Planung, Aufbau und Evaluation einer Membranadsorber-basierten Periodic-Counter-Current-Chromatographie (PCCC)-Anlage für die kontinuierliche Aufreinigung von Proteinen

Von der Naturwissenschaftlichen Fakultät der

Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktorin der Naturwissenschaften

(Dr. rer. nat.)

genehmigte Dissertation

von

Chantal Marie Brämer, M.Sc.

[2019]

Referent:	Prof. Dr. rer. nat. Thomas Scheper
Korreferent:	PD Dr. rer. nat. Sascha Beutel
Tag der Promotion:	18.12.2019

“*Remember: Your potential is endless*”

- unknown

Danksagung

Ich möchte mich ganz herzlich bei all denen bedanken, die mich während der Zeit meiner Promotion unterstützt haben.

Mein besonderer Dank gilt Herrn Prof. Dr. Thomas Scheper und PD Dr. Sascha Beutel. Danke für die Bereitstellung dieses spannenden Promotionsthemas und die Unterstützung während meiner gesamten Zeit am TCI. Ich bin sehr dankbar für die Möglichkeit der Promotion am TCI; es hätte nicht schöner sein können.

Herrn PD Dr. Ulrich Krings danke ich herzlich für die Übernahme des Prüfungsvorsitzes.

Ein großer Dank gilt Thorleif Hentrop, Martina Weiß und Ulrike Dreschel.

Meinem Büro 165 danke ich für die tolle Büroatmosphäre und gegenseitige Unterstützung. Besonders danke ich Alina dafür, dass sie darüber hinaus so eine gute Freundin ist.

Auch allen anderen lieben Kollegen danke ich für die schöne Zeit am TCI. Ich bin froh, so viele tolle Menschen kennengelernt zu haben. Sandra, ich freue mich vor allem dich kennengelernt zu haben.

Zuletzt möchte ich meiner Familie und meinem Freund danken. Danke Niclas, dass du mich immer bedingungslos unterstützt und mir zutraust alles schaffen zu können. Mama, Lisa und Ruben, ich habe euch sehr lieb.

Kurzfassung

Obwohl Batch-Chromatographie viele Nachteile wie die ineffiziente Nutzung der Kapazität und hohe Leerlaufzeiten aufweist, überwiegt bisher der Einsatz im Vergleich zur kontinuierlichen Chromatographie. Vor allem unter dem Gesichtspunkt der hohen Prozesskosten im Bereich des Downstream Processings, würde der Einsatz von kontinuierlichen Methoden zu Produktivitätssteigerungen und damit zur Senkung der Prozesskosten führen.

In dieser Arbeit wurde ein kontinuierliches Chromatographie-System entwickelt und nach dem Periodic-Counter-Current-Chromatographie (PCCC)-Prinzip in Betrieb genommen. Das System wurde für (Einweg-)Membranadsorber als Alternative zu Säulen ausgelegt und soll damit aufgrund des erhöhten konvektiven Massentransports zu weiteren Produktivitätssteigerungen führen.

Zunächst wurde das System mit drei Membranadsorbern und schließlich mit vier Membranadsorbern zur Optimierung des Systems und Steigerung der Flexibilität betrieben. Durch das Beladungsprinzip der PCCC können die Membranadsorber über die dynamische Bindungskapazität hinaus beladen werden. Das führt z. B. zur besseren Kapazitätsnutzung, verringertem Pufferverbrauch und zur Steigerung der Raum-Zeit-Ausbeute. Im Rahmen dieser Arbeit wurden drei Applikationsbeispiele zur kontinuierlichen Aufreinigung mit dem PCCC-System untersucht: die Aufreinigung einer *Candida antarctica* Lipase B, einer Patchoulolsynthase und eines monoklonalen Antikörpers.

Das System eignet sich vor allem für die Aufreinigung mittels Affinitätschromatographie, da hier das Prinzip der PCCC bestmöglich ausgenutzt wird. Für die drei Applikationsbeispiele wurden Produktivitätssteigerungen zwischen 20 und 50 % sowie eine Steigerung der Kapazitätsnutzung von 20 % erreicht. Generell kann jedoch keine pauschale Aussage über die zu erzielenden Steigerungen getroffen werden, sodass jeder Prozess individuell bewertet werden sollte.

Schlagwörter: *Candida antarctica* Lipase B, Kontinuierliche Chromatographie, Membranadsorber, Monoklonale Antikörper, Patchoulolsynthase, Periodic-Counter-Current-Chromatographie

Abstract

Although batch chromatography has many disadvantages, such as inefficient capacity utilization and long downtimes, its use has so far predominated over continuous chromatography. Particularly in regard to the high process costs in the field of downstream processing, the use of continuous methods would lead to productivity increases and thus to a reduction of process costs.

In this work, a continuous chromatography system was developed and put into operation according to the principle of periodic counter-current chromatography (PCCC). The system was designed for (disposable) membrane adsorbers as an alternative to columns which could further increase the productivity due to higher convective mass transport.

Initially, the system was operated with three membrane adsorbers and subsequently with four membrane adsorbers for system optimization and increased flexibility. Due to the loading principle of the PCCC the membrane adsorbers can be loaded beyond the dynamic binding capacity. This results, for example, in better capacity utilization, reduced buffer consumption and increased space-time yield. Three application examples for continuous purification with the system were presented: the purification of *Candida antarctica* lipase B, patchoulol synthase and a monoclonal antibody.

The system is particularly suitable for purification by affinity chromatography, since the principle of PCCC is then optimally exploited. For the three application examples, productivity increases of 20 to 50 % and an increase in capacity utilization of 20 % were obtained. In general, however, no general statement can be made about the increases to be achieved, so that each process should be evaluated individually.

Keywords: *Candida antarctica* lipase B, continuous chromatography, membrane adsorbers, monoclonal antibodies, patchoulol synthase, periodic counter-current chromatography

Inhaltsverzeichnis

Danksagung ..... **I**

Kurzfassung ..... **II**

Abstract ..... **III**

Inhaltsverzeichnis ..... **IV**

Abbildungsverzeichnis ..... **V**

Abkürzungsverzeichnis ..... **VI**

1 Einleitung **1**

1.1 Zielsetzung 3

2 Theoretische Grundlagen **5**

2.1 Grundlagen der Chromatographie 5

2.2 Kontinuierliche Chromatographie 6

2.3 Membranadsorber 10

2.4 Aufbau und Funktionsweise der entwickelten PCCC-Anlage 11

3 Experimenteller Teil **22**

3.1 Kontinuierliche Aufreinigung von *Candida antarctica* Lipase B aus *E. coli*-Lysat 25

3.2 Kontinuierliche Aufreinigung von Patchoulolsynthase aus *E. coli*-Lysat 39

3.3 Kontinuierliche Aufreinigung eines monoklonalen Antikörpers aus Chinese Hamster Ovary-Zellüberstand 52

4 Zusammenfassung und Ausblick **71**

5 Literaturverzeichnis **74**

6 Anhang **80**

6.1 Betreute Abschlussarbeiten 80

6.2 Veröffentlichungen und Konferenzbeiträge 80

6.3 Lebenslauf 84

Abbildungsverzeichnis

Abbildung 1: Der Effekt des pI-Wertes auf die Proteinoberflächennettoladung; in Anlehnung an [15]. ... 6

Abbildung 2: Exemplarische Durchbruchskurve; in Anlehnung an [22]. ... 7

Abbildung 3: Schematische Darstellung der Counter-Current-Chromatographie; übersetzt nach [23]. ... 8

Abbildung 4: Schematische Darstellung des Massentransports in einem Chromatographie-Partikel und einem Membranadsorber; in Anlehnung an [28]. ... 10

Abbildung 5: Blockly-Methoden-Designer. Über den Webbrowser können Methoden erstellt und gespeichert werden. ... 24

Abbildung 6: GUI der 4MA-PCCC-Software zur Steuerung und Überwachung des Chromatographie-Prozesses. ... 24

Abbildung 7: Dreidimensionale Struktur der *Candida antarctica* Lipase B (PDB ID: 4K6G). ... 25

Abbildung 8: Schematischer Aufbau eines IgG-Antikörpers; in Anlehnung an [57]. ... 52

Abkürzungsverzeichnis

AEX	Anionenaustauschchromatographie
AU	Absorption Units
BFM	Biofeuchtmasse
BSA	Bovine Serum Albumin
CalB	*Candida antarctica* Lipase B
CEX	Kationenaustauschchromatographie
CHO	Chinese Hamster Ovary
CV	Column Volume
CWW	Cell Wet Weight
DB	Dynamische Bindungskapazität
DNA	Desoxyribonukleinsäure
DoE	Design of Experiments
DSP	Downstream Processing
E. coli	*Escherichia coli*
Fab	Antigen-bindendes Fragment
Fc	kristallisierbares Fragment
FDA	Food and Drug Administration
FPP	Farnesyldiphosphat
GC-FID	Gaschromatographie mit Flammenionisationsdetektor
HC	Schwere Kette
HCP	Host Cell Proteine
HIC	Hydrophobe Interaktionschromatographie
His	Histidin
HPLC	Hochleistungsflüssigkeits-Chromatographie
IMAC	Immobilisierte-Metallionen-Affinitätschromatographie
IPTG	Isopropyl-β-D-thiogalactopyranosid
LB-Medium	Lysogeny Broth-Medium
LC	Leichte Kette
LOD	Limit of Detection
LOQ	Limit of Quantification
MA	Membranadsorber
mAb	Monoklonaler Antikörper
MW	Molecular Weight

OD	Optische Dichte
PAT	Process Analytical Technology
P-CAC	Preparative Continuous Annular Chromatography
PCCC	Periodic-Counter-Current-Chromatographie
pI	Isoelektrischer Punkt
p-*NPA*	para-Nitrophenolacetat
PTS	Patchoulolsynthase
SC1	Switching Condition 1
SC2	Switching Condition 2
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Größenausschluss-Chromatographie
SiLA 2	Standardization in Lab Automation 2
SMB	Simulated Moving Bed
TB-Medium	Terrific Broth-Medium
TMB	True Moving Bed
USP	Upstream Processing
UV	Ultraviolett

1 Einleitung

Proteine tauchen überall in unserem Alltag auf. Sie dienen nicht nur als wichtiger Baustoff vieler Strukturen im menschlichen Körper, sondern finden auch in vielen Bereichen des täglichen Lebens eine Anwendung. In Waschmittel werden beispielsweise Proteine in Form von Enzymen eingesetzt, um die Waschleistung zu erhöhen.

Für das oben genannte Beispiel werden oftmals rekombinant hergestellte Proteine verwendet. Die rekombinante Proteinproduktion ist seit den 1970er Jahren möglich [1]. Hierfür werden Bakterien, Hefen oder Säugetierzellen eingesetzt, wobei die Komplexität des Proteins die Wahl des Produktionsorganismus beeinflusst.

Die rekombinanten Proteine finden Anwendung in verschiedenen Bereichen der Biotechnologie wie z. B. der roten Biotechnologie (Medizinische Biotechnologie, Antikörper) oder der weißen Biotechnologie (Industrielle Biotechnologie, Enzyme für die Lebensmittel-, Kosmetik- oder Textilindustrie). Die Herstellung eines biotechnologisch hergestellten Proteins setzt sich aus der Zellanzucht/Zellproduktion (Upstream Processing, USP) und der Aufreinigung des Proteins (Downstream Processing, DSP) zusammen. Wobei die genauen Prozessschritte stark vom jeweiligen Produkt bzw. Protein abhängen. In den letzten Jahren konnten erhebliche Produktivitätssteigerungen im Bereich des Upstream Processings erzielt werden, ohne dass die Kosten signifikant ansteigen. Die Kosten für das Downstream Processing eines biotechnologischen Produkts können daher bis zu 80 % der Herstellungskosten betragen [2, 3].

Proteine werden am häufigsten mit chromatographischen Methoden aufgereinigt, da diese oftmals zur gewünschten Reinheit und Qualität des Produkts führen und damit die regulatorischen Ansprüche erfüllen. Bei der Chromatographie handelt es sich um eine Methode zur Auftrennung von Molekülen anhand ihrer Eigenschaften. Das Prinzip der (Flüssig-)Chromatographie beruht auf spezifischen Interaktionen, wie z.B. Ladung, Affinität oder Hydrophobizität. Die aufzutrennenden Substanzen werden in einer flüssigen Phase (meist wässrige Pufferlösung) durch eine stationäre, feste Phase transportiert und interagieren dadurch mit dieser [4].

Die Chromatographie wird hauptsächlich satzweise (im Batch, diskontinuierlich) durchgeführt, da diese im Vergleich zu kontinuierlichen Methoden einfacher zu handhaben ist. Der Batch-Prozess bringt jedoch viele Nachteile mit sich: einen erhöhten

Aufwand beim Betreiben sowie Starten einer neuen Charge, eine Fehleranfälligkeit durch Starten und Stoppen, sowie dass nur ein Teil der Kapazität der Chromatographie-Materialien genutzt wird.

Um durch Produktivitätserhöhungen die hohen Kosten für das Downstream Processing zu senken, könnten daher kontinuierliche Methoden eingesetzt werden. Durch die Verschaltung mehrerer Einheiten bei der kontinuierlichen Chromatographie kann zusätzliche Kapazität genutzt werden. Gleichzeitig kann die Produktivität des Prozesses gesteigert werden, indem die Raum-Zeit-Ausbeute erhöht und die Prozesszeit gesenkt wird. Dies führt zur Senkung der Prozesskosten und letztendlich zur Senkung des Preises des hergestellten Produkts. Die FDA (Food and Drug Administration, USA) befürwortet die kontinuierliche Prozessführung und führt neben diesem Punkt der Kostensenkung weitere Vorteile wie höhere Sicherheit und Effizienz an [5]. Viele weitere Aspekte sprechen für die Anwendung einer kontinuierlichen Prozessführung: schnellere und effizientere Prozesse, verbesserte Qualitätssicherung, größere Kontrollierbarkeit [6], vereinfachte Maßstabsvergrößerung (Scale-up) [7], Möglichkeit der Automatisierung, keine Batch-zu-Batch-Schwankungen und Kosteneinsparungen [8]. Viele Industrien haben Batch-Prozesse erfolgreich durch kontinuierliche Prozesse ersetzt, dazu gehören: die Stahlguss-Industrie, die Chemie- und vor allem die Petrochemie- sowie die Lebensmittelindustrie. Die biotechnologische und pharmazeutische Industrie ist in dieser Hinsicht eher ein Nachzügler. Erst in den letzten 10 Jahren stieg das Interesse daran kontinuierliche Prozesse einzuführen, wodurch bisher nur kleine Fortschritte verzeichnet wurden [9, 10]. Um den Ansprüchen der biotechnologischen Industrie gerecht zu werden, müssen voll-automatisierte kontinuierliche Chromatographie-Anlagen eingesetzt werden, welche mit einer intelligenten Steuerung im Sinne der PAT (Process Analytical Technology)-Initiative ausgestattet sind, um den Prozess direkt zu überwachen [11].

1.1 Zielsetzung

Ziel dieser vorliegenden kumulativen Dissertationsarbeit ist die Etablierung einer automatisierten kontinuierlichen Chromatographie-Anlage nach dem Periodic-Counter-Current-Chromatographie (PCCC)-Prinzip unter Verwendung von (Einweg-)Membranadsorbern. Die Membranadsorber werden dafür seriell und zyklisch beladen, um die Kapazitätsnutzung zu steigern und damit die Raum-Zeit-Ausbeute und somit die Produktivität des Prozesses zu erhöhen. Das System wird zunächst nach folgenden Ansprüchen ausgelegt, geplant und gebaut. Dabei soll vor allem berücksichtigt werden, dass nur notwendige Komponenten in das System integriert werden, bei gleichzeitiger Flexibilität des Systems. Das System-Setup soll zunächst mit einem 3-Membranadsorber-Setup getestet und in Betrieb genommen werden. Für die Testung wird ein Gemisch aus BSA (Bovine Serum Albumin) und Lysozym gewählt und dieses soll mit der Anlage kontinuierlich aufgereinigt werden. Die Chromatographie-Bedingungen sollen so gewählt werden, dass BSA an einen Anionenaustausch-Membranadsorber bindet. Mit dem Modellgemisch sollen unter anderem Aussagen über die Kapazitätsnutzung, Pufferverbrauch, Produktivität sowie den Langzeitbetrieb getroffen werden.

Im Rahmen dieser Arbeit werden drei Anwendungsbeispiele vorgestellt. Zwei Anwendungsbeispiele betreffen die Aufreinigung eines löslichen Proteins aus *E. coli*-Lysat. Bei dem ersten Protein handelt es sich um *Candida antarctica* Lipase B (CalB), ein für die Biokatalyse relevantes Protein, welches beispielsweise in Waschmittel eingesetzt wird. CalB soll mittels Ionenaustauschchromatographie aufgereinigt werden. Daher sollen geeignete Bedingungen gefunden und auf die kontinuierliche Chromatographie-Anlage übertragen werden. Das zweite Anwendungsbeispiel ist die Aufreinigung des Enzyms Patchoulolsynthase (PTS). Dieses wird zur biotechnologischen Produktion von Patchouli (Duftstoff) eingesetzt. Da diese PTS einen His-Tag besitzt, soll Immobilisierte-Metallionen-Affinitätschromatographie (IMAC) verwendet werden, um PTS aus dem Lysat zu isolieren. Hierbei sollen verschiedene Bedingungen (Puffersystem, Metallionen) unter Berücksichtigung der Enzymaktivität untersucht werden. Die optimierte Batch-Methode soll ebenso auf das kontinuierliche PCCC-System übertragen werden.

Im Laufe der Arbeit soll die Anlage weiterentwickelt und auf vier Membranadsorber Einheiten ergänzt werden, um die Funktionalität des Systems zu erweitern. Im Rahmen

der Weiterentwicklung soll die Automatisierung (intelligente Steuerung) und Digitalisierung abgeschlossen werden. Dafür werden die Benutzeroberfläche (GUI) und andere Teile der Programmierung dahingehend verbessert, dass das System benutzerfreundlicher und robuster wird. Des Weiteren soll die Geräteansteuerung mithilfe von SiLA 2 (Standardization in Lab Automation 2) standardisiert werden.

Mit der weiterentwickelten PCCC-Anlage soll eine Anwendung gezeigt werden, bei der ein sekretierter Antikörper aus einem Zellkulturüberstand aufgereinigt wird. Für die Aufreinigung des Antikörpers soll die Protein-A-Chromatographie (Affinitäts-chromatographie) eingesetzt werden, da diese die Standard-Methode zur Aufreinigung von Antikörpern darstellt. Der Antikörper soll zukünftig als aktiver Wirkstoff in einem Medikament eingesetzt werden. Daher wird die Chromatographie unter Berücksichtigung der Qualität optimiert und auf die PCCC-Anlage übertragen.

Aus den Ergebnissen der drei Anwendungsbeispiele sollen Schlussfolgerungen getroffen werden, inwieweit die Verwendung der kontinuierlichen Chromatographie mit Membranadsorbern zur Steigerung der Produktivität bzw. zur Steigerung der Kapazitätsnutzung im Vergleich zum Batch-Prozess führt. Weiterhin soll der Einsatz von Membranadsorbern im Generellen als Alternative zu Chromatographie-Säulen diskutiert werden. Schließlich soll eine Empfehlung gegeben werden, für welche Systeme und Produkte sich die kontinuierliche Chromatographie eignet.

2 Theoretische Grundlagen

Im ersten Abschnitt dieser Arbeit werden die theoretischen Grundlagen der Chromatographie vorgestellt sowie in das Thema der kontinuierlichen Chromatographie mit Fokus auf der verwendeten Periodic-Counter-Current-Chromatographie (PCCC) eingeführt.

