codifica una proteína del rVWF;
 - (d) cultivar una o más células en el medio de cultivo de células suplementadas con cobre de manera que el rVWF se expresa y excreta de las células en un sobrenadante del cultivo de en donde el contenido de NH₄⁺ del sobrenadante de cultivo celular se mantiene a una concentración por debajo de 10 mM.; y
 - (e) recuperar al menos una porción del sobrenadante del cultivo, en donde el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 30 mU/µg del rVWF.
2. El método de conformidad con la reivindicación 1, caracterizado porque además comprende la etapa de suplementar el medio de cultivo de células basales con un hidrolizado previo a cultivar una o más células.
3. El método de conformidad con la reivindicación 2, caracterizado porque el hidrolizado es un hidrolizado de planta.
4. El método de conformidad con la reivindicación 3, caracterizado porque el hidrolizado es un hidrolizado de soya.
5. El método de conformidad con cualquiera de las reivindicaciones 1 a 4, caracterizado porque el medio de cultivo de células basales es un medio de cultivo libre de proteína animal.
6. El método de conformidad con cualquiera de las reivindicaciones 1 a 4, caracterizado porque el medio de cultivo de células basales es un medio de cultivo libre de proteína.
7. El método de conformidad con cualquiera de las reivindicaciones 1 a 6, caracterizado porque el medio de cultivo de células basales es un medio de cultivo libre químicamente definido.

8. El método de conformidad con cualquiera de las reivindicaciones 1 a 7, caracterizado porque la concentración final de cobre del medio de cultivo de células basales suplementado con cobre es al menos 4 µg/L de cobre.
9. El método de conformidad con cualquiera de las reivindicaciones 1 a 8, caracterizado porque la concentración final de cobre del medio de cultivo de células basales suplementado con cobre es entre 2.4 µg/L y 20 µg/L de cobre.
10. El método de conformidad con cualquiera de las reivindicaciones 1 a 9, caracterizado porque el medio de cultivo de células basales suplementado con cobre se proporciona como una sal de cobre, un quelato de cobre, o una combinación de los mismos.
11. El método de conformidad con la reivindicación 10, caracterizado porque la sal de cobre se selecciona del grupo que consiste de sulfato de cobre, acetato de cobre, carbonato de cobre, cloruro de cobre, hidróxido de cobre, nitrato de cobre y óxido de cobre.
12. El método de conformidad con cualquiera de las reivindicaciones 1 a 11, caracterizado porque una o más células son células de mamífero.
13. El método de conformidad con la reivindicación 12, caracterizado porque las células de mamífero son células CHO.
14. El método de conformidad con cualquiera de las reivindicaciones 1 a 13, caracterizado porque cultivar una o más células comprende cultivación por lotes de las células.
15. El método de conformidad con cualquiera de las reivindicaciones 1 a 13, caracterizado porque cultivar una o más células comprende cultivación continua de las células.
16. El método de conformidad con la reivindicación 15, caracterizado porque la cultivación continua de células se realiza en modo quimiostático.
17. El método de conformidad con la reivindicación 15, caracterizado porque la cultivación continua de células se realiza en modo de perfusión.
18. El método de conformidad con cualquiera de las reivindicaciones 1 a 17,

caracterizado porque una o más células se cultivan en al menos 100 L del medio de cultivo suplementado a base de células basales.

19. El método de conformidad con cualquiera de las reivindicaciones 1 a 18, caracterizado porque la densidad celular se mantiene a menos de 2.5×10^6 células por mL durante la etapa de cultivar una o más células.

20. El método de conformidad con la reivindicación 19, caracterizado porque la densidad celular se mantiene a menos de 2.0×10^6 células por mL durante la etapa de cultivar una o más células.

21. El método de conformidad con la reivindicación 19, caracterizado porque la densidad celular se mantiene a menos de 1.5×10^6 células por mL durante la etapa de cultivar una o más células.

22. El método de conformidad con cualquiera de las reivindicaciones 1 a 21, caracterizado porque la etapa de recuperar al menos una porción del sobrenadante del cultivo comprende filtración o centrifugación para remover las células de la porción del sobrenadante del cultivo.

23. El método de conformidad con cualquiera de las reivindicaciones 1 a 22, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 40 mU/ μ g del rVWF.

24. El método de conformidad con la reivindicación 23, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 50 mU/ μ g del rVWF.

25. El método de conformidad con la reivindicación 23, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 60 mU/ μ g del rVWF.

26. El método de conformidad con la reivindicación 23, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 70 mU/ μ g del rVWF.

27. El método de conformidad con la reivindicación 23, caracterizado porque el sobrenadante recuperado tiene una actividad del cofactor de ristocetina específico del rVWF de al menos 80 mU/ μ g del rVWF.

28. El método de conformidad con cualquiera de las reivindicaciones 1 a 27, caracterizado porque al menos 10% del rVWF en el sobrenadante está presente en un multímero del VWF de alto peso molecular de más de 10 dímeros.

29. El método de conformidad con cualquiera de las reivindicaciones 1 a 27, caracterizado porque al menos 20% del rVWF en el sobrenadante está presente en un multímero del VWF de alto peso molecular de más de 10 dímeros.

30. El método de conformidad con cualquiera de las reivindicaciones 1 a 27, caracterizado porque al menos 30% del rVWF en el sobrenadante está presente en un multímero del VWF de alto peso molecular de más de 10 dímeros.

31. El método de conformidad con cualquiera de las reivindicaciones 1 a 30, caracterizado porque el sobrenadante contiene multímero del VWF de alto peso molecular de 14 a 22 dímeros.

32. El método de conformidad con la reivindicación 1, caracterizado porque el contenido de NH_4^+ del sobrenadante de cultivo celular se mantiene a una concentración por debajo de 4 mM

33. El método de conformidad con cualquiera de las reivindicaciones 1 a 32, caracterizado porque el rVWF es co-expresado con el Factor VIII recombinante (rFVIII).

34. El método de conformidad con la reivindicación 33, caracterizado porque además comprende una etapa de purificación del rVWF lejos de al menos 50% del rFVIII presente en el sobrenadante recuperado.

35. El método de conformidad con la reivindicación 34, caracterizado porque la relación del rVWF a rFVIII después de la etapa de purificación es al menos 10:1.

36. El método de conformidad con cualquiera de las reivindicaciones 1 a 35, caracterizado porque el método además comprende una etapa de enriquecimiento del rVWF.

37. Un sobrenadante de cultivo celular que comprende el Factor Von Willebrand recombinante (rVWF), caracterizado porque el sobrenadante se prepara por un método de conformidad con cualquiera de las reivindicaciones precedentes.

38. Un sobrenadante de cultivo celular que comprende el Factor Von Willebrand recombinante (rVWF), caracterizado porque al menos 20% del rVWF en el sobrenadante está presente en un multímero del VWF de alto peso molecular de más de 10 dímeros.

39. Un sobrenadante de cultivo celular que comprende el Factor Von Willebrand recombinante (rVWF), caracterizado porque el sobrenadante contiene al menos 0.5 IU de actividad del cofactor de ristocetina por mL.

40. Una composición del Factor Von Willebrand recombinante (rVWF) caracterizada porque se prepara por un método de conformidad con cualquiera de las reivindicaciones 1 a 36.

41. La composición de conformidad con la reivindicación 40, caracterizada porque la composición además comprende el Factor VIII recombinante (rFVIII).

42. La composición de conformidad con la reivindicación 41, caracterizada porque la relación del rVWF a rFVIII es al menos 10:1.

Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine

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The formulation of the culture medium for a Chinese hamster ovary (CHO) cell line has been investigated in terms of the simultaneous replacement of glucose and glutamine, the most commonly employed carbon and nitrogen sources, pursuing the objective of achieving a more efficient use of these compounds, simultaneously avoiding the accumulation of lactate and ammonium in the medium. The key factor in this process is the selection of compounds that are slowly metabolized. Among the different compounds studied, galactose and glutamate provide the best results, allowing support of cell growth with an optimal balance between nutrient uptake and cell requirements and the generation of minimal quantities of lactate and ammonium. The attained results also highlight the capacity of the cells to redistribute their metabolism as a response to the changes in medium composition.

Introduction

The formulation of optimal culture media for animal cell growth has received a lot of interest for many years as a result of its critical impact on the development of bioprocesses based on animal cell technology (Sato, 1975; Kovar and Franek, 1986; Castro et al., 1992; Keen and Rapson, 1995; Lao and Shalla, 1996). Undoubtedly, the replacement of fetal calf serum is the most noteworthy advancement, particularly if it can be achieved by media containing low protein concentration or media that are completely protein-free. When this is achieved, the process can be operated with a medium that is well defined, that is, with constant quality and composition, and this has a major impact in terms of process reproducibility, downstream processing, and sometimes in the medium cost. Also, from the point of view of regulation and acceptability of the final product, especially if it has a therapeutic use, it will be an increasingly important requisite to grow the cells in media without any components originated from an animal source, such as fetal calf serum. All of these reasons have generated a lot of interest, and various successful formulations have been developed, leading to a number of protein-free and serum-free media that are commercially available today. In addition to these efforts, the reformulation of media with different types of compounds, such as vitamins, growth factors, hormones, lipids, trace elements, or amino acids, has been addressed. An important criterion in the development of new media is, in addition to the supplementation of all the necessary nutrients, to do it in a balanced way, avoiding an unnecessary increase in the osmolarity of the medium and also in the generation of metabolic waste products (Castro et al., 1992; Xie and Wang, 1994; Lao and Shalla, 1996).

In this effort for medium reformulation, it has been generally accepted that the compounds to use in the medium as main carbon and energy sources are glucose and glutamine. However, the rapid glucose and glutamine metabolism that animal cells exhibit when they are cultured in vitro leads to a very inefficient use, causing

their rapid depletion from the culture medium and eventually the accumulation of lactate and ammonium ions in the medium. These products often have inhibiting effects for the cells, specially in the case of ammonium (Hansen and Emborg, 1994; Ozturk et al., 1992; Ryll et al., 1994). It has previously been demonstrated in chemostat cultures (Sanfeliu et al., 1997) that cells cultured in media with high glucose and glutamine concentrations exhibit much higher uptake rates than those strictly necessary for their metabolism. The solution to this problem has been addressed in many different ways. From the macroscopic point of view, that is, the definition of the extracellular conditions in the bioreactor where cell growth takes place, the fed-batch addition of glucose and glutamine to the medium (Ljunggren and Häggström, 1994; Xie and Wang, 1994; Bibila and Robinson, 1995; Zhou et al., 1995; Zhou et al., 1997) or the in situ removal of the inhibitory compounds (Matsumara and Nayve, 1995) are two possible strategies that generally require a high degree of instrumentation to be performed in an optimal way. From the microscopic point of view, which is at the cellular level, one of the findings to highlight is the modification of hybridoma and myeloma cells with the glutamine synthetase gene (Bibila and Robinson, 1995; Zhou et al., 1997; Paredes et al., 1999). In this way, cells can grow in medium with glutamine being replaced by glutamate, which is consumed in a more efficient way, producing a lower ammonium accumulation and allowing the extension of fed-batch cultures for a longer time. In this case it is interesting to point out that the glucose consumption and lactate accumulation is also reduced, showing how glutaminolysis and glycolysis pathways are interconnected in the metabolism of these cells. An intermediate possibility to improve cell growth patterns is to find alternative compounds for the cells to use as carbon and energy source, in theory without the need to undertake any genetic modification. The basic requirements for this substitution would be, first, that cells could grow in the new conditions, and second, that the metabolic uptake of the new compounds would be markedly

lower than glucose and glutamine uptake. In this way, it could be envisaged that the cells would use the main compounds of the medium more efficiently, generating a smaller amount of metabolic waste products, particularly lactate and ammonium, and therefore allowing the establishment of fed-batch or high-density perfusion cultures without inhibition limits.

The simultaneous substitution of both glucose and glutamine in the medium formulations has not been addressed previously, and only partial substitutions have been discussed, although in most cases not as a result of an analysis at the process level, linked to the metabolic patterns of the cells. Indeed, for some cells, particularly for hybridomas, it has been shown that this substitution is not possible (Wagner, 1997), at least not without genetic modification of the cells, for example, with the glutamine synthetase approach already discussed. For other cell lines, more studies have been reported. Wagner et al. (Wagner et al., 1991) showed that human tumor kidney cells could be grown on microcarriers, with slower cell death, using a mixture of glucose and galactose. Kurano et al. (Kurano et al., 1990), after discussing the inhibitory levels of ammonium and lactate, replaced glutamine by asparagine for the culture of CHO cells, obtaining a clear reduction in ammonium accumulation and the improvement of cell growth. These results were also confirmed by the work of Hansen and Emborg (Hansen and Emborg, 1994). Reitzer et al. (1979) in their very interesting discussion on the identification of glutamine as the major energy source for HeLa cells showed that cells can grow in a similar way on galactose or fructose as the main sugar in the medium instead of glucose, providing remarkably lower amounts of lactate. Marquis et al. (1996) provided data on the growth of a lymphoblastoid cell line on a medium with glucose replacement by galactose, with lower lactate production.

In addition to the changes in the primary metabolism that the substitution of glucose and glutamine would generate in the cells, it is also very interesting to study other metabolic modifications, for example, at the level of amino acids consumption or excretion, to generate hypotheses about the overall changes introduced in the cell behavior. In the present work, the substitution of glucose and glutamine in a low-protein medium for the culture of a Chinese hamster ovary (CHO) cell line producing t-PA in batch systems is analyzed from the point of view of the physiological changes observed in the cells and their internal metabolic redistribution. This substitution has been addressed by selecting a slowly metabolized sugar compound to replace glucose as carbon source. This compound should be a hexose sugar, as the cells used (a CHO cell line) do not perform gluconeogenesis activity. The entry of the sugar molecule into the glycolytic pathway should therefore be upstream of the metabolic step generating phosphogluconate, which in turn is essential to generate pentose molecules for the nucleic acids synthesis. With regards to glutamine, the objective of this work is to investigate its replacement by glutamate, as CHO cells do have the glutamine synthetase activity expressed constitutively. In addition to the fact that glutamate has only one amino group in its structure, the fact that glutamate is transported into the cell at a lower velocity should provide a more efficient use of this compound with lower generation of ammonium, because spontaneous decomposition would also then be minimized, with respect to the situation where glutamine is used.

Material and Methods

Cell Culture. The cell line CHO TF 70R, kindly provided by Pharmacia & Upjohn (Stockholm, Sweden), has been manipulated genetically to produce t-PA. The cell cultures were carried out in spinner flasks (Techne) with a working volume of 125 mL (or 250 mL) and stirred at 50 rpm in a CO₂ incubator (Forma Scientific CO₂ incubator), at 37.0 °C, with 96% relative humidity in an atmosphere of 5% CO₂ in air.