2.1 Grundlagen der Chromatographie

Für die Aufreinigung von Proteinen ist Chromatographie die Methode der Wahl [12], da fast jede Trennaufgabe damit bewältigt werden kann und verschiedene Proteineigenschaften (z. B. Nettoladung, Affinität, Größe, Hydrophobizität) zur Trennung genutzt werden können. In dieser Arbeit werden die Ionenaustausch-chromatographie sowie die Affinitätschromatographie verwendet.

Die Ionenaustauschchromatographie (IEX) erlaubt das Auftrennen von geladenen Molekülen auf der Grundlage von Unterschieden in den Ladungseigenschaften der Moleküle. Durch die vielen Vorzüge ist die Ionenaustauschchromatographie die am meisten verwendete Technik in der Flüssigchromatographie [13], dazu gehören ein breiter Anwendungsbereich, moderate Kosten, ein gutes Auflösungsvermögen, einfache Maßstabsvergrößerung sowie die leichte Automatisierung. Wie bei anderen Formen der Flüssigchromatographie wird zwischen der mobilen und der stationären Phase unterschieden. Die mobile Phase ist eine wässrige Pufferlösung, in der das zu trennende Substanzgemisch vorliegt. Die stationäre Phase besitzt funktionelle Gruppen, die je nach Anwendung eine positive (Anionenaustauschchromatographie, AEX) oder negative (Kationenaustauschchromatographie, CEX) Ladung tragen [14]. Um ein Protein an einen Ionenaustauscher zu binden, muss das Protein eine Nettoladung aufweisen (vgl. Abbildung 1). Bei einem pH-Wert oberhalb des isoelektrischen Punkts (pI) ist ein Protein negativ geladen (Anion) und kann an einen Anionenaustauscher gebunden werden. Bei einem pH-Wert unterhalb des isoelektrischen Punkts (pI) ist ein Protein positiv geladen (Kation) und kann an einen Kationenaustauscher gebunden werden. Der pH-Wert des Puffersystems sollte mindestens eine halbe pH-Einheit von dem pI gewählt werden, um eine ausreichend starke Bindung an den Ionenaustauscher zu garantieren [15].

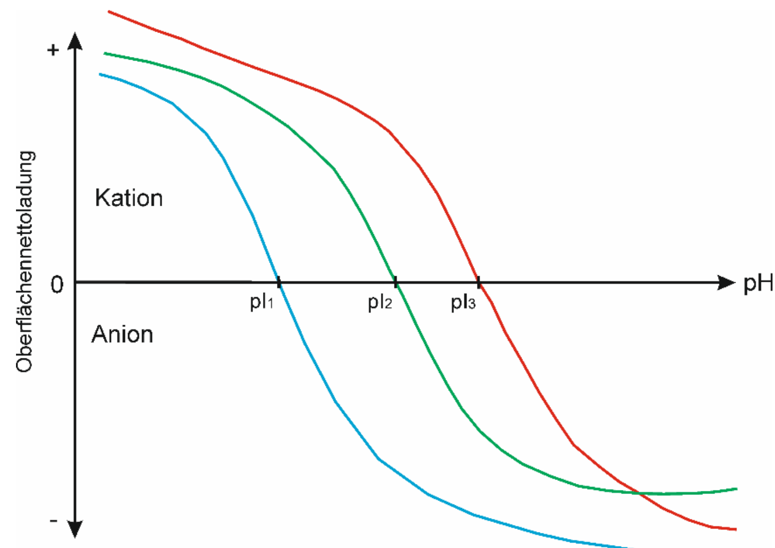


Abbildung 1: Der Effekt des pI-Wertes auf die Proteinoberflächennettoladung; in Anlehnung an [15]**.**

Die Affinitätschromatographie ist nach der IEX die zweithäufigst verwendete Chromatographie-Methode [13] und trennt ein Flüssigkeitsgemisch nicht nach der Größe der einzelnen Moleküle oder deren Ladung auf, sondern nutzt die spezifischen Struktureigenschaften von Proteinen für eine selektive Auftrennung. Verwendet wird dabei die Interaktion zum Beispiel zwischen Enzymen und Substraten, Rezeptoren und ihren Liganden oder die Bindung zwischen Antigen und Antikörper [16]. Diese reversible Bindung kann mithilfe von Denaturierungsmitteln, Konkurrenzanaloga oder durch Änderung der umgebenden Bedingungen wie pH-Wert, Ionenstärke und Polarität gelöst werden, um das gewünschte Protein zu gewinnen [17]. Beispiele sind die Protein-A-Chromatographie zur Aufreinigung von Antikörpern oder die Immobilisierte-Metallionen-Affinitätschromatographie (IMAC) zur Aufreinigung von Proteinen mit einem His-Tag.

2.2 Kontinuierliche Chromatographie

Der Wechsel vom Batch-Prozess zum kontinuierlichen Prozess führte in vielen Industrien zu erheblichen Verbesserungen [18]. Dabei ist der kontinuierliche Prozess definiert als eine Grundoperation, bei der ein kontinuierlicher Fluss für längere Zeiträume verarbeitet wird. Ein kontinuierlicher Gerätebetrieb hat ein minimales internes Haltevolumen, das kontinuierlich oder zyklisch in kleineren Mengen abgeführt werden kann [19].

Dennoch wird zur chromatographischen Aufreinigung hauptsächlich der Batch-Betrieb genutzt. Denn obwohl die kontinuierliche Prozessführung viele Vorteile

(Produktivitätssteigerung, kleinere Chromatographie-Anlagen/-Materialien, Kosteneinsparungen) mit sich bringt, müssen noch einige Hürden genommen werden. Das sind beispielsweise die hohen Anschaffungskosten für die komplexen Anlagen oder der hohe Aufwand für die Automatisierung. Deswegen scheint es derzeit noch einfacher zu sein, den Batch-Prozess in die Produktion zu übertragen, als den kompletten Prozess kontinuierlich auszulegen [19]. Daher wurden im Bereich der Chromatographie im Vergleich zu anderen Bereichen bisher eher kleinere Erfolge erzielt [2].

Die erste kontinuierliche multi-column Chromatographie wurde in den 1920ern durchgeführt (Merry-Go-Round-Installation): Zwei Säulen wurden in Reihe beladen, während die dritte Säule regeneriert wurde. Der Produktdurchbruch wurde auf der zweiten Säule aufgefangen [20]. Abbildung 2 zeigt wie dieses Beladungsprinzip die Kapazitätsnutzung der ersten Säule steigert und somit deutlich effizienter ist als der Batch-Prozess [21].

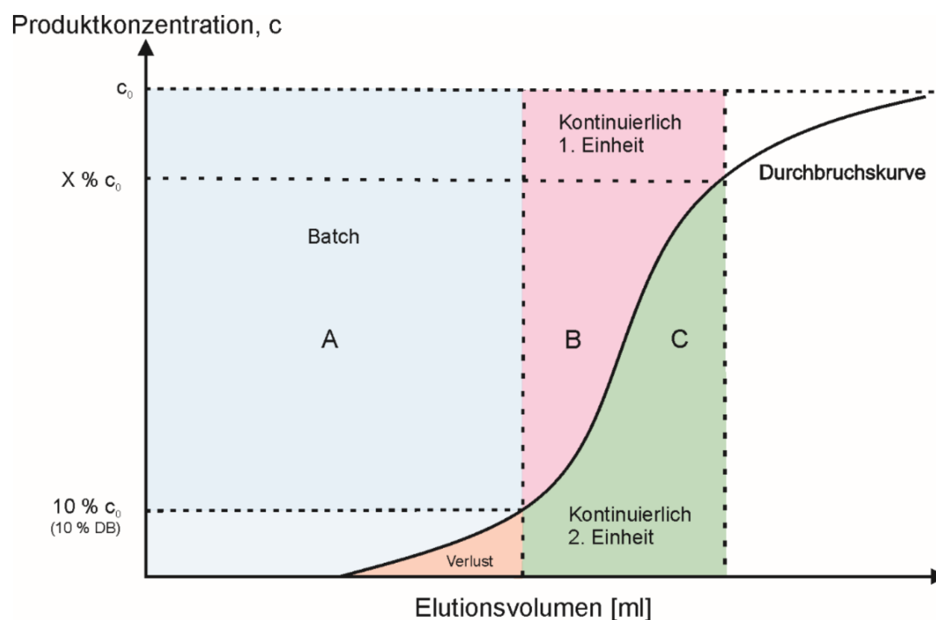


Abbildung 2: Exemplarische Durchbruchskurve; in Anlehnung an [22]**.**

Zu sehen ist eine Produktdurchbruchskurve, wobei die Produktkonzentration gegen das Elutionsvolumen aufgetragen wurde. Die Flächen A und B über der Durchbruchskurve entsprechen der Menge an Produkt, die an die erste Säule bzw. Chromatographie-Einheit gebunden wurde. Die Fläche C unter der Durchbruchskurve entspricht der Menge an Produkt, die am Ausgang der Chromatographie-Einheit gemessen wird. Beim Batch-Prozess endet die Beladung des Chromatographie-Materials in der Regel bei Erreichen von 10 % des Produktdurchbruchs (10 % c_0 oder 10 % DB). Dieser Punkt wird auch als

Dynamische Bindungskapazität (DB) bezeichnet und gibt die Menge an Produkt an, die unter den gegebenen Bedingungen bindet, bevor ein signifikanter Durchbruch (bzw. Verlust) an ungebundenem Produkt auftritt. Gängige Werte des Produktdurchbruchs liegen zwischen 1 und 10 % der aufgetragenen Produktkonzentration. Bei der Batch-Chromatographie wird demnach nur ein Teil der Kapazität (Fläche A) genutzt. Durch das Verschalten von zwei Chromatographie-Einheiten in der kontinuierlichen Chromatographie, wird die Kapazitätsnutzung gesteigert und es wird zusätzlich Fläche B an die erste Chromatographie-Einheit gebunden sowie die Fläche C auf der zweiten Chromatographie-Einheit aufgefangen. Dies führt z. B. zu verringertem Pufferverbrauch und einer kürzeren Prozesszeit.

2.2.1 True Moving Bed Chromatographie

In den 1950er Jahren wurde der Counter-Current-Ansatz eingeführt und für die True Moving Bed (TMB) Chromatographie zur Aufreinigung von (binären) Flüssig-Flüssig-Gemischen verwendet. Abbildung 3 stellt das schematische Prinzip der Counter-Current-Chromatographie mit einer stationären (festen) und einer mobilen (flüssigen) Phase dar: Die stationäre und mobile Phase bewegen sich in entgegengesetzter Richtung. Die aufzutrennende Probe (Feed) wird in der Mitte der Säule aufgetragen und die aufgetrennten Substanzen können in Abhängigkeit ihrer Affinität zur stationären Phase an den Enden der Säule (Extrakt und Raffinat) aufgefangen werden [23].

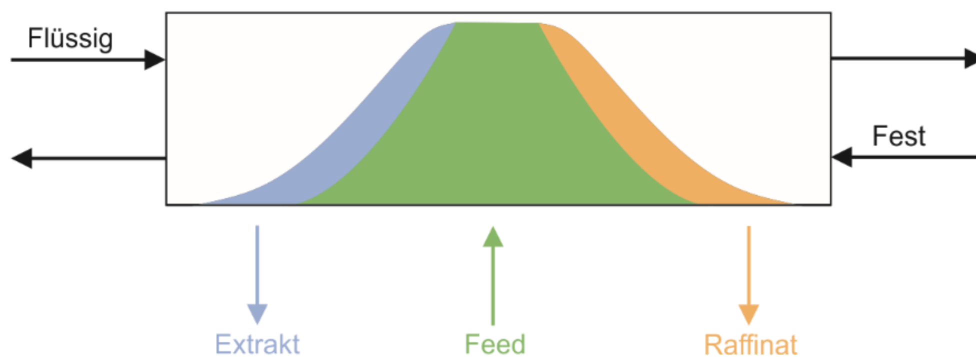


Abbildung 3: Schematische Darstellung der Counter-Current-Chromatographie; übersetzt nach [23].

2.2.2 Simulated Moving Bed Chromatographie und Periodic-Counter-Current-Chromatographie

Der Ansatz der TMB Chromatographie wurde zur quasi-kontinuierlichen Simulated Moving Bed (SMB) Chromatographie weiterentwickelt und im Jahr 1961 als Anwendung

für Festbettsäulen zur Auftrennung binärer Stoffgemische (fest-flüssig) vorgestellt. Dabei wird eine kontinuierliche Gegenstrombewegung der festen Phase durch periodisches Schalten von Ventilen simuliert [20, 23]. Eine weitere kontinuierliche Methode ist die Periodic-Counter-Current-Chromatographie (PCCC). Sie ist wie die SMB eine quasi-kontinuierliche Chromatographie-Methode, bei der der Gegenstrom durch Schaltung von Ventilen simuliert wird. Grundlegend basieren SMB und PCCC also auf dem Counter-Current-Prinzip und der Verschaltung mehrerer Chromatographie-Einheiten. Der Unterschied der Methoden liegt zum einen in der Prozessauslegung und zum anderen in der Prozesssteuerung; denn bei der SMB wird der Prozess mithilfe von Adsorptionsisothermen ausgelegt und bei der PCCC durch Aufnahme der Durchbruchskurve, wobei der Prozess weiterhin dynamisch gesteuert werden kann. Eine geeignete Prozesskontrollstrategie ist ein wesentliches Element eines kontinuierlichen Chromatographie-Prozesses und ist im Sinne der PAT-Initiative. Durch die Überwachung des UV-Signals während der PCCC kann die Qualität während des Prozesses überwacht werden. Diese Überwachung ist ein Schlüsselelement in der industrialisierten Produktion von bspw. Biopharmazeutika. Weiterhin wird die SMB zur Auftrennung von binären Stoffgemischen verwendet, da nur in zwei Fraktionen getrennt werden kann [24, 25]. Die PCCC kann zur Auftrennung von mehreren Komponenten verwendet werden, wobei der Prozess flexibel durch Ergänzung von Chromatographie-Einheiten erweitert werden kann. Damit eignet sich die PCCC vor allem für die Aufreinigung von Proteinen aus komplexen Gemischen wie Lysat oder Zellkultur. Aufgrund der zuvor genannten Vorteile der PCCC wurde das Prinzip für die Auslegung einer kontinuierlichen Chromatographie-Anlage gewählt. Das Funktionsprinzip sowie das Anlagen-Setup werden detailliert in 2.4 beschrieben.

2.2.3 Preparative Continuous Annular Chromatographie

Neben diesen zwei quasi-kontinuierlichen Chromatographie-Methoden ist die kontinuierliche Preparative Continuous Annular Chromatographie (P-CAC) zu erwähnen. Die P-CAC wurde ebenfalls in den 1950er Jahren entwickelt. Bei der P-CAC sitzt die stationäre Phase in einem Ring, welche um ihre Achse rotiert. Die aufzutrennende Lösung wird oben an einer festen Position auf die Säule aufgetragen und die Komponenten abhängig von der Interaktion mit der Säule aufgetrennt. Anders als bei den oben vorgestellten Methoden, tritt hier ein Cross-Current von mobiler und stationärer Phase auf [11, 12, 26]. Damit ist die Auftrennung komplexer Gemische möglich.

Dennoch ist ein entscheidender Nachteil, dass das einheitliche Packen der Säulen nicht sehr reproduzierbar ist [26].

2.3 Membranadsorber

Neben der oben beschriebenen Hardware-Entwicklung wurden neue Chromatographie-Materialien und Liganden entwickelt, um die Limitierung des Massentransports durch Diffusion bei herkömmlichen Säulen zu überwinden. Neuste Entwicklungen haben besonders die Membranchromatographie sehr interessant für die Aufreinigung von Biomolekülen gemacht [9]. In Abbildung 4 ist eine schematische Darstellung eines Chromatographie- Partikels sowie eines Membranadsorbers gezeigt. Wie zu erkennen ist, wird das Erreichen der Bindungsstellen im Chromatographie-Partikel durch Porendiffusion limitiert. Dagegen können die Bindungsstellen auf den Membranadsorbern unmittelbar durch den konvektiven Fluss erreicht werden. Hier tritt lediglich Filmdiffusion auf [27].

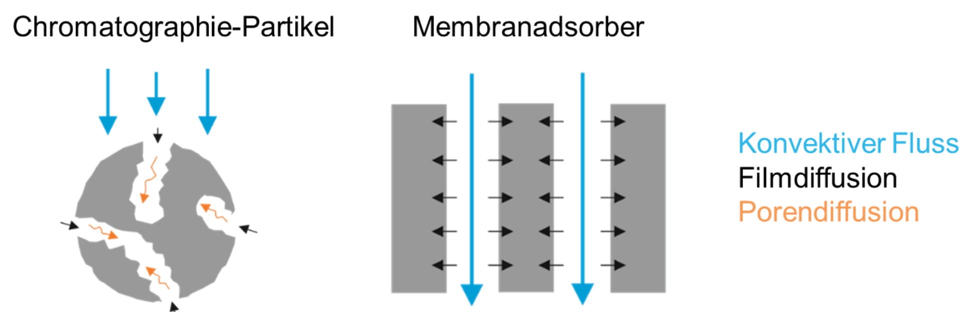


Abbildung 4: Schematische Darstellung des Massentransports in einem Chromatographie-Partikel und einem Membranadsorber; in Anlehnung an [28]**.**

(Einweg-)Membranadsorber (MA) können in kontinuierlichen Chromatographie-Systemen im Niederdruckbereich verwendet werden [20]. Membranadsorber weisen neben hoher mechanischer Stabilität auch eine starke Salz- und pH-Toleranz auf [29]. Die Kapazität von Membranadsorbern ist unabhängig von der Durchflussrate [30] und ermöglicht so höhere Durchsätze respektive die Prozessierung niedriger Produkttiter. Trotzdem sind die Durchbruchkapazitäten von Säulen und Membranadsorbern vergleichbar [31]. Darüber hinaus ist der up- und down-scale von Membranadsorbern einfach, da die Kapazität nur von der Membranoberfläche abhängt [32].

2.4 Aufbau und Funktionsweise der entwickelten PCCC-Anlage

Im Rahmen dieser Arbeit wurde eine kontinuierliche Chromatographie-Anlage nach dem Periodic-Counter-Current-Chromatographie (PCCC)-Prinzip geplant, aufgebaut und in Betrieb genommen. In der PCCC werden bis zu zwei Chromatographie-Einheiten in Reihe geschaltet, um den Produktdurchbruch der ersten Chromatographie-Einheit auf der zweiten Chromatographie-Einheit aufzufangen. Das UV-Signal der Durchbruchskurve dient als dynamische Steuerung für die Automatisierung. Diese Betriebsart ermöglicht eine Beladung der Chromatographie-Einheit über der dynamischen Bindungskapazität und nahe der statischen Bindungskapazität (verfügbare Kapazität der Chromatographie-Einheit im Gleichgewicht) und führt somit zu einer höheren Kapazitätsnutzung der Chromatographie-Einheit [6, 33]. Im Gegensatz zu kommerziell erhältlichen Chromatographie-Systemen, ist diese Anlage auf Membranadsorber ausgelegt, da diese, wie in 2.3 beschrieben, Vorteile gegenüber Säulen aufweisen.

Das System wurde zunächst für drei Membranadsorber-Einheiten (3MA-PCCC) aufgebaut und schließlich auf vier Einheiten erweitert (4MA-PCCC). Mit der 3MA-PCCC konnte ein einfacher Prozessablauf aus Beladen, Waschen, Eluieren und Regenerieren abgebildet werden. Durch Erweiterung der Anlage auf vier Membranadsorber, konnte zusätzlich dazu der Waschschritt über den vierten Membranadsorber geleitet werden. Dies ist wichtig, um Produktverluste zu vermeiden und wird als interconnected wash bezeichnet.

Das 4MA-PCCC-Setup wurde erfolgreich mit dem Modellproteingemisch aus BSA und Lysozym getestet, wobei BSA (Produkt) an den Membranadsorber Sartobind® Q75 gebunden wurde. Die Anlage wurde sowohl zeitlich als auch dynamisch über das UV-Signal gesteuert. Der kontinuierliche Betrieb erzielte im Vergleich zum Batch-Prozess eine gesteigerte Raum-Zeit-Ausbeute, eine Steigerung der Kapazitätsnutzung von 20 %, sowie Puffereinsparungen.

Auf die genaue Funktionsweise der verwendeten PCCC und das Anlagen-Setup wird im nachfolgenden Artikel „*Development and Testing of a 4-Columns Periodic Counter-Current Chromatography System Based on Membrane Adsorbers*“ (2019, Separations) eingegangen. Die zuvor beschriebenen Zusammenhänge werden nochmals aufgegriffen und die Periodic-Counter-Current-Chromatographie detailliert anhand eines Beispiels erläutert.

Communication

Development and Testing of a 4-Columns Periodic Counter-Current Chromatography System Based on Membrane Adsorbers

Chantal Brämer [1], Frank Lammers [2], Thomas Scheper [1] and Sascha Beutel [1,*]

[1] Institute of Technical Chemistry, Callinstraße 5, 30167 Hannover, Germany; braemer@iftc.uni-hannover.de (C.B.); scheper@iftc.uni-hannover.de (T.S.)

[2] Sanofi-Aventis Deutschland GmbH, 65926 Frankfurt am Main, Germany; frank.lammers@sanofi.com

* Correspondence: beutel@iftc.uni-hannover.de; Tel.: +49-511-762-2868

Received: 8 October 2019; Accepted: 20 November 2019; Published: 22 November 2019

Abstract: Continuous chromatography can surmount the disadvantages of batch chromatography like low productivities and extensive usage of consumables. In this work, a 4-column continuous chromatographic system based on the principle of periodic counter-current chromatography (PCCC) was developed and tested with a model protein mixture of BSA and lysozyme. The PCCC system was specially designed for membrane adsorbers as an alternative to conventional columns to facilitate the use of disposable process units and to further increase the productivity due to higher convective mass transport in the membrane adsorber. Membrane adsorber Sartobind® Q was used to continuously purify BSA from the protein mixture. The usage of PCCC led to an increased capacity utilization (here 20%) and higher space–time-yields, and thus to a remarkable productivity increase and cost savings.

Keywords: membrane adsorber; continuous chromatography; periodic counter-current chromatography

1. Introduction

The biotechnological industry produces biopharmaceuticals for the treatment of a wide variety of diseases like cancer or autoimmune diseases [1], enzymes for the detergent industry to achieve better cleaning performance [2], or for the production of fragrances for expensive perfumes [3]. The manufacturing process of a biotechnological product consists of cell cultivation (upstream processing), mostly of genetically modified microorganisms, yeasts, or mammalian cells and purification of the product (downstream processing). To date, the batch process is predominantly used for production, especially since it is easier to handle. Nevertheless, the batch has disadvantages such as the increased effort involved in operating, emptying, and refilling to start a new batch. This leads to long downtimes between batches. Thus, the batch process is not very efficient. Therefore, the trend is moving toward continuous processing as a continuous process is more efficient and achieves higher space–time-yields [1,4,5]. When considering the cost distribution for the production of a biotechnological product, it is noticeable that the purification of the product is unequally distributed with up to 80% of the total production costs [6]. Therefore, the interest in continuous processes is increasing, especially in the field of chromatography as the method of choice [7]. Continuous chromatography is intended to remedy limited productivities by increasing the capacity utilization and decreasing the usage of consumables, thus leading to higher space–time-yields [5,8–10]. However, the introduction of continuous processes involves hurdles: planning, setup, and handling are much more complex than for batch processes [1,11].

The first continuous multi-column chromatography was carried out in the 1920s (merry-go-round installation). The system consisted of three chromatography units, of which two were always loaded and one was regenerated. The two chromatography units were connected in a row so that the product breakthrough of the first chromatography unit was captured on the second chromatography unit. This method allowed an improvement in capacity utilization and continuous sample introduction. In the 1950s, various methods for continuous chromatography were developed, mainly to purify metals or wastewater by liquid–liquid extraction [12]. These (chromatography) methods were based on the counter-current principle: the mobile and stationary phases move in opposite directions. The sample to be separated is applied in the middle of the chromatography unit and the separated substances can be collected at the ends of the chromatography unit. First, true moving bed (TMB) liquid–liquid chromatography was implemented, and finally simulated moving bed (SMB) chromatography was developed [10]. Further examples of continuous methods are continuous annular chromatography [7] and periodic counter-current chromatography [4,8,13,14]. SMB [15] and periodic counter-current chromatography (PCCC) are pseudo-continuous methods, since the counter-current is simulated using the column switching approach. Usually, these continuous methods are operated with chromatographic columns. In this work, the PCCC was run with membrane adsorbers, which will be explained in more detail shortly. In PCCC, up to two chromatography units are loaded in series to capture the product breakthrough of the first chromatography unit on the second chromatography unit. The UV signal of the breakthrough curve serves as the dynamic control strategy for automation (see Figure 1, operated with membrane adsorbers). This mode of operation allows for the chromatography unit to be loaded close to the static binding capacity (available capacity of the chromatography unit in equilibrium) and thus results in a higher capacity utilization of the chromatography unit [1,2,8,9].

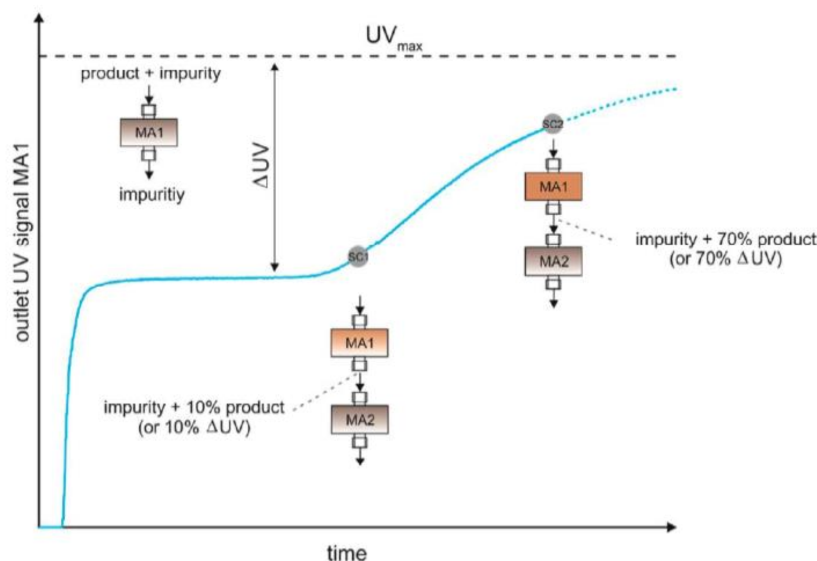


Figure 1. Loading principle of periodic counter-current chromatography operated with membrane adsorbers.

M. Hall and K. Lacki (GE Healthcare Bio-Sciences AB) patented (WO 2008/153472 A1) a semi-continuous chromatography method based on the PCCC principle. The system can be operated with three (3C-PCC) or four (4C-PCC) chromatography units. GE Healthcare offers the commercial system ÄKTA™ pcc 75, which is based on the ÄKTA™ avant system and is operated with Unicorn software. The system can be operated with flow rates up to 75 mL/min and contains a UV measurement after each chromatography unit (at 280 nm) as well as sensors for pH and conductivity measurement.

Some examples of 3C-PCC or 4C-PCC application can be found in the literature, mainly for the purification of monoclonal antibodies [16–19]. In addition, a few other applications are used such as

desalting, virus purification, plasma protein purification [16], purification of unstable proteins [8], lipase [2], and a sesquiterpene synthase purification [3].

In addition to the hardware development described above, new chromatography materials and ligands have been developed to overcome the limitation of mass transport in conventional columns by diffusion. Recent developments have made membrane chromatography particularly interesting for the purification of biomolecules [20]. (Disposable) membrane adsorbers (MA) [12] can be used in continuous low-pressure chromatography systems. The ligands are attached to the membrane surface so that the mass transport is mainly due to convection and not to pore diffusion [21]. The capacity of membrane adsorbers is independent of the flow rate [14] and thus enables higher throughputs or the processing of lower product titers up to a few g/L. Nevertheless, the breakthrough capacities of conventional columns and membrane adsorbers are comparable [22]. In addition, the up- and down-scale of membrane adsorbers is simple because the capacity depends only on the membrane surface [23]. A continuous chromatography system, in which MA can be operated as an alternative to classical columns, could further increase productivity of a purification process [24,25]. This applies particularly to the purification of special pharmaceuticals or highly valuable proteins from complex mixtures such as antibodies, proteins from human serum, growth factors, etc.

In this work, a new PCCC system was developed and tested using MA devices instead of conventional chromatography columns. The MA offers the advantages of being disposable and can be easily exchanged. Furthermore, cleaning and validation costs are eliminated through the use of disposables. The PCCC system setup in this study was designed for four MA units and was equipped with a flexible UV measurement in the range from 230–650 nm. The 4MA-PCCC system can be operated with the interconnected wash, which is very important to avoid product loss [26], in comparison to the previously developed 3MA-PCCC system. In the course of its development, the PCCC system was redesigned to be compact and portable. In addition, a GUI (graphical user interface) was developed to increase usability. In this work, the continuous purification of a model protein mixture of BSA (bovine serum albumin) and lysozyme was performed to evaluate the continuous chromatography system. BSA (pI = 4.7) and lysozyme (pI = 11.4) were chosen because of their different pI (isolelectric point) values, which makes purification by ion exchange chromatography very easy. Therefore, the anion exchange membrane adsorber Sartobind® Q was used to bind BSA at pH 7 due to its negative surface net charge.

2. Materials and Methods

2.1. Materials

BSA and lysozyme were purchased from Sigma-Aldrich (now Merck, Darmstadt, Germany) of a technical purity grade. All other chemicals were bought from Carl-Roth (Karlsruhe, Germany).

2.2. Methods

2.2.1. Protein Purification

The batch purification was performed using the commercial system ÄKTA™ pure (GE Healthcare, Uppsala, Sweden). In this study, the membrane adsorber (MA) Sartobind® Q (anion-exchange chromatography) was used to bind BSA. The membrane adsorber has a surface area of 75 cm^2 and a membrane volume of 2.1 mL. The dynamic binding capacity for BSA was 54.2 mg and the static binding capacity was 73.5 mg per unit.

The model proteins BSA and lysozyme were diluted in binding buffer (20 mM KH_2PO_4-buffer pH 7.0) to the desired concentration. Elution was performed by adding 0.5 M NaCl. For continuous purification, a chromatographic (PCCC) system with four membrane adsorbers was used. The PCCC system and procedure is further described in Section 3.1.

Separations **2019**, 6, 55

2.2.2. Protein Quantification

For protein quantification, Bradford assay was performed using the reagent Roti®-Quant (Carl-Roth). A 20 µL sample was mixed with 300 µL of the reagent in a 96-well plate. Incubation was performed for 5 min. At 595 nm, detection was carried out with Multiskan GO. BSA and lysozyme standards in the range of 0.0125–0.2 g/L BSA and 0.0125–0.75 g/L lysozyme were measured for calibration.

2.2.3. Qualitative Analysis Using SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used for qualitative analysis of the purification. The gel consisted of a stacking gel (6%) and a separating gel (15%). The gel was run for 15 min at 100 V and 1.5 h at 150 V. Samples were diluted 1:1 with Lämmli buffer and boiled for 10 min at 95 °C. A sample of 5 µL of the marker (Unstained Protein Molecular Weight Marker, Thermo Fisher Scientific, Waltham, MA, USA) and 10 µL of the sample was applied onto the gel.

3. Results

In previous work, a PCCC system was developed and tested with three membrane adsorbers (3MA-PCCC) and a simple schedule. This system was further used for the purification of two industrial relevant proteins *Candida Antarctica* lipase B [2] and patchoulol synthase [3]. In these experiments, product was lost in the washing steps. To recover more product and increase effectivity, the interconnected wash [2,18,26] was implemented, meaning that the wash fraction is passed through another MA. Therefore, a fourth chromatography unit with measurement system was added to the PCCC system. After installation and reconstruction, the new 4MA-PCCC device was tested with the model proteins BSA and lysozyme and put into operation for the separation of a simple two-component system. Due to the different isoelectric points of BSA and lysozyme, BSA can be separated specifically by an anion-exchange membrane adsorber (Sartobind® Q).

3.1. System Setup

The 3MA-PCCC system components (pump, valves, UV measurement) were described in Brämer et al., (2018) whereas the 4MA-PCCC fraction collector was changed to a BioFrac™ Fraction Collector and 16 additional 3–2-way valves (The Lee company, Westbrook, CT, USA) were added. Furthermore, a fourth UV measurement unit and UV flowthrough cuvettes with a pathlength of 2 mm (Hellma Analytics, Müllheim, Germany) can also be used in the system. Different pathlengths are useful when maintaining different purification tasks. In this work, 2 mm cuvettes were used. The PCCC system circuit diagram is shown in Figure 2.

3.2. Integration of SiLA2 and Blockly

The PCCC system consists of various components that are addressed in the program scripts using python™. Due to the complexity of the device, the program script is very long. To simplify the operation for the user, Blockly was implemented. Blockly is a graphical tool developed by Google Developers that uses simple graphical tools that create syntactically correct source code. The user can create a method by selecting the required blocks and entering values, for example, pump rates. To guarantee standardized device communication, SiLA 2 was implemented. SiLA 2 is a standard driver protocol and was developed to ensure compatibility of laboratory systems and here, enables the user to access the functionality of the system in a uniform way.

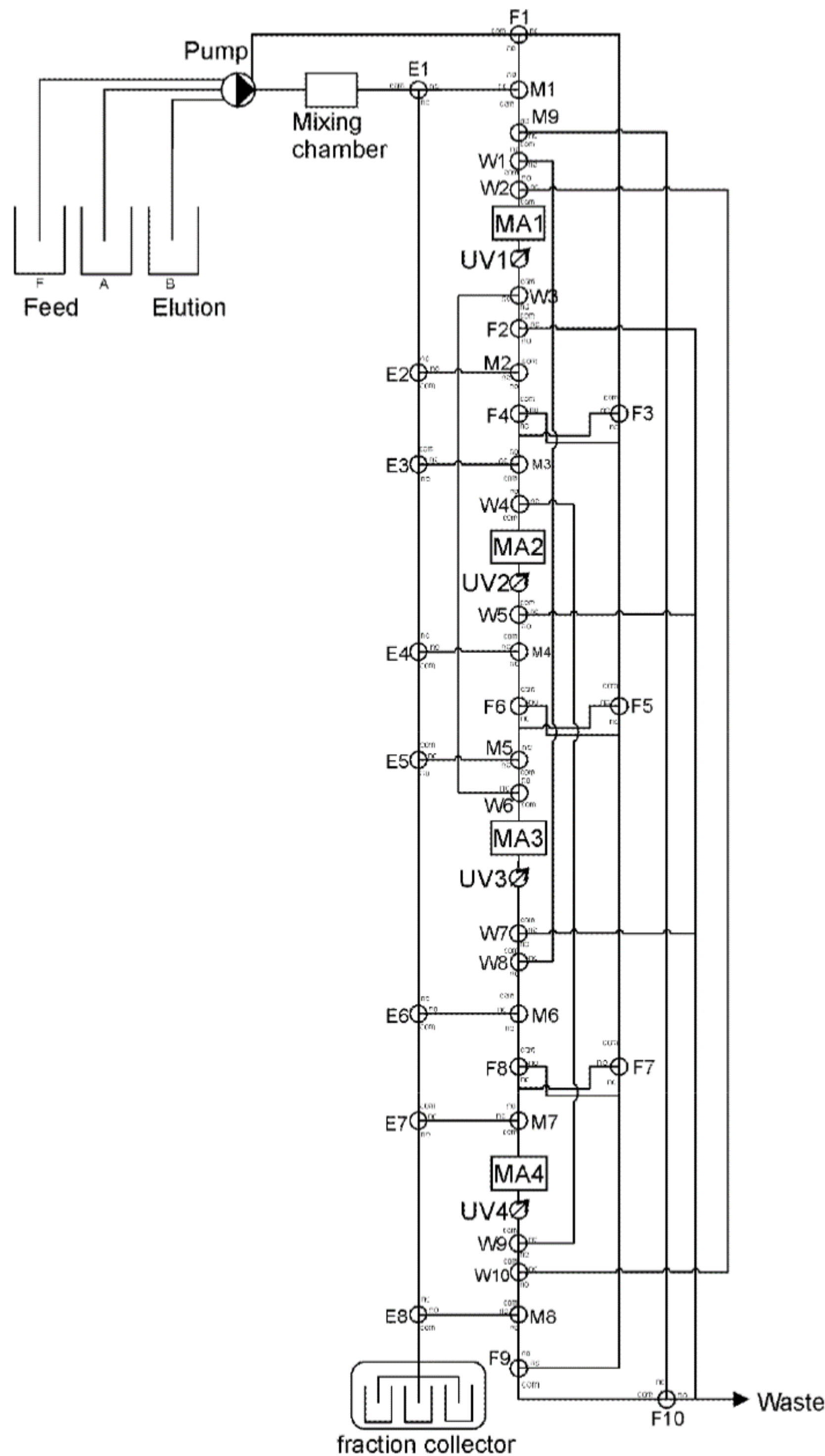


Figure 2. Circuit diagram of the periodic counter-current chromatography system with four membrane adsorber units.

3.3. Continuous Chromatography with Model Proteins

Protein concentrations of the model purification task were chosen according to the calibration data (Appendix A, Figure A1) for BSA and lysozyme. A total of 5 g/L BSA and 1.25 g/L lysozyme was used to achieve a double breakthrough curve so that the UV signal could be used to control the process. By continuously applying the protein mixture onto a MA, a double breakthrough can be recorded at the outlet of the MA (see Figure 1). The impurity (here lysozyme) breaks through while

BSA binds to the MA. When the MA is slowly saturated with BSA, BSA (here the product) also breaks through and can be measured at the outlet. The aim is to represent the PCCC process as displayed in Figure 3.

As described in Figure 1, in step 1, MA 1 is loaded until a certain amount of the product breaks through (here 10%, SC1). The process switches to step 2 and MA 2 is added to the load. The breakthrough from MA 1 is then bound to MA 2. The second step stops when MA 1 is saturated with product [4,5] (here 70%, SC2). The MA is decoupled from the circuit, the product is eluted, and the MA is regenerated and fed back into the circuit. The procedure described for MA 1 is continued with MA 2 to 4, resulting in the process schedule in Figure 3 [13]. By washing, eluting, and regenerating (displayed in grey) in parallel to the loading (displayed in light blue), the process can begin again at step 1 after step 8 [8]. Steps 1 to 8 show the nested cyclic process of the 4MA-PCCC. By linking two MA units, the loading can be carried out quasi-continuously. Furthermore, by linking the units, loading takes longer and thus more capacity is used without product being lost. In contrast to the 3MA-PCCC system [2], this 4MA-PCCC system can be used with the interconnected wash. This means that the wash after loading is applied on a further MA to increase the product yield while the feed stream is applied continuously. This can be seen, for example, in step 3 for MA 1. After loading in steps 1 and 2, a wash step is performed in step 3. Unbound product is captured on MA 3 and the loss is consequently reduced. As already described [17], there are the following degrees of freedom for the operation of the PCCC: the pump rates of the feed and the buffers A and B.