Culture Medium. The basal medium used in all of the cultures was a proprietary serum-free and low-protein medium denominated BIOPRO1, kindly provided by Bio Whitaker Europe (Verviers, Belgium). BIOPRO1 was supplemented with vitamins (Sigma), lipids and cholesterol (GibcoBRL), proline, serine, and aspartic acid (Sigma). This medium was also supplemented with 20 mM glucose, fructose, mannose, or galactose and 7 mM glutamine or glutamic acid in the different experiments, as indicated in Figures 1 and 3. The culture of the cells in the different media was carried out without previous adaptation. Cells used for inoculum ($0.18\text{--}0.20 \times 10^6$ cells/mL) were centrifuged to remove the culture medium, particularly to ensure that neither remaining glucose nor glutamine from inoculum preparation medium was present. This step was repeated 3–5 times, keeping the cells subcultured in the corresponding carbon and nitrogen source combination under study, before the realization of the experiment. In those passages the growth of the cells was very reproducible.

Analytical Methods. Cell Number. Cells were counted using a hemacytometer (Neubauer improved, Brand). Cell viability was determined by the trypan blue exclusion method (1:1 mixture of 0.2% trypan blue in normal saline and cell sample). After cell counting, the remainder of each sample was centrifuged (5000g, 1 min) to remove the cells, and the supernatant was frozen for further analysis.

Metabolite Determinations. Glucose and lactate concentrations were determined with a YSI 2700 automated glucose and L-lactate analyzer. Ammonium concentration was determined by a flow injection analysis system as previously described (Campmajó, 1994). Amino acids concentrations were measured by HPLC (Hewlett-Packard 1090) using a reversed-phase column (aminoquant 200 mm $\times$ 2.1 mm) after derivatization of the samples with *O*-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) for the analysis of both primary and secondary amino acids. The concentration of t-PA was quantified by enzyme immunoassay (Biopool Imulysse t-PA kit, Diagnostic International, Hannover, Germany).

Specific Rate Calculations. The specific growth rate (μ) in the exponential growth phase was calculated by linear regression of the logarithm of the concentration of viable cells, X_v , as a function of time, t , (eq 1)

$$X_v = X_{v0} \exp(\mu t) \quad (1)$$

In the above expression, X_{v0} is the initial cells number (10^6 cell mL⁻¹). The specific consumption/production rates, q , in nmol h⁻¹ 10⁻⁶ cells, were calculated using the following expressions

$$S = S_0 + \frac{q_s 10^{-3} X_{v0}}{\mu} [\exp(\mu t) - 1] \quad (2)$$

where S is the concentration of the different compounds

investigated (carbon and nitrogen sources, and products) and S_0 refers to its initial concentration (mM).

In the case of glutamine, this spontaneously decomposes, releasing ammonia following first-order kinetics. Its rate constant, k_d , has been evaluated in the experimental conditions used in this work, obtaining a value of $2.01 \times 10^{-3} \text{ h}^{-1}$. Thus equations for glutamine and ammonium were modified conveniently to take into account this fact:

$$\text{Gln} = \text{Gln}_0 \exp(-k_d t) + \frac{q_{\text{Gln}} 10^{-3} X_{v0}}{k_d + \mu} [\exp(\mu t) - \exp(-k_d t)] \quad (3)$$

$$\text{NH}_4^+ = \text{NH}_4^+_0 + \frac{X_{v0} 10^{-3}}{\mu} \left[q_{\text{NH}_4^+} + \frac{k_d q_{\text{Gln}}}{\mu + k_d} \right] [\exp(\mu t) - 1] + \left[\text{Gln}_0 - \frac{q_{\text{Gln}} 10^{-3} X_{v0}}{\mu + k_d} \right] [1 - \exp(-k_d t)] \quad (4)$$

Results and Discussion

Substitution of Glucose by a Hexose. In a first series of batch experiments, the glucose substitution in the formulation of the culture medium was investigated, with glutamine maintained in all cases as nitrogen source. Three different compounds were tested: fructose, galactose, and mannose. Glucose was used as the control system. The results are presented in Figure 1. It can be observed (Figure 1a) that the maximal cell concentration achieved for glucose and mannose is similar, reaching a level of 1.2×10^6 cells/mL, whereas for galactose and fructose it is lower, 0.8×10^6 and 0.5×10^6 cells/mL, respectively. The specific growth rate on glucose and mannose was almost the same, as reflected in Table 1, and higher than for galactose and fructose. With respect to the consumption of the carbon source (Figure 1c), two groups of compounds can be distinguished, with marked differences: glucose and mannose are consumed at a high rate, whereas fructose and galactose are consumed much more slowly. The specific consumption rates are provided in Table 1, and it should be highlighted that the values observed for the consumption of fructose and galactose are around 25% of that for glucose. As a consequence of these different consumption rates, the generation of lactate also differs greatly. For glucose and mannose, the high consumption rate leads to high lactate generation, and lactate concentration reaches levels of about 16 mM (Figure 1d), with high values for the specific production rate (see Table 1). On the other hand, when cells are grown on fructose and galactose, the lactate production decreases drastically, and final lactate concentrations are below 1 mM, the specific lactate production rates being about 22% of the values corresponding to glucose. When the glutamine consumption is analyzed (Figure 1e) a similar profile is observed for all of the cultures; however, taking into account the different level of cells generated, it can be calculated that the specific consumption of glutamine is notably higher when galactose and fructose are used, as reflected in Table 1. It can be observed that in the case of the medium formulation with galactose, an abrupt cell concentration decline occurs at the point when glutamine is exhausted. These profiles are linked to those corresponding to the ammonium ion generation in the medium (Figure 1f), which are higher for the compounds providing a higher specific consumption of glutamine (fructose and galactose), reaching levels of 13 mM for ammonium, and lower when glucose or mannose

are used, reaching 9 mM for ammonium. Finally it should be mentioned that the profile of the t-PA concentration follows that of the cell growth (Figure 1b), and therefore a higher t-PA level is obtained when a higher cell concentration is generated.

The analysis of the previous results from the metabolic approach can be done at different levels. The scheme of the most relevant metabolic pathways for the metabolism of sugar compounds and glutamine in animal cells is presented in Figure 2. It can be observed that mannose enters into the glycolytic pathway at the level of fructose-6-P, whereas galactose does so at the level of glucose-6-P. Nevertheless, mannose is consumed at a much faster speed than fructose, with a rate similar to that for glucose.

The observation of the experimental data suggests some hypotheses with respect to the transport of these compounds into the cells. Mannose and glucose seem to be transported at the same velocity into the cell, either through the same transporter or a different one. On the other hand, fructose is probably transported more slowly into the cells, as a result of a lower affinity of the fructose transporter. With respect to galactose, in theory one might expect to find a consumption rate similar to glucose, as both sugar molecules are incorporated into the glycolysis at the level of glucose-6-P. However, the observed rate is considerably slower, and again this fact could be explained by a low transport rate of galactose into the cells. Indeed, it has been previously reported that, for several mammalian cell lines (Plagemann et al., 1981) galactose is taken up into the cells through the same transporter as glucose but with an affinity which is eight times lower. A second possibility that could explain the different consumption rate of the compounds studied is a lower affinity of the enzyme hexokinase for galactose and fructose (Wagner, 1997).

For the quickly metabolized sugars, glucose and mannose, the cell converts most of them into pyruvate through the glycolytic pathway, and probably a small percentage is cycled through the pentose phosphate cycle to obtain the necessary molecules to build compounds essential for the cell viability. It can also be observed from the experimental results that a high percentage of pyruvate is metabolized to lactate. The explanation for this fact may be found in the need for the cell to balance its redox potential (Paredes et al., 1998). It should also be mentioned that it has been previously demonstrated that this pattern has been observed in other cell lines, independently of the oxygen level in the culture system (Sanfeliu et al., 1996), and in consequence it cannot be attributed to a lack of oxygen.