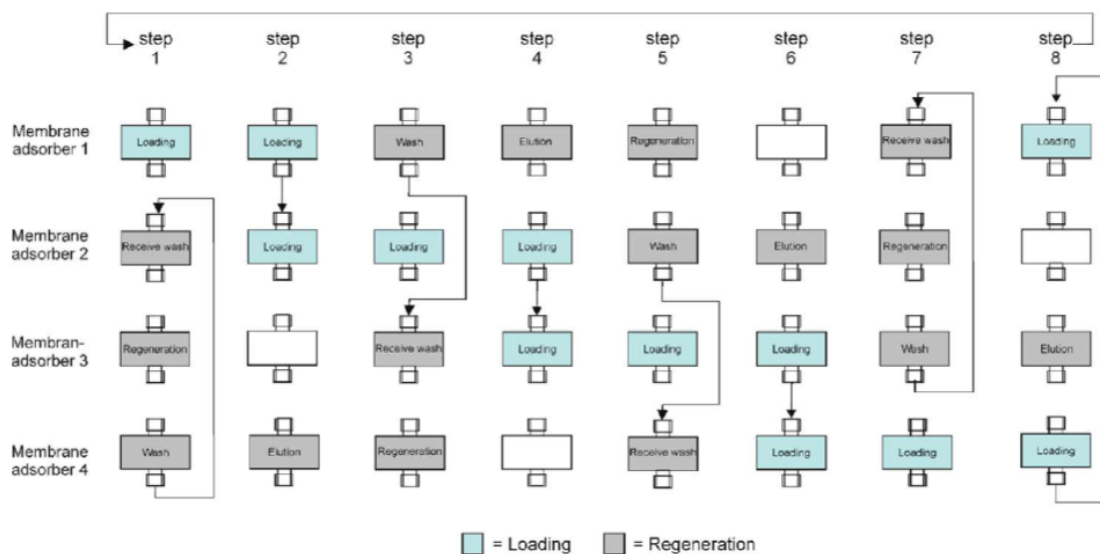


Figure 3. Schematic schedule for the continuous chromatography with four membrane adsorbers. Four membrane adsorbers are loaded, washed, and regenerated in eight steps based on the periodic counter-current principle. Thus, quasi-continuous processing is realized by loading the membrane adsorbers in a sequential and cyclic manner.

3.3.1. Time-Controlled Process (Static Control)

At first, a simplified experiment was performed to check if the 4MA-PCCC system setup (especially the complex circuit and program script) fulfills the requirements for PCCC. Therefore, a time-controlled (static control) experiment was performed, which can be seen in Figure 4a. The feed rate was set to 0.5 mL/min as the BSA concentration was quite high with 5 g/L. The pump rates for buffer A and B were set to 8.6 mL/min (maximum pump rate with utilized tubing). The chromatography was run at room temperature. Each step of the continuous process from Figure 3 was maintained for 5 min. During the experiment, 105 mL feed containing 5 g/L BSA and 1.25 g/L lysozyme were purified within 3.5 h and four PCCC-cycles. The chromatogram in Figure 4a shows the absorption at 280 nm of the four sequentially loaded membrane adsorbers. Uniform breakthroughs and elution peaks can be seen, which indicate that the circuit and programming was

correctly implemented: both the loading of the four MA as well as the implementation of the interconnected wash were successful.

3.3.2. Dynamic Control with UV-Signal

The 4MA-PCCC system is to be used by running it with the dynamic control using the UV-signal at 280 nm. The UV-signal provides information on how much of the MA's capacity has already been used. The capacity can be used more efficiently by setting the switching conditions SC1 and SC2 [4,8]. SC1 corresponds to the dynamic binding capacity whereas SC2 corresponds to a nearly saturated MA (Figure 1). These were calculated as follows:

$$SC1 = 10\% \cdot \Delta UV + UV_{lysozyme/impurity} = 0.51\ AU \quad (1)$$

$$SC2 = 70\% \cdot \Delta UV + UV_{lysozyme/impurity} = 0.84\ AU \quad (2)$$

where ΔUV is the difference between the UV-signal of the protein mixture or feed (UV_{max}) and the UV-signal of lysozyme (here $UV_{lysozyme/impurity}$).

A total of 80 mL protein solution with 5 g/L BSA and 1.25 g/L lysozyme (feed) was used for the dynamically controlled experiment. This required 1.8 L buffer (equilibration and elution buffer) for the washing, elution, and regeneration phases. Two PCCC cycles were performed in 2.75 h. Product recovery was over 90%.

The two cycles are shown in Figure 4b. The expected double breakthroughs of the four MA can be seen. The elution peaks were above 1.5 AU in the non-linear range. Therefore, no statement can be made about the different heights of the peaks between membrane adsorbers 1, 2, 3, and 4. The capacity utilization under these conditions was increased by about 20% (see Appendix A, Figure A2). Compared to the batch process, a cost saving of about 17% with regard to the chromatography unit could be achieved.

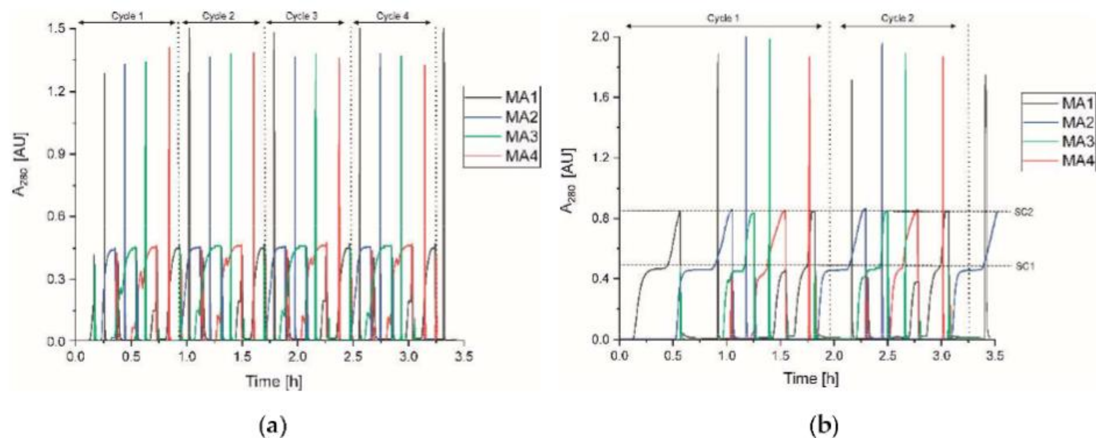


Figure 4. Chromatogram of continuous run with temporal control, duration of one phase is 5 min (**a**) and dynamic control (**b**).

4. Discussion

This study depicts the successful establishment of a PCCC device operated with four membrane adsorbers, enabling the use of disposable process units. The measurement components were optimized for the intended use so that the UV measurement is very flexible and changeable flowthrough cuvettes (2 mm and 10 mm) can be used, depending on the purification process. The optimized 4MA-PCCC system was successfully tested with BSA and lysozyme as the model purification task, whereas BSA was bound to the MA Sartobind Q® MA75. This strong anion exchange membrane adsorber was selected as BSA has a negative surface net charge in the selected buffer and thus binds to the membrane adsorber. With the system static, dynamic control is possible and protein concentrations in the absorption range of 0.01–1.5 AU can be measured.

Separations **2019**, *6*, 55

The application example with model proteins enabled a higher space–time-yield compared to batch chromatography, which was reached by increased capacity utilization (20%). Furthermore, buffer and time savings were achieved using the PCCC. The feasibility and performance depend on the individual process, respective product, and impurities to separate, and therefore needs to be investigated in detail before applying the continuous system. It is important for the PCCC to have a high product concentration in the feed for the dynamic control via UV-signal. It must be sufficiently high and measurable in relation to the impurities, so that the dynamic process control is possible. Here, a high concentration with 5 g/L BSA was used, whereas only 1.25 g/L lysozyme were added as an impurity. It should be noted that this concentration ratio is further dependent on the extinction coefficient at 280 nm, which differs for every product. Furthermore, the feed to be purified may contain various contaminants. These can be host cell proteins, media components, DNA, etc.; all of these have different absorption behaviors. Specific online measurement methods could be used to detect the product (e.g., fluorescence for either aromatic amino acid-rich proteins or fluorescent proteins). The PCCC is particularly suitable for the purification of secreted proteins from mammalian cells or microorganisms, whereby the ratio of product to impurity is decisive. In particular, the purification of highly valuable products leads to significant process cost savings.

5. Conclusions

In this study, a model protein mixture of BSA and lysozyme was purified with an advanced 4MA-PCCC. The system had decisive advantages over the previously developed 3MA-PCCC. Due to the extended design, the yield could be increased, particularly because the so-called interconnected wash was implemented. Due to the graphical user interface and the integration of a method editor, the implementation was easy for the user and thus meets the current standards. In particular, the use of SiLA2 is advantageous, because the connection to other devices is possible via this standardized device control. This is crucial with regard to integrated or continuous processes from upstream and downstream processing.

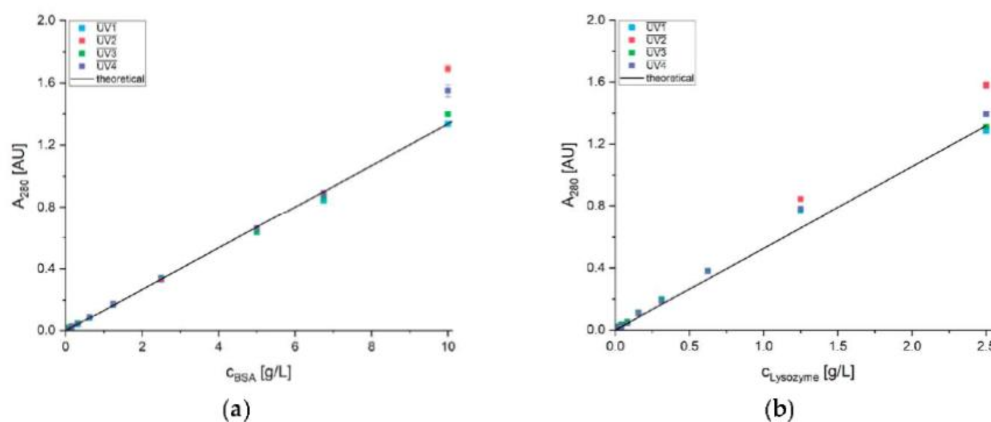
Author Contributions: Conceptualization, C.B. and S.B.; Methodology, C.B.; Software, C.B.; Formal analysis, S.B.; Investigation, C.B.; Resources, T.S. and S.B.; Writing—original draft preparation, C.B.; Writing—review and editing, F.L., T.S., and S.B.; Visualization, C.B.; Supervision, T.S. and S.B.; Project administration, S.B.; Funding acquisition, S.B.

Funding: Part of this work was funded by the German Federal Ministry of Education and Research (BMBF, funding number: 031B0463C).

Acknowledgments: The authors would like to thank Laura Niemeyer for her work on Blockly GUI. The publication of this article was funded by the Open Access fund of Leibniz Universität Hannover.

Conflicts of Interest: The authors have declared no conflicts of interest.

Appendix A



(a) (b)

Figure A1. Calibration data of the four UV measurements at 280 nm with BSA (bovine serum albumin) (**a**) and lysozyme (**b**) using a pathlength of 2 mm.

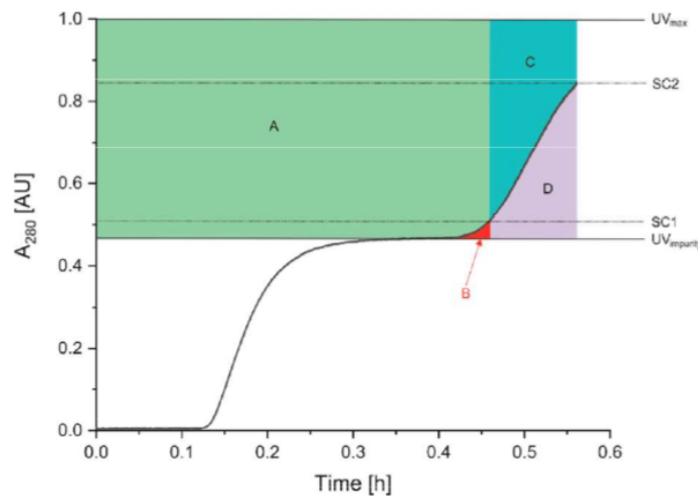


Figure A2. Capacity utilization in periodic counter-current chromatography vs. batch chromatography. (**A**) Bound product batch; (**B**) product loss batch; (**C**) further product bound in continuous chromatography on first chromatography unit; (**D**) product bound on second chromatography unit.

References

1. Ozturk, S.S. Opportunities and Challenges for the Implementation of Continuous Processing in Biomanufacturing. *Contin. Process. Pharm. Manuf.* **2014**, 457–478, doi:10.1002/9783527673681.ch18.
2. Brämer, C.; Schreiber, S.; Scheper, T.; Beutel, S. Continuous purification of Candida antarctica lipase B using 3-membrane adsorber periodic counter-current chromatography. *Eng. Life Sci.* **2018**, *18*, 414–424.
3. Brämer, C.; Ekramzadeh, K.; Lammers, F.; Scheper, T.; Beutel, S. Optimization of continuous purification of recombinant patchoulol synthase from Escherichia coli with membrane adsorbers. *Biotechnol. Prog.* **2019**, 1–10. doi:10.1002/btpr.2812.
4. Warikoo, V.; Godawat, R.; Brower, K.; Jain, S.; Cummings, D.; Simons, E.; Johnson, T.; Walther, J.; Yu, M.; Wright, B.; et al. Integrated continuous production of recombinant therapeutic proteins. *Biotechnol. Bioeng.* **2012**, *109*, 3018–3029.
5. Heeter, G.A.; Liapis, A.I. Perfusion chromatography: Performance of periodic countercurrent column operation and its comparison with fixed-bed operation. *J. Chromatogr. A* **1995**, *711*, 3–21.
6. Pathak, M.; Ma, G.; Bracewell, D.G.; Rathore, A.S. Re-use of protein A resin: Fouling and economics. *BioPharm Int.* **2015**, *28*, 28–33.
7. Wolfgang, J.; Prior, A. Continuous Annular Chromatography. *Modern Advances in Chromatography* **2002**, *76*, 233–255.
8. Godawat, R.; Brower, K.; Jain, S.; Konstantinov, K.; Riske, F.; Warikoo, V. Periodic counter-current chromatography–design and operational considerations for integrated and continuous purification of proteins. *Biotechnol. J.* **2012**, *7*, 1496–1508.
9. Janson, J.-C. *Protein Purification: Principles, High Resolution Methods, and Applications*; John Wiley & Sons: Hoboken, NJ, USA, 2012; Volume 151.
10. Rajendran, A.; Paredes, G.; Mazzotti, M. Simulated moving bed chromatography for the separation of enantiomers. *J. Chromatogr. A* **2009**, *1216*, 709–738.
11. Whitaker, S.C.; Francis, R.; Siegel, R.C. Validation of Continuously Perfused Cell Culture Processes for Production of Monoclonal Antibodies. In *Validation of Biopharmaceutical Manufacturing Processes*; American Chemical Society: Washington, DC, USA, 1998; Volume 698, pp. 3–28.
12. Bisschops, M. Bio SMB ™ Technology: Continuous Countercurrent Chromatography Enabling a Fully Disposable Process. *Biopharm. Prod. Technol.* **2012**, *1*, 769–791.
13. Steinebach, F.; Müller-Späth, T.; Morbidelli, M. Continuous counter-current chromatography for capture and polishing steps in biopharmaceutical production. *Biotechnol. J.* **2016**, *11*, 1126–1141.

14. Skoglar, H.; Blom, H.; Mathiasson, L.; Akerblom, A.; Łącki, K. The use of dynamic control in periodic counter-current chromatography. *Bioprocess Int.* **2015**, *13*, 29150261.
15. Heuer, C.; Küsters, E.; Plattner, T.; Seidel-Morgenstern, A. Design of the simulated moving bed process based on adsorption isotherm measurements using a perturbation method. *J. Chromatogr. A* **1998**, *827*, 175–191.
16. Mathiasson, L.; Skoglar, H.; Berg, M.; Sichting, M.; Chmielowski, R.; Forma, E.; Nordvarg, H. Continuous chromatography beyond affinity capture of monoclonal antibodies. **2017**.
17. Baur, D.; Angarita, M.; Müller-Späth, T.; Steinebach, F.; Morbidelli, M. Comparison of batch and continuous multi-column protein A capture processes by optimal design. *Biotechnol. J.* **2016**, *11*, 920–931.
18. Pollock, J.; Bolton, G.; Coffman, J.; Ho, S.V.; Bracewell, D.G.; Farid, S.S. Optimising the design and operation of semi-continuous affinity chromatography for clinical and commercial manufacture. *J. Chromatogr. A* **2013**, *1284*, 17–27.
19. El-Sabbahy, H.; Fagan, L.; Nancollis, V. Factors affecting the productivity of 4-Column Periodic Counter Current Chromatography (4C-PCC). **2015**.
20. Rathore, A.S.; Kateja, N.; Agarwal, H. Continuous Downstream Processing for Production o fBiotech Therapeutics. Continuous Biomanufacturing: *Innov. Technol. Methods* **2017**, 261–288, doi:10.1002/9783527699902.ch10.
21. Knudsen, H.L.; Fahrner, R.L.; Xu, Y.; Norling, L.A.; Blank, G.S. Membrane ion-exchange chromatography for process-scale antibody purification. *J. Chromatogr. A* **2001**, *907*, 145–154.
22. Gebauer, K.H.; Thömmes, J.; Kula, M.R. Breakthrough performance of high-capacity membrane adsorbers in protein chromatography. *Chem. Eng. Sci.***1997**, *52*, 405–419.
23. Demmer, W.; Nussbaumer, D. Large-scale membrane adsorbers. *J. Chromatogr. A* **1999**, *852*, 73–81.
24. Mothes, B.; Pezzini, J.; Schroeder-Tittmann, K.; Villain, L. Accelerated, seamless antibody purification. *Bioprocess. Int.* **2016**, *14*, 5.
25. Zobel-Roos, S.; Stein, D.; Strube, J. Evaluation of Continuous Membrane Chromatography Concepts with an Enhanced Process Simulation Approach. *Antibodies* **2018**, *7*, 13.
26. Mahajan, E.; George, A.; Wolk, B. Improving affinity chromatography resin efficiency using semi-continuous chromatography. *J. Chromatogr. A* **2012**, *1227*, 154–162.

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

3 Experimenteller Teil

Dieses Kapitel setzt sich aus drei wissenschaftlichen Publikationen zusammen, die sich mit Anwendungsbeispielen für die kontinuierliche Chromatographie mit der vorgestellten PCCC-Anlage (aus Kapitel 2.4) beschäftigen.

Der Schwerpunkt liegt auf der Etablierung von kontinuierlichen Aufreinigungsmethoden für die Zielproteine unter Verwendung der entwickelten PCCC-Anlage. Als Alternative zu herkömmlich verwendeten Chromatographie-Säulen werden (Einweg-) Membranadsorber verwendet. Diese sind kommerziell erhältlich und können mit verschiedenen Liganden bestückt gekauft werden. In dieser Arbeit werden Membranadsorber der Sartobind® Serie (Q75, IDA75, Protein A 2 mL) verwendet.

Die PCCC-Anlage wurde zunächst mit einem einfachen Setup unter Verwendung von drei Membranadsorbern entwickelt. Dieses Setup wird in den ersten zwei Publikationen verwendet. Der Systemaufbau wird in der ersten Publikation mit dem Titel „*Continuous purification of Candida antarctica lipase B using 3-membrane adsorber periodic counter-current chromatography*“ (2018, Engineering in Life Sciences) vorgestellt. In dieser Veröffentlichung wird die Methodenentwicklung zur Aufreinigung von *Candida antarctica* Lipase B (CalB) beschrieben, sowie die Übertragung der Batch-Methode auf die PCCC-Anlage dargestellt. CalB wurde mittels Anionenaustauschchromatographie (AEX) aus dem *E. coli*-Lysat isoliert. Die kontinuierliche Aufreinigung mit der optimierten Methode hat sich hierbei als praktikabel erwiesen, allerdings ist die nicht-selektive Bindung der AEX unvorteilhaft. Im Zuge der weiteren Arbeiten wird daher Affinitätschromatographie angewandt.

Die zweite Veröffentlichung „*Optimization of continuous purification of recombinant patchoulol synthase from E. coli with membrane adsorbers*“ (2019, Biotechnology Progress) beschäftigt sich mit der kontinuierlichen Aufreinigung von Patchoulolsynthase aus *E. coli*-Lysat mittels Immobilisierte-Metallionen-Affinitätschromatographie (IMAC). Diese ist aufgrund des angebrachten His-Tags möglich. Da das aufzureinigende Enzym eher instabil ist, wurde bei der Methodenentwicklung besonders die Enzymaktivität berücksichtigt. Nach der erfolgreichen Pufferoptimierung wurden verschiedene Metallionen getestet und die besten Bedingungen gefunden. Auch hier wurde die Batch-Methode auf die PCCC-Anlage übertragen und konnte erfolgreich abgebildet werden. Aufgrund der niedrigen Produktkonzentration wurde die PCCC-Anlage zeitlich

gesteuert; eine hohe Produktkonzentration wäre für die optimale Durchführung der PCCC jedoch wünschenswert.

Daher befasst sich die dritte Veröffentlichung „*Membrane Adsorber for the Fast Purification of a Monoclonal Antibody Using Protein A Chromatography*“ (2019, Membranes) mit der Aufreinigung eines monoklonalen Antikörpers (mAb) aus Chinese Hamster Ovary (CHO)-Zellüberstand. Im aufzureinigenden Überstand wurden 0,6 g/L monoklonaler Antikörper nachgewiesen. Diese Produktmenge liefert optimale Voraussetzungen für die PCCC-Anlage und die intelligente Steuerung anhand des UV-Signals. Der Fokus der Aufreinigung lag neben der Chromatographie-Performance des Capture-Schrittes auf der Qualität und Stabilität des mAb unter den durchgeführten Bedingungen. Die Batch-Methode wurde dahingehend optimiert und auf die PCCC-Anlage mit vier Membranadsorbern übertragen. Die PCCC-Anlage wurde auf vier Membranadsorber-Einheiten erweitert, um u.a. weitere Sequenzen in die kontinuierliche Methode zu integrieren. Damit wird eine weitere Prozessphase ermöglicht, der interconnected wash, durch den wie in 2.4 beschrieben, die Produktausbeute erhöht werden kann.

Da die Komplexität und Automatisierung von kontinuierlich betriebenen Anlagen oftmals abschreckend wirken, dennoch sehr wichtig für die Industrialisierung sind, wurde flankierend im Rahmen eines Digitalisierungsprojektes die Digitalisierung der PCCC-Anlage vorgenommen (BMBF-Projekt DigInBio FKZ 031B0463C). Das bedeutet erstens, dass die Geräteansteuerung mittels SiLA 2 standardisiert wurde, also alle verbauten Gerätekomponenten miteinander kommunizieren können. Zweitens wurde die Bedienung vereinfacht, indem ein Methoden-Editor und eine leicht verständliche Benutzeroberfläche implementiert wurden. Der Methoden-Editor wurde mit Blockly Software (Google Developers, USA) realisiert und erlaubt die einfache Erstellung von Methoden im Webbrowser. Der Benutzer gibt, wie in Abbildung 5 zu sehen ist, die gewünschten Prozessparameter (z. B. Flussraten) ein und kann die Methode dann speichern. Über die Benutzeroberfläche (Graphical User Interface, GUI) in Abbildung 6 kann die erstellte Methode aufgerufen und gestartet werden. Die aktuellen Prozessparameter wie UV-Werte, Druckwerte und die Fraktionsnummer werden laufend geloggt und können ebenfalls abgelesen werden. In den weiteren Tabs können die Daten exportiert sowie das Chromatogramm des aktuellen Laufs verfolgt werden.

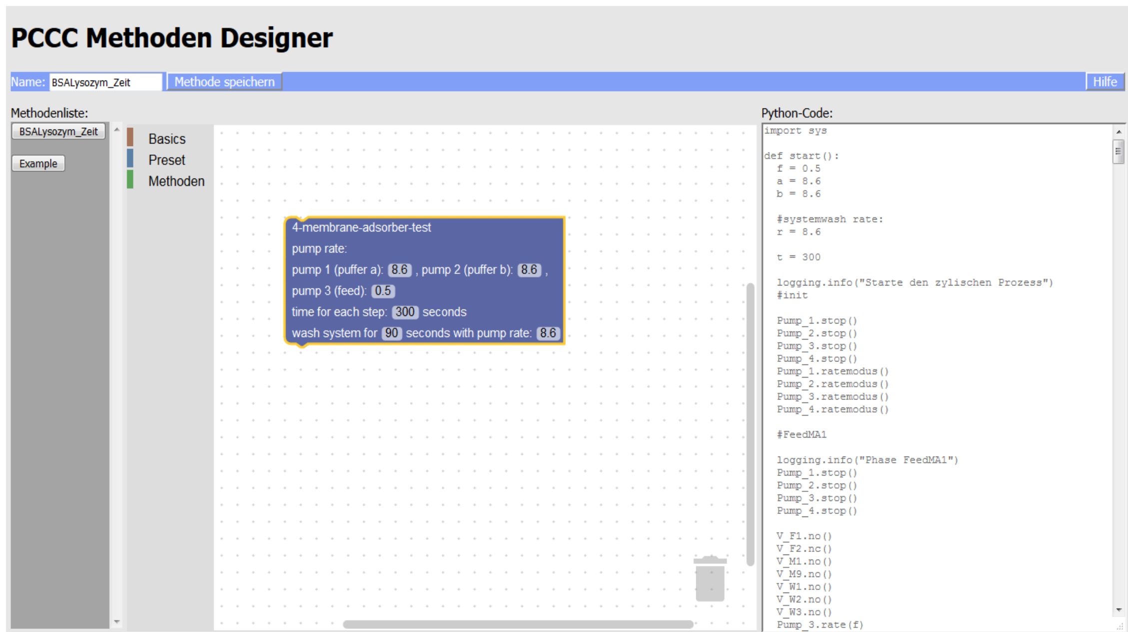


Abbildung 5: Blockly-Methoden-Designer. Über den Webbrowser können Methoden erstellt und gespeichert werden.

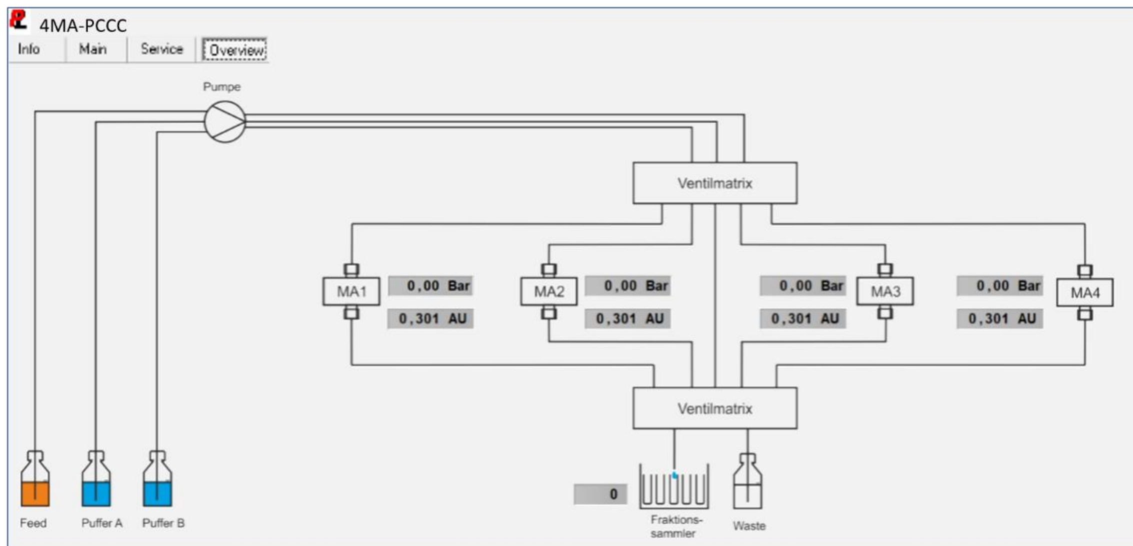


Abbildung 6: GUI der 4MA-PCCC-Software zur Steuerung und Überwachung des Chromatographie-Prozesses.

3.1 Kontinuierliche Aufreinigung von *Candida antarctica* Lipase B aus *E. coli*-Lysat

In diesem Kapitel wird die Aufreinigung von industriell relevanter *Candida antarctica* Lipase B (CalB) beschrieben. Durch den Einsatz von (Einweg-)Membranadsorbern in Kombination mit der PCCC-Anlage soll die Aufreinigung von CalB etabliert und verbessert werden. Dafür wurden Hydrophobe Interaktionschromatographie (HIC) und Anionenaustauschchromatographie (AEX) unter Verwendung verschiedener Puffer im Batch-Betrieb getestet und mit bereits veröffentlichen Methoden verglichen [34–37]. Um die Produktivität der Chromatographie zu steigern, wurde die etablierte Methode auf die PCCC-Anlage mit drei Membranadsorbern übertragen. Der Batch-Prozess wurde mit dem kontinuierlichen Prozess in Bezug auf die Qualität und Quantität der Aufreinigung, den Verbrauch von Verbrauchsmaterialien und die Prozesszeit verglichen.

CalB (EC 3.1.1.3) ist eine der meist eingesetzten Lipasen in der Biokatalyse [38] und wird der Familie der Serinhydrolasen zugeordnet [38]. Die dreidimensionale Struktur von CalB ist in Abbildung 7 zu sehen. CalB besteht aus 317 Aminosäuren und weist ein Molekulargewicht von 33 kDa auf [39].

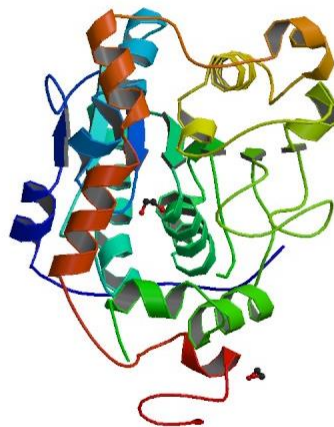


Abbildung 7: Dreidimensionale Struktur der *Candida antarctica* Lipase B (PDB ID: 4K6G).

Aufgrund der vielen vorteilhaften Eigenschaften wie breites Substratspektrum, hohe Temperaturstabilität bis 60°C und pH-Stabilität im Bereich von pH 3–10 [40], findet CalB in verschiedenen Bereichen Anwendung. Das sind bspw. die Anwendung in der Waschmittelindustrie [38], bei der Herstellung von Fruchtestern oder L-Menthol [41, 42] sowie im Lebensmittelbereich zur Entfernung von strengem Geruch in Schaf- und

Ziegenmilchprodukten [43, 44]. Der weite Stabilitätsbereich lässt sich auf den breiten pI-Bereich der CalB von pH 5–8 zurückführen [34].

Die Aufreinigung von CalB ist jedoch aufgrund des breiten pI-Bereichs problematisch, sodass mit konventioneller Säulenchromatographie noch keine optimale Strategie entwickelt wurde. Hauptsächlich wurde die Ionenaustauschchromatographie eingesetzt, die eine Ladung der CalB voraussetzt. Aufgrund des pI-Bereichs von CalB muss unterhalb von pH 5 (positiv geladen) oder oberhalb von pH 8 (negativ geladen) gearbeitet werden. Darüber hinaus sollte CalB in diesen pH-Bereichen in seiner Aktivität nicht beeinträchtigt werden.

In dieser Arbeit wurde eine Methode für die Aufreinigung der CalB mit dem AEX-Membranadsorber Sartobind® Q75 entwickelt. Der Puffer der Wahl war 20 mM TRIS-HCl, pH 8,5 und die Elution von CalB erfolgte mit 0,2 M NaCl (20% Puffer B) und gebundene Verunreinigungen wurden mit 1 M NaCl (100% Puffer B) eluiert. Die dynamische Bindungskapazität betrug 56 µg/cm^2 CalB und die statische Bindungskapazität betrug 84 µg/cm^2 CalB (mit MA Sartobind® Q75). Die Aufreinigung von CalB aus *E. coli*-Lysat führte zu einer hohen spezifischen Aktivität von 223 U/mg (wobei die Aktivität des Lysats vor der Aufreinigung 55 U/mg betrug) und einer Wiederfindung von 97 %. Darüber hinaus zeigten die CalB-Fraktionen eine hohe Reinheit. Im Vergleich zu den veröffentlichten Methoden mit Säulen wurden eine hohe Wiederfindung, eine hohe Aktivität und ebenfalls hohe Reinheit erzielt (vgl. [34–37]). Diese vielversprechende Methode zur Aufreinigung von CalB wurde auf die 3MA-PCCC-Anlage übertragen. Die Werte für die intelligente Prozessteuerung mittels UV-Signal wurden mit 10 % Produktdurchbruch (Switching Condition 1, SC1) und 70 % Produktdurchbruch (Switching Condition 2, SC2) definiert und ermittelt.

Im Rahmen der durchgeführten PCCC wurden 0,9 L *E. coli*-Lysat mit 0,37 g/L Gesamtprotein in 6 h und fünf Zyklen aufgereinigt. Da die AEX-Methode für die Aufreinigung von CalB aus Lysat auch Verunreinigungen gebunden hat, wurde die Elution in zwei Schritten mit 20 % und 100 % Puffer B durchgeführt. CalB wurde aktiv und in hoher Reinheit mit einer Konzentration von bis zu 1 g/L eluiert. Die Ausbeute betrug 0,22 g bei einer Wiederfindung von 80 %. Im Vergleich zur Batch-Chromatographie führte der Einsatz der PCCC hier zu einer Produktivitätssteigerung von 36 %.

Die Methode zur Aufreinigung von CalB mit der PCCC erwies sich damit als praktikabel, jedoch unter der Einschränkung, dass die Anzahl der Zyklen aufgrund einer Verschlechterung der Performance durch bindende, nicht einfach durch Waschschritte zu entfernende Verunreinigungen begrenzt war. Die nicht-selektive Bindung von CalB war nicht optimal für die Anwendung der PCCC.

Die PCCC-Anlage sollte daher vor allem für Anwendungen mit Affinitätswechselwirkungen eingesetzt werden, z. B. mit spezifisch-bindenden Proteinen wie Antikörpern oder His-getaggten Proteinen.

Im folgenden Artikel „*Continuous purification of Candida antarctica lipase B using 3-membrane adsorber periodic counter-current chromatography*" werden die Ergebnisse detailliert beschrieben und diskutiert.

Engineering in Life Sciences

Eng. Life Sci. **2018**, *0*, 1–11 www.els-journal.com

Chantal Brämer
Sarah Schreiber
Thomas Scheper
Sascha Beutel

Institute of Technical Chemistry, Gottfried Wilhelm Leibniz University of Hanover, Hanover, Germany

Research Article

Continuous purification of *Candida antarctica* lipase B using 3-membrane adsorber periodic counter-current chromatography

Batch chromatography has several disadvantages, such as insufficient utilization of the capacity of the resin, high buffer consumption and discontinuity. Considering the high costs for downstream processing, a continuously working chromatographic system with three membrane adsorber units was designed, tested and put into operation. The basic principle of the setup is periodic counter-current chromatography (PCCC). The PCCC system was used for capturing and purifying *Candida antarctica* lipase B (CalB) directly from cell lysate in one single unit operation. The best purification result was achieved by means of anion-exchange chromatography. The dynamic binding capacity with Sartobind® Q 75 amounted to 4.2 mg (56 g/cm^2). After transferring the method to the 3MA-PCCC, 0.22 g CalB (73 U/mg) were obtained from 0.9 L *E. coli* lysate within 6 h and a recovery of 80%. Compared to the batch process, the productivity could be increased by 36% and the buffer consumption could be reduced by about 20%. Although the purification of CalB from lysate by means of anion-exchange chromatography was not selective and quantitative using the 3MA-PCCC device, it could be shown that the concept of the system was successfully implemented and led to a significant improvement of CalB purification.

Keywords: *Candida antarctica* lipase B / Continuous chromatography / Membrane adsorber / Multi column chromatography / Periodic counter-current chromatography

Received: September 25, 2017; *revised:* March 23, 2018; *accepted:* March 26, 2018

DOI: 10.1002/elsc.201700159

1 Introduction

Downstream processing plays a key role in providing pure and safe biopharmaceuticals. Therefore, about 80% of the whole process costs have to be invested in the purification of the product [1,2]. In this context, the interest in continuous purification techniques increases because they can improve the productivity. As chromatography can be used for almost every separation task and is often the method of choice, it offers great potential for optimization by continuous operation [3].

Considering the high costs for downstream processing, continuous chromatographic methods, such as periodic counter-current chromatography (PCCC), could replace conventional batch chromatography [4]. Periodic counter-current chromatography is a pseudo-continuous chromatographic method that uses a column switching approach.

In PCCC, three or more columns are used to carry out the chromatography continuously [5]. Two columns are connected in series during the loading steps, so that the product breakthrough of the first column is guided directly to a second column [5,6]. This approach allows for a loading of the column up to nearly its static binding capacity [5] and results in a higher capacity utilization of the resin [4,6]. In contrast to batch chromatography, the loading can be carried out beyond the dynamic binding capacity [5,6].

The introduction of continuous chromatography has few disadvantages concerning mostly acquisition and setup, but as shown in Table 1, its advantages compared to batch chromatography prevail. Furthermore, the disadvantageous aspects mainly refer to the non-recurring costs while significantly reducing the running costs.

As an alternative to conventional bed columns, disposable membrane adsorbers (MA) can be used in continuous

Correspondence: Dr. Sascha Beutel (beutel@iftc.uni-hannover.de) Institute of Technical Chemistry, Gottfried Wilhelm Leibniz University of Hanover, Callinstr. 5, 30167 Hanover, Germany

Abbreviations: A280, absorption at 280 nm; **AEX**, anion-exchange chromatography; **CalB**, *Candida antarctica* lipase B; **CV**, column volume; **CWW**, cell wet weight; **HIC**, hydrophobic interaction chromatography; **MA**, membrane adsorber; **MW**, molecular weight; **PCCC**, periodic counter-current chromatography

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering in Life Sciences
www.els-journal.com
Eng. Life Sci. 2018, 0, 1–11

Table 1. Advantages and disadvantages of the PCCC

Advantages	Disadvantages
• More effective use of resin [31, 32], less buffer consumption, higher productivity • Processing costs savings about 5–30% [26] • FDA advocates continuous techniques [33] • Smaller facilities, higher flexibility [34]	• Higher residence times • Four times higher costs for acquisition [26] • Complex constructions • Planning/Handling more complex [35]

chromatographic systems to further improve the process. Ligands are attached to the membrane surface so that mass transport is mainly based on convection and not on pore diffusion [7]. In addition to high mechanical stability, membrane adsorbers also show a strong salt- and pH-tolerance [8]. The capacity of membrane adsorbers is independent of the flow rate [9] and allows for shorter cycle times. Nevertheless, the breakthrough capacities of columns and membrane adsorbers are comparable [10]. In addition, up- and downscaling the membrane adsorber is much easier, as the capacity only depends on the membrane surface area [11].

PCCC with membrane adsorbers can be used to purify enzymes that are of high biotechnological interest. The enzyme class of hydrolases includes the lipases that have attracted profound scientific interest, as no co-factor is needed for their enzymatic reaction. Lipases can cleave ester bonds in the presence of water, synthesize ester in the absence of water and are stereospecific. Therefore, lipases are used in the detergent, cleaning, and food industries [12–14].