It can be observed that for the sugars presenting a lower glycolytic rate, fructose and galactose, glutamine is consumed at a higher specific rate (see Table 1) and that for similar total consumption values (see Figure 1e), a higher ammonium yield is obtained. (see Figure 1f). The main metabolic intermediate through which glycolysis and glutaminolysis pathways are connected is pyruvate, which also is the key intermediate compound between glycolysis and the tricarboxylic acids cycle. Because of the low specific consumption rate for fructose and galactose, it should be assumed that, in this situation, most of the carbon is diverted to the pentoses synthesis and therefore only a small amount of pyruvate is generated (Reitzer et al., 1979). This fact leads to two consequences: first, the amount of lactate generated in the culture medium is minimal; second, the depletion of pyruvate would stop the TCA cycle, unless the cell could provide more pyruvate from glutaminolysis. One possible

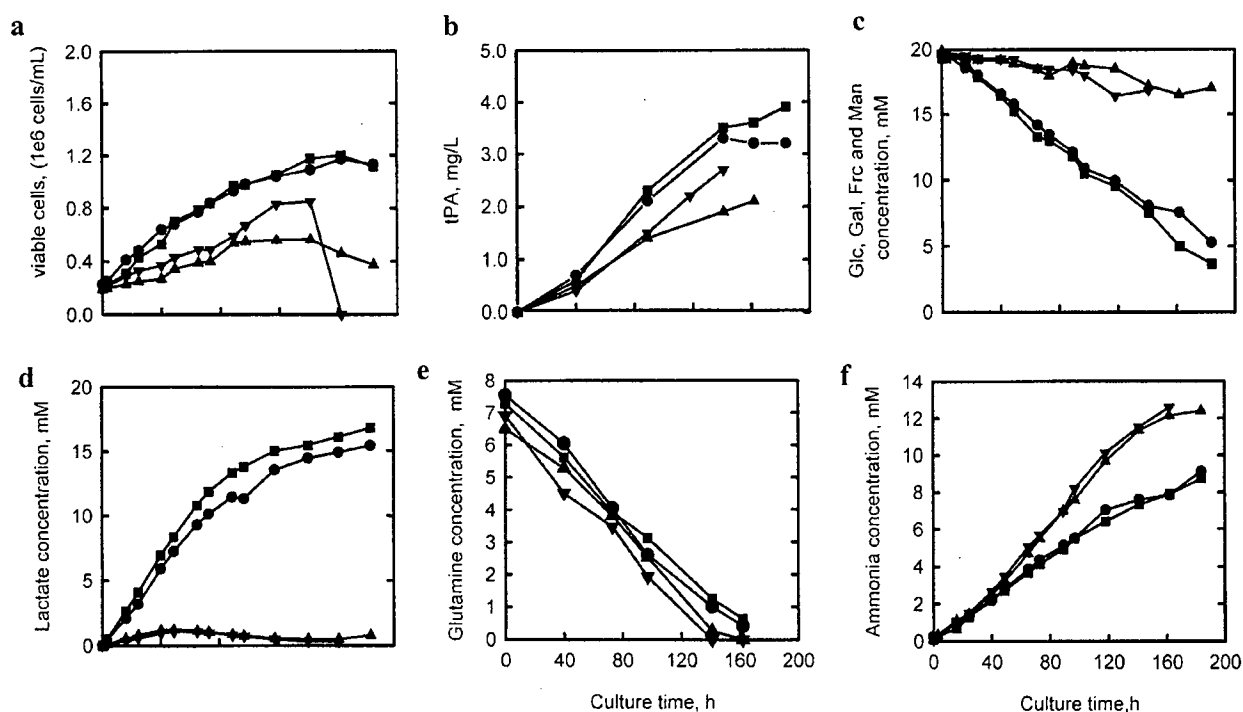


Figure 1. Results of the batch cultures of the CHO cells, using glutamine (7 mM) and four different sugar compounds in the medium formulation: 20 mM fructose (▲), 20 mM galactose (▼), 20 mM mannose (■), and 20 mM glucose (●) (control). (a) viable cells; (b) t-PA concentration; (c) sugar concentration; (d) lactate concentration; (e) glutamine concentration; and (f) ammonium concentration.

Table 1. Values of the Specific Cell Growth Rate and Specific Consumption Rates for the CHO Cell Line Growing on Different Carbon Sources

	glucose	fructose	mannose	galactose
μ (h ⁻¹)	0.0227	0.0109	0.0220	0.0153
q_c	-176.1	-48.7	-203.7	-48.0
q_{Lac}	336.2	78.8	364.7	61.9
q_{Gln}	-65.9	-103.3	-79.9	-115.0
q_{NH_4}	48.7	85.2	52.8	78.5

reaction that the cell can activate to compensate the low generation of pyruvate is that catalyzed by the malic enzyme (Sauer et al., 1980; Zielke et al., 1980), enabling the transformation of malate (that is then exported from TCA) into pyruvate (see Figure 2).

It is also of interest to discuss the differences encountered with respect to alanine production. Indeed, as already mentioned, although glutamine is consumed in almost the same total quantity for the four sugars studied, ammonium is produced in higher amounts when the sugar is consumed slowly (i.e., fructose and galactose), and at the same time alanine is produced in a much lower quantity than for the culture on glucose or mannose (level of alanine at 180 h of culture, 0.7 mM for galactose and fructose and 3.0 mM for glucose and mannose). Alanine is normally obtained in cell culture as a product of the transamination reaction between pyruvate and glutamate. The reaction also produces α -ketoglutarate, and generally this is the most common way to obtain this compound in cells that are proliferating rapidly, simultaneously reducing the ammonium generation (Matsuno et al., 1994). The low alanine production that is observed for the slowly metabolizable sugars should probably be linked to the low availability of pyruvate in the cell due to the slow glycolysis. In that situation, most of the pyruvate provided by the low glycolytic flux must be incorporated by the cells into the TCA cycle, and the transamination to alanine is then less favored. Alternatively, the generation

of α -ketoglutarate is provided by the action of the enzyme glutamate dehydrogenase.

Finally, it should be pointed out that glycine excretion is also changing with the carbon source used. For sugars rapidly metabolized, a lower glycine production is observed when compared to slowly metabolized sugars (level of glycine at 180 h of culture, 1.2 mM and 2.5 mM, respectively). Glycine is related to pyruvate metabolism.

Replacement of Glutamine by Glutamate. In the second series of experiments of this work, the same approach was followed to substitute glutamine in the culture medium, with the main objective of obtaining a more efficient use of the nitrogen source, with a lower release of ammonium ions. In this case, taking into account the previous results published in this respect (Hansen and Emborg, 1994; Ozturk et al., 1992; Ryll et al., 1994) and considering the glutamine metabolism (see Figure 2), glutamate was selected directly as the compound to be used, for three main advantages: first, glutamate does not suffer spontaneous decomposition as does glutamine (ammonium ions will not be generated as a result); second, glutamate can be obtained directly from glutamate by the CHO cells, as they exhibit glutamine synthetase activity; and third, glutamate has only one amino group. With respect to the carbon source used in this second series of experiments, in view of the results presented in the previous section, it was decided to select galactose, as a slowly metabolizable sugar, and glucose as the control system. The results of the four experiments carried out, using glutamine and glutamate as nitrogen sources and glucose and galactose as carbon sources, are presented in Figure 3.