Candida antarctica lipase B (CalB) (EC 3.1.1.3) is one of the lipases most widely used in biocatalysis [15]. CalB consists of 317 amino acids and has a size of 33 kDa [16]. Advantageous characteristics of CalB are e.g. the broad substrate spectrum, the high pH- and temperature stability (until 60°C) as well as the activity in aqueous and organic solvents [17]. Figure 1 shows the

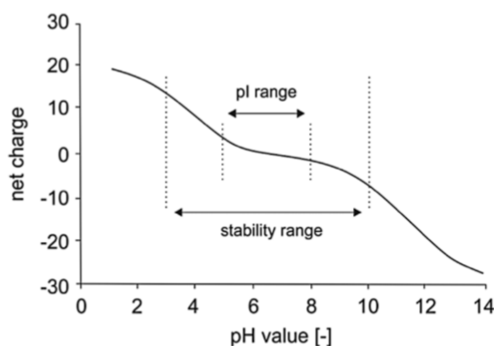


Figure 1. Net charge of CalB as a function of pH, modified according to [18]. CalB is stable from pH 3 to 10 due to the pI range from pH 5 to 8.

net charge of CalB depending on the pH value. At the isoelectric point (pI), a protein is uncharged. Since CalB has no titratable side chains in the range of pH 5–8 [18], the total charge does not change in this pH range, and the net charge is 0 [17]. As a result, CalB is stable in a pH range of pH 3–10. The theoretical *pI* value of CalB is pH 6.0 [18].

CalB is used in the detergent industry [15], in the production of fruit esters or L-menthol, the synthesis of geranyl acetate (odorous component in food and perfume), as well as the removal of malodor of sheep and goat milk products [19–22].

Enzymes like CalB are produced recombinantly in microorganisms such as *E. coli, Aspergillus niger* or *Pichia pastoris* (new: *Komagataella phaffii*). In order to use CalB in different industries for various purposes, it must be partially purified. Different chromatographic purification methods are described in the literature [18], [23–25]. In particular, ion-exchange chromatography is applied to the purification of CalB, which is rather difficult due to the broad range of the pI value. Apart from ion-exchange chromatography, hydrophobic interaction chromatography (HIC) and biomimetic affinity chromatography (B-AC) are used. Even though the methods are described in the literature, each method has disadvantages, such as a low capacity of the resin, low recovery or loss of activity.

In this study, the purification of CalB should be improved by using membrane adsorbers combined with a continuous chromatography device. HIC and AEX (anion-exchange chromatography) membrane adsorbers are tested under different buffer conditions in batch mode and compared to bed column methods [18], [23–25]. To increase the productivity of the process, the established downstream process is further transferred to the continuously operated three membrane adsorber periodic countercurrent chromatography (3MA-PCCC) device. The batch process is compared with the continuous process with regard to the quality and quantity of the purification, the usage of consumables and the process time.

2 Materials and methods

2.1 Materials

Commercial CalB was purchased from c-LEcta, Germany, and pre-filtered before usage with a 0.22 μm filter. *Para*-nitrophenyl acetate (*p*-NPA) and BSA were provided by Sigma Aldrich, USA, whereas lysozyme was provided by Fluka (Sigma Aldrich), USA.

2.2 Methods

2.2.1 CalB production

CalB was expressed *in E. coli* Rosetta 2 (DE3) pLysS::pET26B(+). Therefore, the sequence of an adapted *Candida antarctica* (LF 058) gene was used for lipase B (GenBank Z30645.1). For cloning restriction enzymes and DNA, ligase was chosen. The plasmid was introduced into the bacteria by heat transformation. For the expression, the cryo culture was grown on lysogeny broth(LB)-agar and incubated for 24 h at 37°C. One colony was picked and the pre-preculture in terrific broth(TB)-media (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 100 mL/L

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering in Life Sciences
www.els-journal.com
Eng. Life Sci. 2018, 0, 1–11

of solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) was inoculated (20 mL in 100 mL shake flask) and cultured overnight at 37°C and 180 rpm. The preculture was inoculated using a pre-preculture to a starting OD_{600} = 0.2 AU (100 mL in 500 mL shake flask). The preculture was incubated for 2 h at 37°C and 180 rpm in TB media. The main culture was started with OD_{600} = 0.1 AU (400 mL in 2 L shake flask) in TB-media. The culture was induced after 2 h of cultivation at 37°C and 180 rpm using 0.8 mM IPTG and a temperature shift to 20°C.

Cell lysis was performed four times by sonification for 45 s on ice (amplitude 100%, 100 W, at time intervals of 0.6 s) in equilibration buffer. A centrifugation and sterile filtration step was carried out to yield cell lysate for the purification by chromatography.

2.2.2 *CalB batch purification*

The purification was developed using a commercial FPLC system. In general, a chromatography run consisted of the following phases: 15 CV (column volume) equilibration, 5 mL sample application, 10 CV wash, 25 CV elution and regeneration. Depending on the experiment, the elution phase was adjusted. For AEX, three buffer conditions were tested: 0.02 M TRIS-HCl) was inoculated (20 mL in 100 mL shake flask) and cultured overnight at 37°C and pH 8.5 and elution with 1 M NaCl, 0.02 M TRIS-HCl pH 8.5 and elution at pH 7.0, 0.05 M ammonium acetate pH 8.5 and elution using 1 M NaCl according to Llerena-Suster *et al.*, 2014 [24]. For HIC, one buffer was tested according to Ujiie *et al.*, 2015 [25]: 0.02 M TRIS-HCl, pH 8.0 + 0.8 M ammonium acetate and elution with decreasing salt concentration. The chromatography was run at room temperature at a flow rate of 5 mL/min. The developed batch method was further transferred to a continuous chromatographic system.

2.2.3 *CalB continuous purification*

The PCCC device was tested and placed into operation using the model proteins BSA (as the product) and lysozyme (as the impurity), thus providing a simple two component purification task to evaluate the system. The overall loading and elution cycle was developed with an Äkta pure system for the membrane adsorber capsules. The optimal cycle procedure (buffer system, flow rate, duration of binding and elution) was transferred to the PCCC system and controlled by a UV signal.

Both the limit of detection (LOD) and quantification (LOQ) for the PCCC were determined employing the data of the calibration curve and the following equations:

$$\text{LOD} = \frac{3 \cdot \text{Standard deviation of the lowest concentration}}{\text{Slope of calibration curve}} \quad (1)$$

$$\text{LOQ} = \frac{10 \cdot \text{Standard deviation of the lowest concentration}}{\text{Slope of calibration curve}} \quad (2)$$

The basic PCCC cycle is presented in Fig. 3. In the first step, membrane adsorber 1 is loaded until a certain amount of the product breaks through. The process switches to step two, and membrane adsorber 2 is added to the loading. The breakthrough of membrane adsorber 1 is bound to membrane adsorber 2. Step two stops when membrane adsorber 1 is saturated with product [4, 5]. The membrane adsorber is decoupled from the circuit, the product is eluted separately and the adsorber is regenerated and coupled back into the circuit. The described procedure for membrane adsorber 1 is continued using membrane adsorber 2 and 3 [6]. By parallel washing, elution and regeneration, after step six, the process can start again at step one. To fulfill this cycle, the decoupling time for regeneration and elution of one membrane adsorber must be less than or equal to the loading time [4].

To switch from one step to another, two automated switching events are required for the PCCC [5]:

- The first switching condition (SC1) is the product breakthrough with a predefined loss (defined as the dynamic binding capacity).
- The second switching condition (SC2) indicates the defined saturation of the membrane adsorber [4, 5].

Figure 4 shows the double breakthrough curve with the switching events of the simple two-component purification task using the PCCC device. This switching approach is based upon the UV absorption difference (ΔUV) of the solution to be purified and the membrane adsorber outlet (measured UV signal) [4], [5]. UV_{max} is the UV signal of the unpurified solution of BSA and lysozyme, and $UV_{Lysozyme}$ is the UV signal of the non-binding lysozyme in the solution. The double breakthrough curve was recorded by feeding the solution onto one membrane adsorber. The non-binding lysozyme immediately breaks through, and the characteristic plateau of the impurity is visible (Fig. 4). Gradually, the membrane adsorber is saturated with product so that a product breakthrough can be measured at the outlet of the membrane adsorber.

According to Warikoo *et al.* [5], SC2 was set to 70% in the three-module system. An SC1 at 10% is regarded as a standard value for dynamic binding capacity-controlled processing. To determine these two switching events, the solution of BSA and lysozyme was directly injected into the cuvette and UV_{max} was measured. $UV_{Lysozyme}$ was determined by recording the double breakthrough curve of the solution to be purified. Subsequently, SC1 and SC2 could be calculated using the following equations.

$$\text{SC1} = 0.1 \cdot \Delta\text{UV} + \text{UV}_{\text{Lysozyme}} \quad (3)$$

$$\text{SC2} = 0.7 \cdot \Delta\text{UV} + \text{UV}_{\text{Lysozyme}} \quad (4)$$

Depending on the product, SC1 and SC2 can be customized. To fulfill the principle of the PCCC, SC2 should not lead to a product breakthrough on the second membrane adsorber (see Fig. 3 step 2).

2.2.4 *Analytical methods*

2.2.4.1 Determination of protein content using the Bradford assay. To determine the total protein concentration in an aqueous solution, the colorimetric Bradford method was applied. For concentration determination, 20 μL of the sample were mixed

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering in Life Sciences
www.els-journal.com
Eng. Life Sci. 2018, 0, 1–11

with 300 μL of the Bradford reagent (Quick Start™ Bradford 1× DyeReagent, Bio-Rad) in a microtiter plate, shaken for 30 s and incubated for 5 min. At 595 nm, the samples were measured. BSA and CalB standards of 0.0125–0.2 g/L were used for calibration.

2.2.4.2 Qualitative analysis using SDS-PAGE with silver staining. SDS-PAGE was used for qualitative evaluation. The gel consisted of a collecting gel (6%) and a separating gel (12%). The samples were mixed with Lämmli buffer in the ratio 1:1 and boiled for 10 min at 95°C. 3 to 10 μL of the sample were used for the gel compared to 5 μL of the marker (Unstained Protein Molecular Weight Marker, Thermo Fisher Scientific). The SDS-PAGE was run for 15 min at 100 V and then for 45–60 min at 150 V in TGS buffer. To visualize the protein bands, silver staining was used.

2.2.4.3 Determination of CalB activity. The activity assay with *para*-nitrophenyl acetate (*p*-NPA) was used to determine the activity of *Candida antarctica* lipase B. *p*-NPA is hydrolyzed to *para*-nitrophenol and acetate. The reaction can be measured photometrically at 405 nm (*para*-nitrophenol). The assay was performed in 50 mM phosphate buffer at pH 7.4 and room temperature. The molar extinction coefficient of 18,450 $M^{-1}cm^{-1}$ for *para*-nitrophenol was applied.

2.3 Equipment

2.3.1 Membrane adsorbers

Two types of membrane adsorber units, Sartobind® Q 75 (AEX) and Sartobind® Phenyl nano 3 mL (HIC) from Sartorius Stedim Biotech, Germany, were employed for the experiments in this study.

2.3.2 Äkta pure

For method development, the chromatographic system Äkta pure (GE Healthcare) was used in combination with membrane adsorbers.

2.3.3 3MA-PCCC setup

For the continuous purification of CalB, a three-membrane adsorber periodic counter-current chromatographic device was used. This PCCC device was designed and built at the Institute of Technical Chemistry, Hanover. The circuit diagram can be seen in Fig. 2.

The PCCC device consists of a peristaltic pump (Type Pump Reglo ICC, Ismatec, Germany) with a pump rate of up to 8.6 mL/min, depending on the pump tubing. The channels are used for equilibration buffer/buffer A (A1), elution buffer/buffer B (B1) and feed (L). The mixing chamber (Type Dynamic Mixer GT0387, Hitachi, Japan) can be applied to realize different elution strategies, such as step elution or linear gradient elution. The interconnection of 21 magnetic 3-2-way valves (E1–E6, F1–F8, M1–M7) and three membrane adsorbers (MA1, MA2, MA3) is required to realize the principle of a periodic counter-current chromatography. Valves (The Lee company, USA) designated with E are exclusively used for buffer A or B, valves with F for feed solution and valves with M are necessary to connect the membrane adsorbers. Further components are three UV flow through cuvettes with a 10 mm path length (Hellma Analytics, Germany), three UV spectrometers (Ocean Optics, USA) (UV1–UV3, 190–650 nm), a fraction collector (Type Model 2110, BioRad, USA) and a light source (Type DH-2000-BAL, Ocean Optics, USA) for wavelengths between 230 and 2500 nm. For the experiments, a wavelength of 280 nm was applied for protein detection.

3 Results

3.1 Purification of *Candida antarctica* lipase B

The purification of CalB is problematic due to the wide p*I* range, so that no optimal strategy has yet been developed with conventional bed columns. Mainly, ion-exchange chromatography has been used, which requires the CalB to be charged. Due to the pI range of CalB, it must be operated under pH 5 (positively charged) or above pH 8 (negatively charged). Additionally, CalB should not be affected in its activity in these pH ranges.

In this study, two membrane adsorbers were tested: one HIC MA (Sartobind® Phenyl) and one AEX MA (Sartobind® Q). AEX was run under three different buffer conditions: an ammonium acetate buffer, such as published by Llerena-Suster *et al.*, 2014 [24] and two TRIS buffers with pH elution and salt elution that have not as yet been published. HIC was performed using a TRIS buffer, as described in Ujiie *et al.*, 2015 [25]. The method development, including the binding experiments (using linear elution) and optimization (with elution steps) of the method were carried out by means of commercial CalB. This allowed for a statement regarding recovery and binding capacity. The commercial CalB was not pure and had a high viscosity, so that the dosage did vary during the experiments for method development.

3.2 Downstream processing for commercial CalB

3.2.1 Comparison of methods

Comparing the methods using HIC- and AEX membrane adsorbers and different buffer systems (data not shown), the best result could be achieved using the AEX method and TRIS buffer salt elution. This method had a recovery rate of more than 99% when employing commercial CalB, where the specific activity of CalB in the buffer system was 73 U/mg. Under these conditions, CalB was charged negatively.

The AEX method with pH elution showed comparable results but the activity decreased during elution. The binding experiment for AEX with ammonium acetate buffer did not indicate any binding of CalB, while HIC produced a recovery of only 56%.

3.3 Optimization and binding capacity using commercial CalB

The filtered 0.3 g/L CalB solution (254 U/mg) was placed onto the membrane adsorber, and an elution step of 20% buffer B was added to separate CalB from impurities. The method included

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering
in Life Sciences
www.els-journal.com
Eng. Life Sci. 2018, 0, 1–11

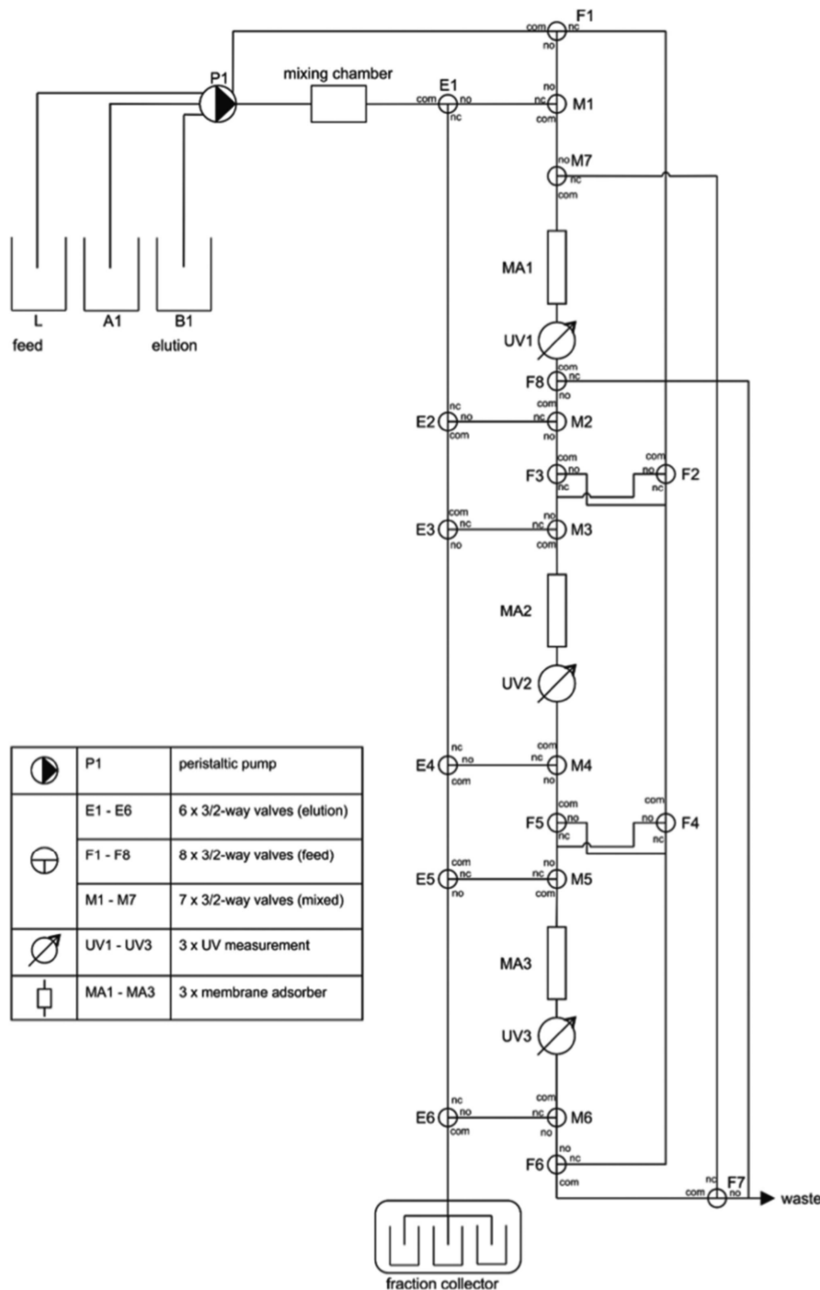


Figure 2. Circuit diagram of the PCCC device with three membrane adsorbers. The system consists of a peristaltic pump, 21 3/2-way valves, 3 membrane adsorbers and 3 UV measurement units. The valves E1 - E6 are used for buffer A and B, the valves F1 - F8 for the feed solution and M1 - M7 to connect the membrane adsorbers according to the principle of the PCCC.

further elution steps at 50 and 100% buffer B. This modification (Fig. 5A) resulted in a high peak of CalB at 20% buffer B due to a weak binding to the adsorber. The peak was very pure (Fig. 5B) and showed a high specific activity of 217 U/mg. Impurities were eluted at 50% buffer B. The recovery of this method was higher than 99%.

To determine the capacity, a breakthrough curve with 0.11 g/L CalB solution was recorded. The dynamic binding capacity at 10% (DB_{10}) was 4.2 mg (56 μg/cm^2) and when

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering in Life Sciences
www.els-journal.com
Eng. Life Sci. 2018, 0, 1–11

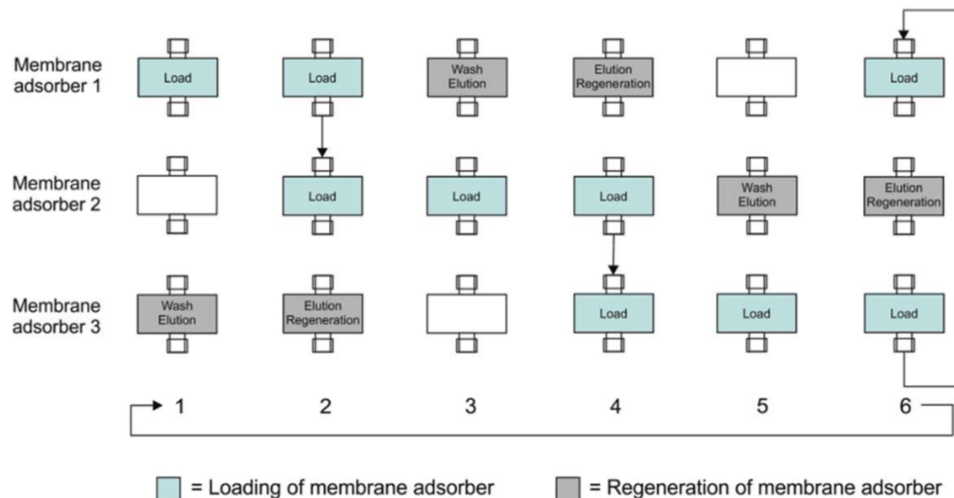


Figure 3. Schematic diagram of a continuous 3MA-PCCC cycle. The three membrane adsorbers are loaded, washed, eluted and regenerated following the principle of periodic counter-current chromatography. The first membrane adsorber is loaded until a certain amount of product breaks through. Then the second membrane adsorber is added to the loading (loading zone, light blue) until saturation of membrane adsorber 1. Membrane adsorber 2 is loaded according to the same principle. Meanwhile membrane adsorber 1 is regenerated (regeneration zone, gray).

compared to the manufacturer's information, with 60 mg BSA per unit, it was quite low. The static binding capacity (DB_{50}) was 6.3 mg (84 μg/cm^2) and was 0.5 times higher than the dynamic binding capacity. Compared to the published capacity of CalB in an affinity column [23], the dynamic binding capacity is 5-fold and the static binding capacity is 7.5-fold higher, even with this far less specific interaction.

3.4 Purification of CalB from *E. coli* lysate

The procedure described in 3.3 for the purification of the CalB was applied to *E. coli* lysate containing 1.7 g/L total protein and 55 U/mg specific activity of CalB. This lysate included host cell proteins and possibly DNA (not tested). The result of the chromatography and the SDS-PAGE are presented in Fig. 6. The breakthrough was not analyzed as the applied amount of CalB was below the dynamic binding capacity at 10% and no product breakthrough was expected. At 20% buffer B, CalB eluted in a sharp peak (Peak 1). On the gel it is evident that CalB fractions are highly pure when compared to cell lysate. Only within the first fraction other bands are visible. In addition, a high specific activity (223 U/mg) could be measured in the fractions of peak 1. The recovery of CalB was 97%. Peaks 2 and 3 did not contain any CalB, however impurities (host cell proteins) are visible in the cell lysate. As the peaks of the elution steps of 50% buffer B and 100% buffer B contained impurities, but no CalB, the method was simplified for the PCCC. The elution step at 50% buffer B was eliminated, so that the method consists of two steps: a 1st elution step of CalB at 20% buffer B and a 2nd step for the elution of all impurities at 100% buffer B. This promising method for capture and purification of CalB had a purification factor between 1.3 and 4.0, and was transferred to the 3MA-PCCC device.

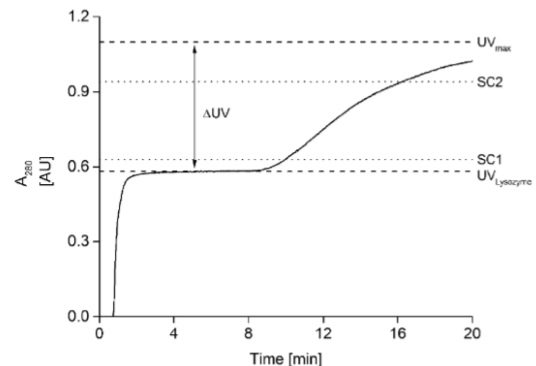


Figure 4. Double breakthrough curve for the determination of the switching conditions with the 3MA-PCCC. The switching conditions 1 and 2 can be determined using the UV signal of the impurity ($UV_{Lysozyme}$) and the UV signal of the solution to be purified (UV_{max}). The difference of UV_{max} and $UV_{Lysozyme}$ is ΔUV. Here SC1 is 10% of ΔUV and SC2 is 70% of ΔUV.

3.4.1 Preparations for PCCC

The developed optimal downstream procedure with step elution at 20% buffer B for CalB and 100% buffer B for impurities was transferred to the PCCC device. The Äkta pure system and the PCCC device have a major difference in the detection system: the

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering in Life Sciences
www.els-journal.com
Eng. Life Sci. 2018, 0, 1–11

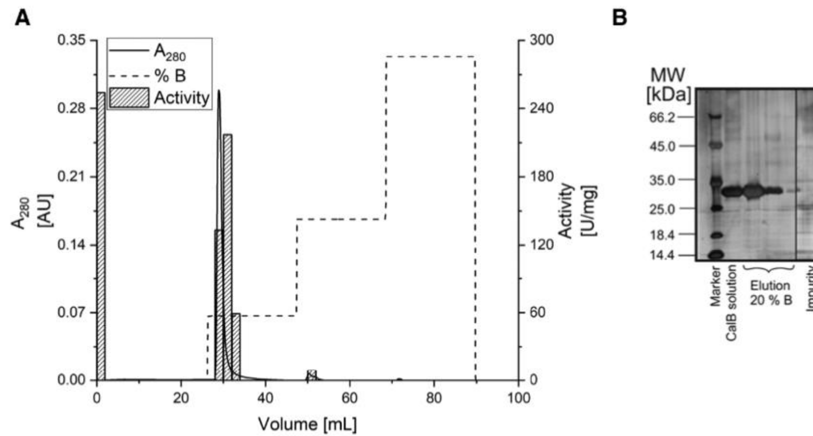
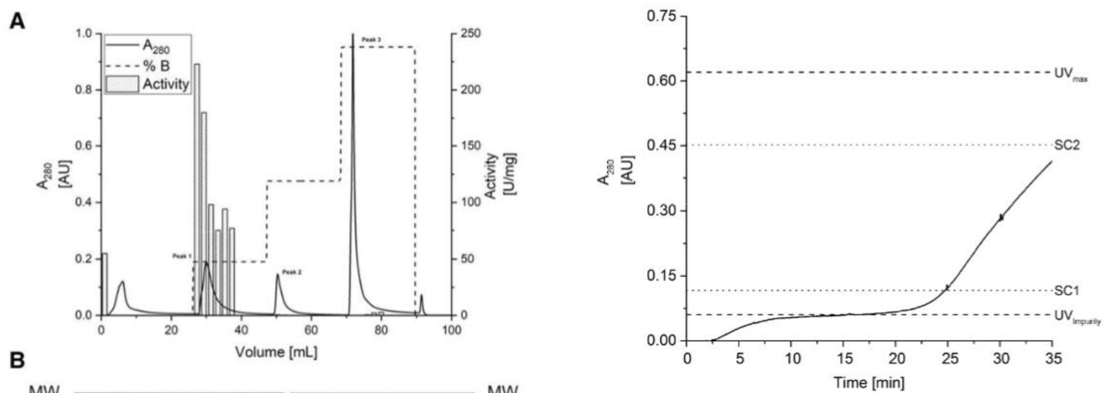


Figure 5. (A) Chromatogram of the binding experiment using AEX-MA–step elution using 20, 50, and 100% buffer B. (B) SDS-PAGE of the binding experiment using AEX-MA.



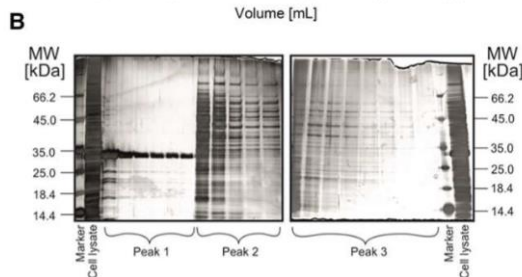


Figure 6. (A) Chromatogram of the method for the purification of CalB in lysate with elution at 20, 50, and 100% buffer B: Sartobind® Q with TRIS-HCl pH 8.5 at 5 mL/min. (B) SDS-PAGE of the method development for the purification of CalB from *E. coli* lysate.

Figure 7. Double breakthrough curve for the determination of $UV_{Impurity}$ for CalB purification. UV_{max} was measured in the cuvette.

Åkta system has a 5-fold smaller path length (2 mm) compared to the PCCC system (10 mm). Since the PCCC system can measure up to 1.5 AU (linear absorption range with a 10 mm cuvette), the lysate was diluted.

To measure CalB in the linear range, a calibration curve was recorded in the range of 0.0625–1.5 g/L commercial CalB. All measuring points were in the linear measuring range below 1.5 AU. As commercial CalB is not highly purified, deviations occurred between the theoretical and practical values. In addition, the buffer system (TRIS buffer) may have disturbed the measurement. The following limits of the measurement were determined from the calibration curve: The LOD for CalB was 0.04 g/L, and the LOQ was 0.13 g/L.

The feed solution for continuous purification consisted of *E. coli* lysate of 0.15 g cell wet weight (CWW)/L containing 0.37 g/L total protein in 20 mM TRIS-HCl buffer of pH 8.5. To determine the switching conditions for the PCCC, UV_{max} was determined in the cuvette and the double breakthrough curve for the determination of $UV_{Impurity}$ was recorded (see Fig. 7). UV_{max} was 0.620 AU and $UV_{Impurity}$ was 0.070 AU. As determined in the PCCC setup, SC1 was 10% (0.116 AU) and SC2 was 70% (0.452 AU). SC1 was above the detection limit (data not shown) and large enough to be measured.

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering in Life Sciences
www.els-journal.com
Eng. Life Sci. 2018, 0, 1–11

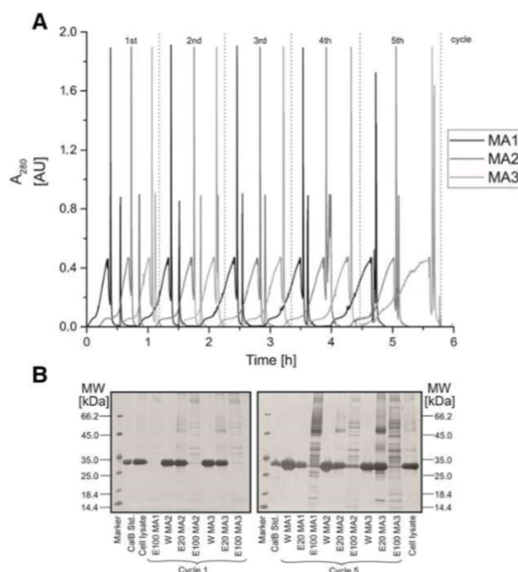


Figure 8. (A) PCCC for the purification of CalB with five cycles (impurity breakthrough, breakthrough of product and other binding components, elution of membrane adsorber 1–3 in two steps: 20% buffer B, 100% buffer B). (B) SDS-PAGE of cycle 1 and 5 of the PCCC run for the purification of CalB (W = Wash; E20 = Elution of CalB at 20% buffer B; E100 = Elution of impurities at 100% buffer B).

3.4.2 PCCC using *E. coli* lysate containing CalB

The PCCC cycle (Fig. 3) was adjusted by setting the switching conditions from 3.4.1. Due to the low binding capacity of CalB to the membrane adsorber, the loading pump rate was reduced to 2.5 mL/min, whereas the regeneration pump rate was increased to 8.6 mL/min. Furthermore, this adaptation guaranteed that the loading lasted longer than the regeneration of the membrane adsorber to enable continuous processing. The capacity of the membrane adsorber did not change as a result of the pump rate. The purification of 0.9 L feed of *E. coli* lysate using 0.15 g CWW/L containing 0.37 g/L total protein took 6 h and needed 3 L buffer (A and B). 0.22 g CalB was achieved over 5 PCCC-cycles with three membrane adsorbers before a visible loss of capacity occurred which might be due to unspecific binding of host cell proteins. The elution was divided into two phases during the purification of CalB: product elution at 20% buffer B, elution of the impurities at 100% buffer B. The result of the PCCC run is shown in Fig. 8A.

The typical double breakthrough curve of the PCCC is more difficult to see in this experiment since the impurity plateau is substantially flatter (0.07 AU). Although the concentration of the lysate was adapted to the PCCC, proteins which bind to the membrane adsorber were present in the lysate. This nonselective purification of CalB is not optimal for the application of the PCCC, but could nevertheless be operated over five cycles. Up to cycle 4, the double breakthrough can be seen with all membrane adsorbers. In cycle 5, the UV signal of membrane adsorber 3 is widened and irregular. The breakthrough curves were broadened during the cycles of the PCCC and the loading time until SC2 increased, while the subsequent membrane adsorber was loaded with the breakthrough. As a result, SC2 led to a product breakthrough on the following membrane adsorber, and the time window for the elution was reduced, which only led to the 100% buffer B elution in the last cycle. Cycles 1 and 5 were sampled. The samples from the phases of washing, elution at 20% buffer B and elution at 100% buffer B were each pooled in a uniform proportion. The pooled samples of the elution which peaked at 20% buffer B had protein concentration values of 0.24–0.31 g/L and showed an increased specific activity compared to the applied cell lysate (Table 2).

Table 2. Bradford analytics and activity of the cell lysate and product peaks at 20% buffer B in cycle 1 (C1) and 5 (C5)

Sample	Protein concentration [g/L]	Activity [U/mL]	Specific activity [U/mg]
Cell lysate	0.37	15.9	43
MA1 C1	n.d.[a]	n.d.	–
MA2 C1	0.27	19.7	73
MA3 C1	0.30	18.5	62
MA1 C5	0.24	14.5	60
MA2 C5	0.31	17.7	57
MA3 C5	n.d.	n.d.	–

[a] n.d.: not determined (technical problems).

To evaluate the quality of the purification, an SDS-PAGE was performed (Fig. 8B). The fractions of the wash steps (W) and the elution steps (E20 or E100) can be seen in the gels. For membrane adsorber 1, the samples of the wash step and the elution are missing at 20% buffer B due to technical problems of the fraction collector. In cycles 1 and 5, thick bands at the size of CalB can be seen during the elution at 20% buffer B. In cycle 1, only impurities and no product eluted at 100% buffer B. In cycle 5, the fractions of the step elution at 100% buffer B contained higher amounts of impurities and had bands at the level of CalB. During the washing step, a product loss occurred, as the protein solution in the tubes, pipes and valves was pumped into the waste. To prevent this loss, the wash step could be applied to a regenerated membrane adsorber (white box in Fig. 3). This complement is called an interconnected wash step [26, 27] and requires a modification of the PCCC device with more valves.

Both gels show that CalB could be eluted highly purified. The CalB concentrations in the eluate were above 1 g/L (calibration curve not shown). The yield was 0.22 g CalB and corresponds to a recovery of 80%. The quality of chromatography decreased during the PCCC, which is clearly evident in the chromatogram and in the SDS-PAGE.

4 Discussion

In this study, a method for the purification of *Candida antarctica* lipase B (CalB) was developed using the AEX membrane adsorber Sartobind® Q 75. The buffer of choice was 20 mM TRIS-HCl, pH 8.5 and the elution of both CalB and bound impurities was carried out using 0.2 M NaCl (20% buffer B) and

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering in Life Sciences
www.els-journal.com
Eng. Life Sci. 2018, 0, 1–11

Table 3. Comparison of methods for the purification of CalB from the literature and the own method

Method	Recovery	Activity	Purity	Capacity	Purification factor
CEX [18]	-	-	-	-	1.5
B-AC [23]	73%	-	91%	0.4 mg/mL CV	-
AEX [24]	47%	18.8 U	-	-	1.38
HIC [25]	23%	90 U	-	-	1.29
Own method	**97%**	**223 U/mg or 91 U/mL, activity of *E. coli* lysate: 55 U/mg or 93 U/mL**	**high**	**2 mg/mL CV**	**Up to 4.0**

Table 4. Comparison of PCCC and batch for purification of CalB

Batch	PCCC
• Yield: 11.25 mg CalB in 0.44 h	• Yield: 0.22 g CalB in 6 h
• Consumption of 150 mL buffer	• Consumption of 3 L buffer
• Recovery of CalB 87% (3% loss of method + 10% dynamic binding capacity)	• Recovery of CalB 80% (90% with interconnected wash step)
• Specific activity of cell lysate: 55 U/mg	• Specific activity of cell lysate: 43 U/mg
• Specific activity of purified CalB: 223 U/mg	• Highest specific activity of purified CalB: 73 U/mg

1 M NaCl (100% buffer B), respectively. The method was developed by applying commercial CalB. The dynamic binding capacity reached 56 $\mu g/cm^2$ commercial CalB and the static binding capacity 84 $\mu g/cm^2$ commercial CalB when the membrane adsorber Sartobind® Q 75 was used. Finally, the purification was performed using *E. coli* lysate. The purification of CalB from lysate led to a high specific activity of 223 U/mg (where lysate with host cell proteins were 55 U/mg) and a high recovery of 97%. The CalB fractions thus showed a high purity. As shown in Table 3, the developed method for CalB had very good characteristics compared to the published methods with conventional bed columns: high recovery, high activity and high purification factor. The purity could not be determined as a numerical value due to the silver staining, but was visually evaluated to be high.

This promising method for capture and purification of CalB was transferred to the 3MA-PCCC device. Within the scope of the PCCC, 0.9 L feed of *E. coli* lysate containing 0.15 g CWW/L and 0.37 g/L total protein was purified within 6 h. CalB could be eluted actively, in high purity (visually evaluated) and with a concentration of up to 1 g/L. The yield was 0.22 g with a recovery of 80%. Using the interconnected washing step, another 10% CalB could be recovered.

The comparison to the batch chromatography is shown in Table 4. In contrast to batch operation, the use of the PCCC led to a productivity increase of 36%[1]. In addition, about 20% of the buffers were saved. This productivity increase cannot be assumed for every purification task. But for these experiments, the productivity increase of 36% is attributed to the loading principle of the PCCC (see Fig. 3) and the usage of membrane adsorbers. As the membrane adsorber is loaded to 70% instead of 10% of the product breakthrough, the capacity of the membrane adsorber is more efficiently utilized. In addition, the loading of the membrane adsorber is combined, so that the product breakthrough is captured on another membrane adsorber. The binding capacity of membrane adsorbers is independent of the flow rate [9]. Hence the loading pump rate could easily be adjusted to fulfill the PCCC principle. This is a major advantage compared to conventional bed columns as here the flow rate strongly affects the capacity due to pore diffusion.

Since the method for the purification of CalB from lysate also binds impurities, elution was carried out in two steps using 20 and 100% buffer B, respectively. This is not optimal for the use of the PCCC, as the binding impurities lead to a gradual decline of the process performance. The quality of the chromatography was influenced because the phases of the method overlapped after 5 cycles. Considering that the lysate contained binding host cell proteins and possibly DNA, a decrease in the performance was predictable. An additional CIP (cleaning in place) step and an intense back-flushing of the whole system would be useful.

The method for the purification of CalB was proved to be feasible in the PCCC, but under the restriction that the number of cycles was limited due to a deterioration in the performance. The nonselective binding of CalB was not optimal for the application of the PCCC. Therefore, the removal of binding impurities should be considered in a previous step, or a chromatographic method which exclusively binds CalB should be taken into account.

In the PCCC washing steps, 10–15% of the product was lost. This problem has already been described in the literature [26,27] and can be solved by changing the system. By passing the wash step onto a further membrane adsorber, the product is not lost. For this modification, additional valves and tubings are necessary and are highly recommended.

The membrane adsorbers were loaded up to five times in the experiments. The manufacturer claims that 100 loadings are possible, before a regeneration step should be carried out [28]. This statement is most likely related to the batch mode (about 10% product breakthrough). In the conducted experiments, the membrane adsorbers were loaded up to a value of 70%. Therefore, it is questionable to what extent the manufacturer's data apply to the use in a PCCC system.

The PCCC device should be operated with specifically binding products such as antibodies or His-tagged proteins (affinity chromatography). The purification of monoclonal antibodies is almost exclusively reported in the literature for PCCC application [4, 26, 29, 30]. Secreted products could be purified more easily using the PCCC, as extracellular products in a supernatant

[1]The productivity was calculated according to Kaltenbrunner *et al.* [36] using the following equations:

$$P_{Batch} = \frac{m_{Cycle}}{V_{Column} \cdot (t_L + t_T)} = \frac{11\ mg}{2.1\ mL \cdot (0.44\ h + 0.15\ h)} = 8.88 \frac{mg}{mL \cdot h}$$

$$P_{PCC} = \frac{m_{Cycle}}{V_{Column} \cdot N_{Column} \cdot t_L} = \frac{44\ mg}{2.1\ mL \cdot 3 \cdot 0.58\ h} = 12.04 \frac{mg}{mL \cdot h}$$

$$\frac{P_{PCC}}{P_{Batch}} = 1.36$$

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering in Life Sciences
www.els-journal.com
Eng. Life Sci. 2018, 0, 1–11

do not contain host cell proteins or DNA, which influence the performance of the chromatography. In any case, the product has to be detected with sufficient accuracy to fulfill the principle of the PCCC. The long-term goal is to integrate the system into a continuous manufacturing process.