In Figure 3a it can be observed that the final cell density attained growing on glucose-glutamate is the highest, being more than two times the concentration attained with the combination galactose-glutamate (1.8×10^6 and 0.8×10^6 cells/mL respectively), and also notably higher than the final cell concentration for the

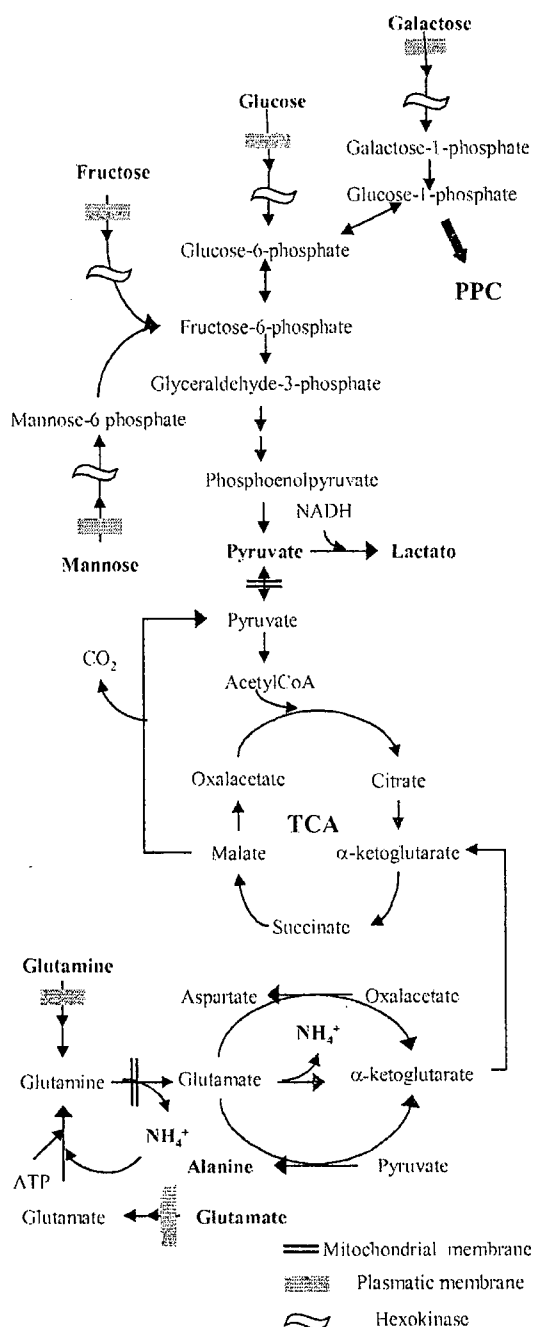


Figure 2. Scheme of the most relevant metabolic pathways for the metabolism of sugar compounds and glutamine in animal cells.

control culture, glucose–glutamine. The cell profiles for the growth on galactose–glutamine and galactose–glutamine are quite similar, with the main difference being that the abrupt drop in the cell concentration observed for galactose–glutamine is not observed for galactose–glutamine, probably because of the very much lower ammonium level generated, as will be discussed below, and also because glutamine is exhausted more rapidly in the first case, that of galactose–glutamine formulation. Regarding t-PA concentration profiles, as in the previous section, there is a clear proportionality between cell growth and product formation.

Figure 3c reflects the profiles for the consumption of the carbon source. It can be observed that galactose is consumed more slowly than glucose, as discussed in the

previous section, and also that in the presence of glutamate, the consumption of the carbon source is always lower than in the presence of glutamine, both for glucose and galactose, as reflected in Table 2. As previously mentioned this fact highlights the relationship existing between glycolysis and glutaminolysis. When the glutaminolysis rate is decreased by the replacement of glutamine by glutamate, the cells possibly have a lower need for pyruvate to be incorporated into the TCA cycle, and therefore the glycolysis rate is also reduced. This behavior is also found in other cell lines; the most evident example is for hybridoma cells modified genetically with the introduction of the glutamine synthetase gene. These transformed cells can then be grown on medium with glutamine being replaced by glutamate, and the pattern observed is a lower glutamate consumption rate, together with a decrease of about 50% in the consumption of glucose (Bibila and Robinson, 1995; Paredes et al., 1999).

With respect to the generation of lactate, Figure 3d shows that it is minimal for the media formulated with galactose, whereas for the media containing glucose, a clear difference can be observed between the control culture, glucose–glutamine (16 mM of lactate) and the experiment with glucose–glutamate (7 mM), clearly as a consequence of the lower consumption rate for glucose when glutamine is replaced by glutamate.

The data for the consumption of the nitrogen source are presented in Figure 1e. The trends for the consumption of glutamine and glutamate can clearly be distinguished, the later being a compound more slowly metabolized by the cells. The specific uptake rate values for the different combinations of media formulation are given in Table 2. The most relevant consequence of this different uptake profile is the ammonium generation. Indeed, it is markedly lower when glutamate is used instead of glutamine. It is also interesting to appreciate that the carbon source also affects, although to a minor extent, the ammonium generation, the general trend being that for any of the two nitrogen sources, ammonium concentration always reaches higher values for galactose than for glucose. Again, this fact also reflects the flexibility of the metabolic network. When the cells are cultured in galactose, the low uptake rate is most probably the limiting factor for cell metabolism, and it is compensated by an increase of the nitrogen source uptake (either glutamate or glutamine), generating a higher amount of ammonium. When glutamate is used in the medium, the alanine production decreases for any of the sugars tested (level of alanine at 180 h, glucose–glutamate culture, 0.7 mM; and galactose–glutamate culture, 0.1 mM) probably as a consequence of a lower availability of the key intermediate, pyruvate, which is transaminated to alanine and α-ketoglutarate in a much lower extent. On the other hand, the glycine production reaches similar levels to those obtained when glutamine is used, independently of the carbon source used (glucose or galactose).

Conclusions

The results presented demonstrate the possibility of regulating the metabolic pattern of CHO cells by substitution of the most commonly used main compounds in the medium formulation, glucose and glutamine, by other compounds that are taken up at significantly lower rates. As most of these compounds are incorporated to the central metabolism, it is most probable that transport rate into the cell is the major step controlling this process; or alternatively it could also be due to the different affinity of the hexokinase enzyme for the different

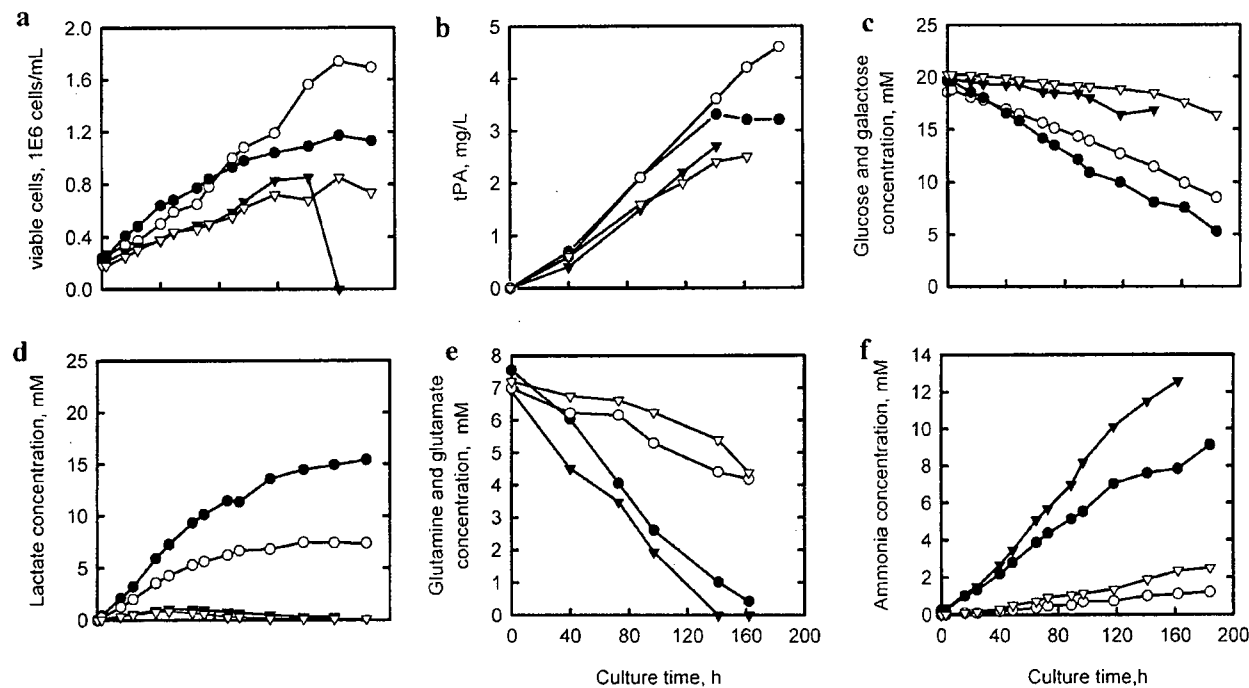


Figure 3. Results of the batch cultures of the CHO cells, using glutamine (7 mM) or glutamate (7 mM) as nitrogen sources and galactose (20 mM) or glucose (20 mM) as carbon sources in the medium formulation: galactose–glutamine (▼), galactose–glutamate (▽), glucose–glutamate (○), and glucose–glutamine (●) (control). (a) viable cells; (b) t-PA concentration; (c) sugar concentration; (d) lactate concentration; (e) glutamine concentration; and (f) ammonium concentration.

Table 2. Values of the Specific Cell Growth Rate and Specific Consumption Rates for the CHO Cell Line Growing on Different Nitrogen Sources

	glucose/ glutamine	glucose/ glutamate	galactose/ glutamine	galactose/ glutamate
μ	0.0227	0.0186	0.0160	0.0160
q_C	-176.1	-113.7	-48.0	-37.5
q_{Lac}	326.2	209.3	61.9	47.0
q_{Gln}	-65.0		-115.0	
q_{Glu}	9.7	-32.5	15.3	-37.5
q_{NH_4}	48.7	13.2	78.5	25.0

compounds. From the different possibilities studied, the combination of galactose and glutamate allows the maintenance of cells at an optimal situation with regard to minimal consumption of nutrients and minimal generation of lactate and ammonium, although a lower cell growth is obtained. Alternatively, glucose and glutamate allow the obtaining of maximized cell growth, significantly higher than the control culture with glucose and glutamine, with very low ammonium accumulation and reduced lactate levels. These conditions offer the possibility for optimization of fed-batch cultures, as it will be possible to expand the time span of the cultures because the limiting conditions in terms of osmolarity and toxic compound accumulation will occur later than in the conventional medium formulation with glucose and glutamine.

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IN SITU REMOVAL OF AMMONIUM IONS FROM HYBRIDOMA CELL CULTURE MEDIA: SELECTION OF ADSORBENT

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ABSTRACT

Various ion-exchange resins and zeolite Phillipsite-Gismondine were tested for their potential application as adsorbents for the *in situ* removal of ammonium ions from animal cell culture media in attempting to increase hybridoma cell density and MAb productivity. A zeolite, Phillipsite-Gismondine demonstrated the best performance in terms of NH_4^+ adsorption capacity, selectivity and autoclavability among the various adsorbents tested. The biocompatibility of Phillipsite-Gismondine was also tested and the result showed improvement in cell density, viability, and antibody productivity.

INTRODUCTION

One of the most challenging problems in animal cell culture is the accumulation of waste metabolites because these metabolites such as NH_4^+ and lactate have been shown to inhibit both cell growth and product formation (Glacken *et al.*, 1988; Glacken, 1988; Miller *et al.*, 1988). The reactor productivity can be dramatically increased by reducing the concentration of those inhibitory waste metabolites as they are formed during cultivation of cells.

Mammalian cells produce NH_4^+ and lactate as waste metabolites of glutamine and glucose catabolism. In a culture with good pH control, ammonium ions are the most detrimental to cell growth (Jeong and Wang, 1990; Miller *et al.*, 1988).

The critical inhibitory concentrations of NH_4^+ for strain L mouse cells, chorioallantoic tissue, mouse ascites tumor cells, canine kidney cells (MDCK), and hybridoma cells have been reported to be 0.5 mM, 10 mM, 40 mM, 5-8 mM, and 3-5 mM respectively (Glacken, 1987). Furthermore, NH_4^+ also inhibits product formation. For example, interferon production by human fibroblast cells is inhibited by 50 % at 2 mM of NH_4^+ (Ito and McLimans, 1981). Vaccine production can also be inhibited by NH_4^+ . The growth of influenza (Jensen and Liu, 1961), Newcastle Disease (Eaton and Scala, 1961), and Columbia SK (Furusawa and Cutting, 1952) viruses were also reported to be inhibited by NH_4^+ .

It is important, therefore, to develop a method for removing NH_4^+ from the media in order to increase the productivities of bioreactors (Glacken, 1988). The simplest and the most promising method for the *in situ* removal of NH_4^+ from the cell culture media is by adsorption (Iio *et al.*, 1985; Jeong and Wang, 1990). To do so, it is most critical to select an adsorbent which is autoclavable, non-toxic and which has high adsorption capacity and high selectivity for NH_4^+ . Several tests were performed to select a good adsorbent based on the requirements mentioned above. *In situ* removal of NH_4^+ from the cell culture media with the selected adsorbent was performed, and the system performances are discussed in this report in terms of cell density, cell viability, and MAb productivity.

MATERIALS AND METHODS

CELL LINE

The mouse-mouse hybridoma cell line VIII H-8 used in this study was kindly given by New Brunswick Sci. Co.. These cells produce mouse immunoglobulin, IgG_{2a} which is specific for the whole cell of bacterium *Rhizobium japonicum* NR-7.

CULTURE MEDIA AND CELL PROPAGATION

The cells were maintained in DMEM (Dulbecco's Modification of Eagle's Medium) containing 4.5 g/l of glucose, supplemented with 0.94 g/l of NCTC 135 (National Cancer Tissue Culture), oxaloacetate (150 mg/l), insulin (75.5 µg/l), sodium bicarbonate (40 mM), streptomycin (100 µg/ml), penicillin (100 U/ml), and 5 % (v/v) calf bovine serum. The medium was filtered with 0.22 µm membrane.

Cells were cultivated in 75 cm² T-flask as a stationary culture and in 250 ml spinner flasks (Corning) of which working volume was 150 ml. T-flasks and spinner flasks were incubated at 37⁰ C in 7 % CO₂ atmosphere.

VIABLE CELL COUNTS

Cells were counted under microscopic examination after small amounts of sample was injected into a haemocytometer. Cell viability was determined by Trypan Blue exclusion method.

ANTIBODY ASSAY

The mouse immunoglobulin, IgG_{2a} was analyzed by using RID (Radial Immuno Diffusion) methods. Anti-IgG was immobilized in the agar on the RID plates. A centrifuged sample of 5 µl was injected into each sample hole on RID plates and incubated at 37⁰ C for 36 h. After incubation, ring was visualized by first staining with a buffaloback stain solution and then rinsed with RID wash solution (5% v/v glacial acetic acid, 0.5 % v/v glycerol).

AMMONIUM ION

Ammonium ion concentration was measured using an Orion 9512 ammonium ion electrode (Cambridge MA, U.S.A.).

ADSORPTION EXPERIMENTS

Ammonium ion was adsorbed by 2.5 g each of Amberlite CG-120, Amberlite CG-50, Phillipsite-Gismondine, and Sordolite CW-18 in 50 ml of NH₄⁺ solution (5 mM initial concentration) with 200 rpm rotary shaking for 12 hours at 37⁰ C. Another sets of adsorption tests were performed in water or culture media with adsorbents which were autoclaved at 121⁰ C for 15 minutes before testing. Equilibrium was assured by measuring several NH₄⁺ concentrations until the adsorption kinetic curve leveled off.

RESULTS AND DISCUSSIONS

AMMONIUM ION TOXICITY

Ammonium chloride was added into culture medium to vary initial NH₄⁺ concentration. The cell growth and the concentration of monoclonal antibody produced in the T-flask were analyzed at different intervals. The effect of initial NH₄⁺ level on the specific cell growth rate is shown on Fig.1 and that on the monoclonal antibody production kinetics on Fig.2. Both specific growth rate and the highest accumulated monoclonal antibody concentration decrease as initial NH₄⁺ concentration increases, demonstrating NH₄⁺ toxicity to the hybridoma cell line tested. This NH₄⁺ toxicity could be caused by cytoplasmic acidification resulted from the uptake of the weak acid, NH₄⁺, into cytoplasm (McQueen and Bailey, 1990).

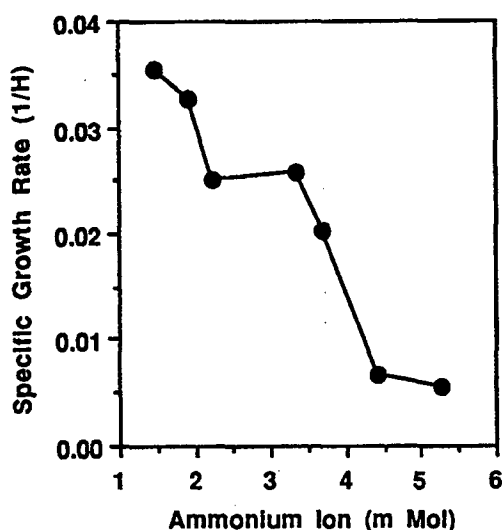


Fig.1 Effect of Ammonium Ion on Specific Growth Rate

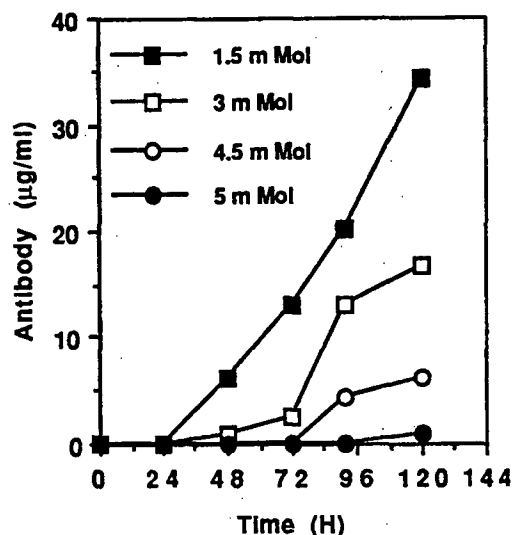


Fig.2 Effect of Initial Ammonium Ion on Antibody Production

SELECTION OF ADSORBENT

Not only high capacity and selectivity for NH_4^+ adsorption but also autoclavability and non-toxicity to hybridoma cells are required for the adsorbents to be used in the *in situ* removal of NH_4^+ in the hybridoma cell culture system. Four adsorbents were tested. Amberlite CG-120 is a strongly acidic cation exchanger, a styrene-divinylbenzene copolymer with a sulfonic group. Amberlite CG-50 is a styrene-divinylbenzene copolymer with a weak acidic carboxyl group. Serdolite CW-18 is a weak acidic cation exchanger with carboxylic group on the basis of polyacrylic acid. Phillipsite-Gismondine is a synthetic zeolite which has an identical structure to natural zeolite 'Merlinolite' (Passaglia *et al.*, 1977).

Fig.3 represents the studies of adsorption capacity and autoclavability of various adsorbents in NH_4^+ solution (initial 5 mM in water). In order to keep the culture system aseptic, adsorbents need to be autoclaved before applying to the media. It is desirable for an adsorbent not to lose its adsorptive capacity after autoclaving. However CG-120, CG-50, and Serdolite exhibit slight decrease in adsorption capacity after autoclaving, implying that their pore structures or functional groups are probably modified by heat. For example, thermal stress will cause the decomposition of sulfonic acid group of CG-120. On the other hand, Phillipsite-Gismondine demonstrates invariant capacity, indicating that the structure of Phillipsite-Gismondine is thermally stable because of its crystalline structure (Passaglia *et al.*, 1977). Therefore, Phillipsite-Gismondine exhibits best performance in autoclavability. In this figure, Phillipsite-Gismondine also shows the highest NH_4^+ adsorption capacity among the adsorbents tested. Fig.4 shows the final NH_4^+ concentration reached after treating 5 mM NH_4^+ solution with various adsorbents. It demonstrates that Phillipsite-Gismondine can easily maintain very low NH_4^+ concentration because it can remove about 98 % of NH_4^+ initially presented under the experimental conditions.

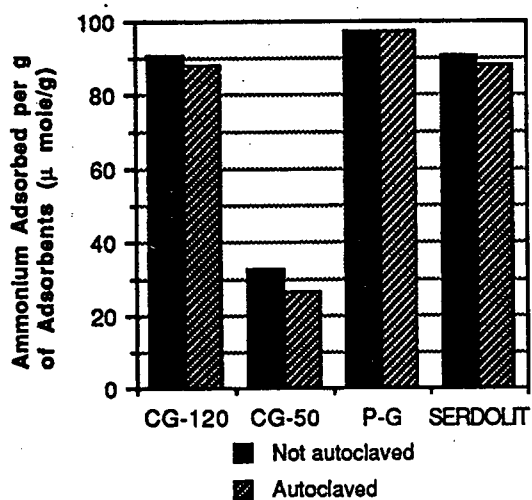


Fig.3 Ammonium Ion Adsorption Capacity Change after Autoclaving

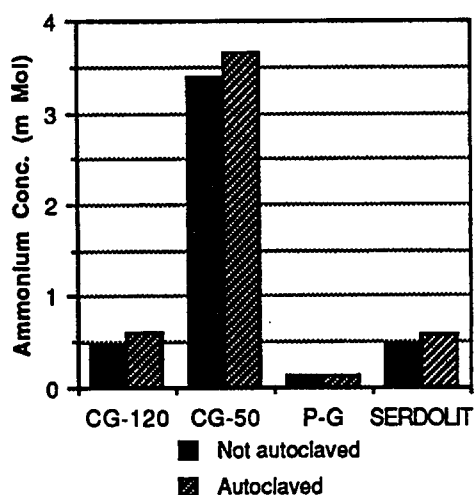


Fig.4 Final Ammonium Ion concentration

Fig.5 shows the studies of NH_4^+ selectivity of various adsorbents in typical animal cell culture media, DMEM and Joklik's medium. There are many kinds of NH_4^+ competitive ions such as Ca^{++} , Na^+ , Mg^{++} , etc., in most animal cell culture. Those competitors bind to the active site of adsorbent, so the available active sites for NH_4^+ adsorption can be easily reduced, consequently low selectivity for NH_4^+ would be expected. Eventhough CG-120 and Serdolite demonstrated relatively high NH_4^+ adsorption capacity in water which is comparable with that of Phillipsite-Gismondine as shown on Fig.3, they show respectively 58 % and 35 % decreases in NH_4^+ adsorption capacity in media. Whereas, Phillipsite-Gismondine shows only 9 % decrease in adsorption capacity. It may be because ion exchangers are usually very sensitive to bivalent ions such as Ca^{++} and Mg^{++} which are subject to strong electrostatic attraction, while zeolite can exclude those ions by size differentials in addition to electrostatic selectivity. Therefore Phillipsite-Gismondine shows the best NH_4^+ selectivity from the culture media of complex ions as well as the best NH_4^+ adsorbing capacity among the adsorbents tested.

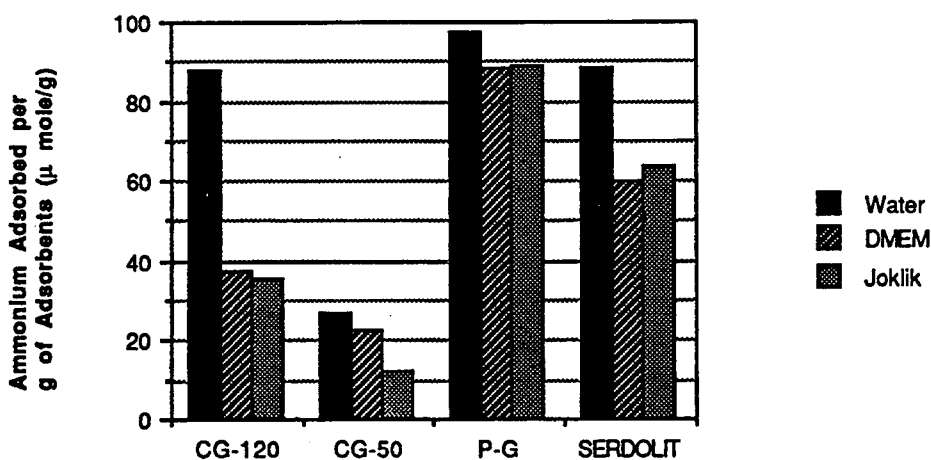


Fig.5 Ammonium Ion Selectivity of Various Adsorbents in Culture Media

BIOCOMPATIBILITY AND IN-SITU REMOVAL OF AMMONIUM ION

Fig.6 shows the result of live cell growth kinetics of hybridoma cells with and without adding zeolite powder in the media. Hybridoma cells were cultured with 0.75 g of Phillipsite-Gismondine powder in a spinner flask of 150 ml working volume at 60 rpm. Fig.7 shows cell viability change and Fig.8 shows NH_4^+ concentration change with respect to time in this run. Applying Phillipsite-Gismondine powder results in 15 % increase in live cell number and 28 % increase in viability after 154 hours. At the same time the difference in NH_4^+ concentration between two spinners becomes significant as shown on Fig.8. So, *in situ* removal of NH_4^+ by Phillipsite-Gismondine can stimulate cell growth and this material is not toxic and it is compatible with hybridoma cells tested. According to Fig.8, the final NH_4^+ concentration of culture with adsorbent is only half of that without adsorbent, demonstrating that Phillipsite-Gismondine can reduce NH_4^+ concentration to below toxic level in later phase of culture. Fig.9 shows the monoclonal antibody (IgG_{2a}) production kinetics in the same run. The enhancement in live cell growth due to maintenance of low NH_4^+ concentration results in 16 % increase in the concentration of monoclonal antibody. Therefore *in situ* removal of NH_4^+ by Phillipsite-Gismondine could stimulate not only cell growth but also monoclonal antibody production.

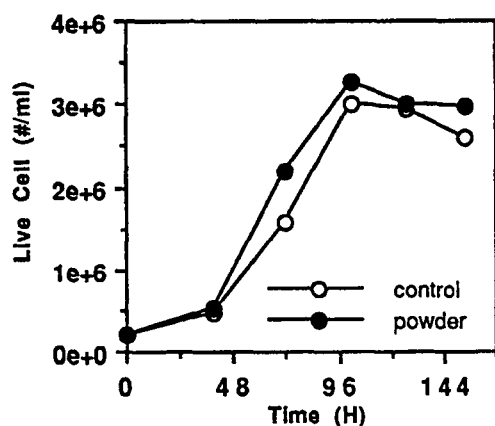


Fig.6 Live Cell Growth Kinetics

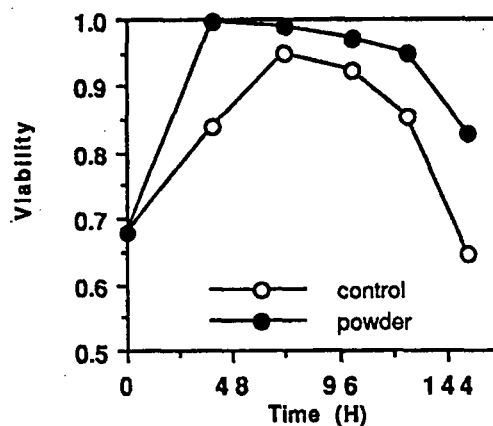


Fig.7 Cell Viability Kinetics

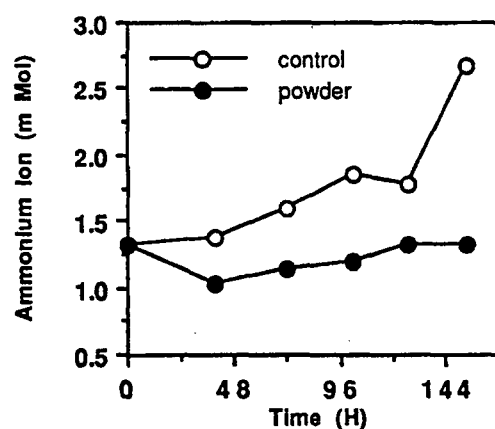


Fig.8 Ammonium Ion Concentration Kinetics

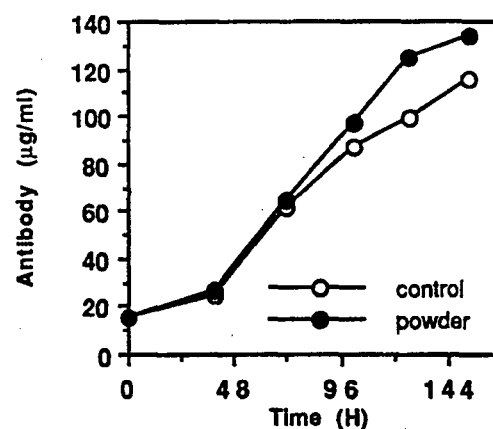


Fig.9 Monoclonal Antibody Kinetics

Although the last few graphs all show positive effects, it is also seen that the effects are rather moderate. This may be caused by following two reasons. The first reason is that the cells in the culture does not produce high enough NH_4^+ to raise up to a seriously toxic level. The final NH_4^+ concentration of culture without adsorbents is only 2.7 mM as shown on Fig.8. According to Fig.1, this level of NH_4^+ is estimated to cause only 30 % decrease in specific growth rate. Another reason is that the nutrient depletion in later phase hampers further increase in cell growth eventhough NH_4^+ accumulation problem has been overcome by adding adsorbents. Nutrient depletion is expected to be more serious in case of culture with adsorbent due to better cell growth and higher antibody production. Therefore, it is suggested that further increase in cell growth and antibody production can be accomplished by combining fed-batch (additional feeding of key nutrients such as glucose and glutamine) and *in situ* removal of NH_4^+ . The combined system which is free of nutrient depletion by fed-batch and free of NH_4^+ accumulation by adsorption showed 60 % increase of final cell density in another experiment. These results will be reported elsewhere very soon.

CONCLUSIONS

NH_4^+ is toxic to the growth of VIII H-8 cells and production of IgG_{2a}. Ammonium ion can be removed *in situ* by adding adsorbent directly to cell culture media. Phillipsite-Gismondine showed the best characteristics in NH_4^+ adsorption capacity, NH_4^+ selectivity, stability in adsorption capacity after autoclaving, and biocompatibility with hybridoma cells. Application of Phillipsite-Gismondine to actual culture system shows better cell growth, higher cell viability, higher antibody productivity and the ability of maintaining low NH_4^+ concentration throughout the culture period. Therefore, Phillipsite-Gismondine can be successfully used as an adsorbent for *in situ* removal of NH_4^+ from animal cell culture media as part of a comprehensive strategy to accomplish high cell density and high monoclonal antibody productivity.

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