Even though small additions to the system are necessary to achieve an optimal result, the periodic counter-current chromatography device was successfully put into operation and further used for the continuous purification of *Candida antarctica* lipase B. It could be confirmed that the productivity was increased compared to batch chromatography for this application.

Practical application

This study presents the establishment of a self-built continuous chromatographic device. The system is operated using three membrane adsorbers according to the principle of periodic counter-current chromatography. The application of this continuous chromatographic method increases the productivity of a purification process by saving consumables, time, and by better utilizing the capacity of membrane adsorbers. The productivity of the purification of *Candida antarctica* lipase B could be enhanced by as much as 36%. In order to evaluate the potential of this continuous chromatographic device, it was operated using affinity chromatography interactions (His-tagged proteins, antibodies). The application of periodic counter-current chromatography is particularly reasonable for very expensive products and resins, such as antibody purification, as its usage may result in significant cost savings for the purification process.

The studies were carried out within the frame of project P38 of the BMBF-Biokatalyse2021-Cluster, hosted by Prof. Garo Antranikian. We would like to thank the German Federal Ministry of Education and Research for their financial support.

The authors have declared no conflict of interest.

5 References

[1] Rathore, A. S., Agarwal, H., Sharma, A. K., Pathak, M., Muthukumar, S., Continuous processing for production of biopharmaceuticals. *Prep. Biochem. Biotechnol.* 2015, *45*, 836–849.

[2] Jungbauer, A., Continuous downstream processing of biopharmaceuticals. *Trends Biotechnol.* 2013, *31*, 479–492.

[3] Wolfgang, J., Prior, A., *Continuous Annular Chromatography* 2002, *76*.

[4] Godawat, R., Brower, K., Jain, S., Konstantinov, K., Riske, F., Warikoo, V., Periodic counter-current chromatography–design and operational considerations for integrated and continuous purification of proteins. *Biotechnol. J.* 2012, *7*, 1496–1508.

[5] Warikoo, V., Godawat, R., Brower, K., Jain, S., Integrated continuous production of recombinant therapeutic proteins. *Biotechnol. Bioeng.* 2012, *109*, 3018–3029.

[6] Steinebach, F., Müller-Späth, T., Morbidelli, M., Continuous counter-current chromatography for capture and polishing steps in biopharmaceutical production. *Biotechnol. J.* 2016, *11*, 1126–1141.

[7] Knudsen, H. L., Fahrner, R. L., Xu, Y., Norling, L. A., Blank, G. S., Membrane ion-exchange chromatography for process-scale antibody purification. *J. Chromatogr. A* 2001, *907*, 145–154.

[8] Wang, J., Macroporous ion-exchange membrane adsorbers: correlation between membrane structure, separation conditions and performance in bioseparation. Universität Duisburg-Essen, Fakultät für Chemie» Technische Chemie, 2009.

[9] Tennikova, T. B., Svec, F., High-performance membrane chromatography: highly efficient separation method for proteins in ion-exchange, hydrophobic interaction and reversed-phase modes. *J. Chromatogr. A* 1993, *646*, 279–288.

[10] Gebauer, K. H., Thömmes, J., Kula, M. R., Breakthrough performance of high-capacity membrane adsorbers in protein chromatography. *Chem. Eng. Sci.* 1997, *52*, 405–419.

[11] Demmer, W., Nussbaumer, D., Large-scale membrane adsorbers. *J. Chromatogr. A* 1999, *852*, 73–81.

[12] Houde, A., Kademi, A., Leblanc, D., Lipases and their industrial applications. *Appl. Biochem. Biotechnol.* 2004, *118*, 155.

[13] Balcão, V. M., Paiva, A. L., Malcata, F. X., Bioreactors with immobilized lipases: state of the art. *Enzyme Microb. Technol.* 1996, *18*, 392–416.

[14] Rahman, N. Z. R. A., Basri, M., *New Lipases and Proteases.* Nova Publishers:New York, 2006.

[15] Uppenberg, J., The three-dimensional structure of lipase B from Candida antarctica, 1994.

[16] Albrich, M. S., *Lipase B from Candida Antarctica in Bicontinuous Microemulsions: A Structural Study.* Shaker Verlag Gmbh, 2014.

[17] Trodler, P., Untersuchung von Lipasen-Elektrostatik, Selektivität und Einfluss von Lösungsmitteln auf Struktur und Dynamik, 2008.

[18] Trodler, P., Nieveler, J., Rusnak, M., Schmid, R. D., Pleiss, J., Rational design of a new one-step purification strategy for Candida antarctica lipase B by ion-exchange chromatography. *J. Chromatogr. A* 2008, *1179*, 161–167.

[19] Ferreira-Dias, S., Sandoval, G., Plou, F., Valero, F., The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries. *Electron. J. Biotechnol.* 2013, *16*, 12.

[20] Jin, Z., Ntwali, J., Han, S.-Y., Zheng, S.-P., Lin, Y., Production of flavor esters catalyzed by CALB-displaying Pichia pastoris whole-cells in a batch reactor. *J. Biotechnol.* 2012, *159*, 108–114.

[21] Berger, R. G., *Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability.* Springer-Verlag:Berlin, Heidelberg, 2007.

[22] Franssen, M. C. R., Alessandrini, L., Terraneo, G., Biocatalytic production of flavors and fragrances. *Pure Appl. Chem.* 2005, *77*, 273–279.

[23] Yao, H., Zhang, T., Xue, H., Tang, K., Li, R., Biomimetic affinity purification of Candida antarctica lipase B. *J. Chromatogr. B* 2011, *879*, 3896–3900.

[24] Llerena-Suster, C. R., Briand, L. E., Morcelle, S. R., Analytical characterization and purification of a commercial extract of enzymes: a case study. *Colloids Surfaces B Biointerfaces* 2014, *121*, 11–20.

[25] Ujiie, A., Nakano, H., Iwasaki, Y., Extracellular production of Pseudozyma (Candida) antarctica lipase B with genuine

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Engineering in Life Sciences
www.els-journal.com
Eng. Life Sci. **2018**, *0*, 1–11

primary sequence in recombinant Escherichia coli. *J. Biosci. Bioeng.* 2016, *121*, 303–309.

[26] Mahajan, E., George, A., Wolk, B., Improving affinity chromatography resin efficiency using semi-continuous chromatography. *J. Chromatogr. A* 2012, *1227*, 154–162.

[27] Pollock, J., Bolton, G., Coffman, J., Ho, S. V., Bracewell, D. G., Farid, S. S., Optimising the design and operation of semi-continuous affinity chromatography for clinical and commercial manufacture. *J. Chromatogr. A* 2013, *1284*, 17–27.

[28] Sartorius Stedim Biotech, Sartobind® IEX MA 15, 75 and 100, 2–3.

[29] Castan, A., Falkman, T., Faldt, E., Persson, T., Blomqvist, L., Forss, A., Process intensification through integration of upstream perfusion cell culture with downstream continuous chromatography in monoclonal antibody production, 2016.

[30] Gjoka, X., Gantier, R., Schofield, M., Transfer of a three step mAb chromatography process from batch to continuous: Optimizing productivity to minimize consumable requirements. *J. Biotechnol.* 2017, *242*, 11–18.

[31] Heeter, G. A., Liapis, A. I., Perfusion chromatography: performance of periodic countercurrent column operation and its comparison with fixed-bed operation. *J. Chromatogr. A* 1995, *711*, 3–21.

[32] Arve, B. H., Liapis, A. I., Biospecific adsorption in fixed and periodic countercurrent beds. *Biotechnol. Bioeng.* 1988, *32*, 616–627.

[33] Chatterjee, S., FDA perspective on continuous manufacturing, in IFPAC Annual Meeting, Baltimore, MD, 2012.

[34] Konstantinov, K. B., Cooney, C. L., White paper on continuous bioprocessing. May 20–21, 2014 Continuous Manufacturing Symposium. *J. Pharm. Sci.* 2015, *104*, 813–820.

[35] Ozturk, S. S., Opportunities and challenges for the implementation of continuous processing in biomanufacturing. G. Subramanian (Ed.) *Continuous processing in pharmaceutical manufacturing*. Wiley-VCH Verlag:Weinheim, 2014.

[36] Kaltenbrunner, O., Diaz, L., Hu, X., Shearer, M., Continuous bind-and-elute protein A capture chromatography: optimization under process scale column constraints and comparison to batch operation. *Biotechnol. Prog.* 2016, *32*, 938–948.

© 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

3.2 Kontinuierliche Aufreinigung von Patchoulolsynthase aus *E. coli*-Lysat

In diesem Kapitel wird die Aufreinigung von Patchoulolsynthase (PTS) mit der 3MA-PCCC beschrieben. Aufgrund der vorherigen Ergebnisse der CalB-Aufreinigung wurde ein Protein gewählt, welches mittels Affinitätschromatographie aufgereinigt werden kann. Da die PTS über einen His-Tag verfügt, kann IMAC für die Aufreinigung aus *E. coli*-Lysat verwendet werden.

Das Enzym Patchoulolsynthase (EC 4.2.3.70) ist an der Biosynthese des Sesquiterpens (-)-Patchoulol beteiligt. (-)-Patchoulol ist besonders für die Kosmetikindustrie interessant, da es dem Patchouliöl seinen charakteristischen holzigen, erdigen Duft verleiht. Patchouliöl ist Bestandteil vieler luxuriöser Kosmetikprodukte wie beispielsweise Parfums oder Cremes und wird aus Pflanzen der Gattung *Pogostemon cablin* mittels Dampfdestillation extrahiert. Das Extrakt enthält 25–60 % des Terpenalkohols (-)-Patchoulol. Darüber hinaus enthält das natürlich gewonnene Öl noch viele weitere Sesquiterpene, welche allerdings nur wenig zum Geruch des Patchouliöls beitragen [45, 46]. Somit ist der Anteil an (-)-Patchoulol ein wichtiges Qualitätsmerkmal [47, 48]. Leider hat die natürliche Gewinnung von Patchouliöl mittels Dampfdestillation viele Nachteile, weil sie hauptsächlich direkt auf dem Feld in Entwicklungsländern durchgeführt wird. Dies hat zur Folge, dass die Qualität des Produkts leidet, da die Produktionsbedingungen oft nicht den geforderten Standards entsprechen. Darüber hinaus sind mit der Produktion sozio-ökologische Konsequenzen verbunden, da bspw. die Anbauflächen für Lebensmittel verdrängt werden [49]. Im Anstieg des Weltmarktpreises auf 150 $/kg [50] ist zu erkennen, dass die Nachfrage steigt, während die natürliche Produktion diese nicht mehr decken kann. Daher wird besonders die biotechnologische Produktion mithilfe von Enzymen wie der Patchoulolsynthase sehr interessant.

Der Aufreinigung und Charakterisierung der PTS wurde bisher wenig Aufmerksamkeit geschenkt. Daher legt dieses Kapitel den Schwerpunkt auf die kontinuierliche Aufreinigung des bioaktiven Enzyms mit (Einweg-)Membranadsorbern. Zunächst wurden die Puffer für die Aufreinigung hinsichtlich Enzymaktivität und der (-)-Patchoulol-Ausbeute optimiert. Das ist wichtig, da schon die Aufreinigung des natürlich-vorkommenden Enzyms durch Munck & Croteau [51] zeigte, dass es während

der chromatographischen Aufreinigung zu Aktivitätsverlusten von mindestens 90 % kommt. Da die IMAC unter neutralen Bedingungen durchgeführt werden kann, wurden verschiedene Puffer im pH-Bereich von pH 7–8 getestet. Bei der PTS handelt es ich um eine Multiprodukt-Sesquiterpensynthase. Durch die Optimierung der Pufferbedingungen konnten die Aktivität maximiert und die Selektivität des Enzyms positiv beeinflusst werden.

Die optimierten Pufferbedingungen (50 mM Triethanolamin-NaOH pH 7, 0,5 M NaCl, 10 % (v/v) Glycerin, 5 mM $MgCl_2$) decken sich größtenteils mit den in der Literatur beschriebenen Ergebnissen [46, 51–53], wobei in dieser Arbeit alle Pufferkomponenten hinsichtlich der maximalen Enzymaktivität optimiert wurden. Mithilfe des optimierten Puffers wurde die Batch-Methode zur Isolierung von PTS aus dem *E. coli*-Lysat unter Verwendung von Co^{2+}-IMAC (Sartobind® IDA75) etabliert. Zur Elution des nativen und bioaktiven Enzyms wurde 250 mM Imidazol verwendet. Die Produktreinheit nach diesem Schritt lag bereits bei 90 %. Die Batch-Methode mit zwei Elutionsstufen bei 25 mM und 250 mM Imidazol wurde auf die PCCC-Anlage mit drei Membranadsorbern übertragen. Im Rahmen des PCCC Laufs wurden 450 mL *E. coli*-Lysat mit 0,17 g/L Gesamtprotein über 2,33 PCCC Zyklen in 1,5 h aufgereinigt. Aufgrund der niedrigen Produktkonzentration von 14,9 mg/L PTS im Lysat, konnte kein Produktdurchbruch detektiert werden. Daher wurde die PCCC-Anlage mittels der flexiblen Programmierung zeitlich gesteuert. Dennoch konnte PTS erfolgreich mit Konzentrationen bis zu 0,33 g/L eluiert werden. Nach dem notwendigen Umpufferungsschritt lag die Reinheit der PTS bei 98 % sowie die Prozessausbeute bei 68 %. Mit 1 µM des aufgereinigten Enzyms und 50 µM des Substrats FPP (Farnesyldiphosphat) konnte 126 mg/L (-)-Patchoulol gebildet werden.

Der Verlust von 32 % PTS ist zum Teil auf den Systemaufbau der PCCC zurückzuführen. Diese Problematik ist ebenfalls im vorherigen Kapitel 3.1 aufgetreten; hier wurden in den PCCC-Waschschritten 10–15 % des Produkts (CalB) verloren. Dieses Problem wurde bereits in der Literatur beschrieben [8, 54] und könnte durch eine Erweiterung des Systems gelöst werden. Durch das Weiterleiten der Waschfraktionen auf einen weiteren Membranadsorber, dem sogenannten interconnected wash, ginge dieser Produktanteil nicht verloren. Nichtsdestotrotz konnte die kontinuierliche Membranchromatographie die Produktivität des Prozesses um 47 % (berechnet nach Kaltenbrunner *et al.* [55]) gesteigert und der Pufferverbrauch um 33 % im Vergleich zum Batch reduziert werden. Der Einsatz

der 3MA-PCCC eignet sich hier vor allem, da die Aufreinigung mit nur einem chromatographischen Schritt durchgeführt wurde. Dies kommt dem Produktionsprozess zugute, da das Enzym eher instabil ist.

Die Nachfrage nach Patchouliöl wird voraussichtlich um 2,8 % pro Jahr steigen [56]. Der biotechnologische Prozess könnte genutzt werden, um diese steigende Nachfrage zu decken. Der vorgestellte Aufreinigungsprozess kann durch Verwendung von Membranadsorbern einfach maßstabsvergrößert werden. Es müssen lediglich die Membranfläche vergrößert und ein geeignetes System (z. B. BioProcess pcc von GE Healthcare) für die large-scale Anwendung eingesetzt werden.

Im folgenden Artikel „*Optimization of continuous purification of recombinant patchoulol synthase from Escherichia coli with membrane adsorbers*” werden die Ergebnisse ausführlich beschrieben und diskutiert.

Received: 14 January 2019 | Revised: 13 March 2019 | Accepted: 21 March 2019
DOI: 10.1002/btpr.2812

RESEARCH ARTICLE

BIOTECHNOLOGY PROGRESS

Optimization of continuous purification of recombinant patchoulol synthase from *Escherichia coli* with membrane adsorbers

Chantal Brämer[1] | Kimia Ekramzadeh[1] | Frank Lammers[2] | Thomas Scheper[1] | Sascha Beutel[1]

[1]Institut für Technische Chemie, Leibniz Universität Hannover, Callinstraße 5, 30167 Hannover, Germany
[2]Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany

Correspondence
Institut für Technische Chemie, Leibniz Universität Hannover, Callinstraße 5, 30167 Hannover, Germany.
Email: beutel@iftc.uni-hannover.de

Abstract

The natural production of patchouli oil in developing countries cannot meet the increasing demand any more. This leads to socioecological consequences, such as the use of arable land, which is actually intended for food. Hence, the world market price increased up to $150/kg. An alternative is the biotechnological production of patchouli oil using a multiproduct sesquiterpene synthase, the patchoulol synthase (PTS). Here, we report the optimization of recombinant PTS purification from *Escherichia coli* lysate using continuous immobilized metal affinity chromatography. First, the purification conditions of the batch process were optimized in regard to optimal buffer composition and optimized chromatographic conditions. The best purification result was achieved with Co^{2+}-immobilized metal affinity chromatography (Sartobind® IDA 75) with a triethanolamine buffer at pH 7, 0.5 M NaCl, 10% [vol/vol] glycerol, 5 mM $MgCl_2$ and 250 mM imidazole for product elution. This optimized method was then transferred to a continuous chromatography system using three membrane adsorber units (surface of 75 cm^2 each). Within 1.5 hr in total, 4.55 mg PTS with a final purity of 98% and recovery of 68% could be gained. The purified enzyme was used to produce 126 mg/L (-)-patchoulol from farnesyl pyrophosphate. Here, for the first time bioactive PTS was successfully purified using membrane adsorbers in a continuous downstream process.

KEYWORDS
membrane chromatography, patchouli oil, patchoulol synthase, periodic counter-current chromatography, sesquiterpenes

1 | INTRODUCTION

Patchouli oil is a widely used ingredient of luxury cosmetic products and is found in many popular perfumes (e.g., Coco Chanel, Tom Ford White Patchouli, Dolce and Gabbana Pour Homme, Giorgio Armani Acqua Di Gio). Patchouli oil consists mainly of several sesquiterpenes and sesquiterpenoids which are produced by plants of the genus *Pogostemon cablin*. Patchouli oil is usually extracted from these plants by steam distillation. The extract contains 25–60% of the terpene alcohol (-)-patchoulol which is characteristic for its wooden earthy scent. The natural oil has a very complex composition of at least 23 different sesquiterpenes.[1,2] Other sesquiterpenes (e.g., α-bulnesene, guaia-5,-11-diene) contribute little to the smell. Thus, the content of (-)-patchoulol is the main quality criterion and should have a minimum value of 27–30%.[3,4]

Abbreviations: CV, column volume; CWW, cell wet weight; IMAC, immobilized metal affinity chromatography; MA, membrane adsorber; MV, membrane volume.

© 2019 American Institute of Chemical Engineers

The preferred natural production (steam distillation) has many disadvantages as it is mainly performed directly at site of the crop in developing countries. A major problem is the displacement of cultivated areas for food or the deforestation (socioecological consequences). A further problem is the quality of the product, as the production conditions in the developing countries often do not follow the guidelines for the cosmetics industry (e.g., substandard quality due to uncontrolled use of pesticides).[5] The world market price (up to \$150/kg[6]) is rising because natural production of patchouli oil cannot meet the demand and quality. The forecast for the end of 2019 is a market value of US\$ 67.5 million.[7] Therefore, alternative production methods for (-)-patchoulol production are considered.[8] Direct chemical synthesis is not economical[9] and therefore biotechnological production gains in importance[8,10,11] using patchoulol synthase (PTS) from *P. cablin* as the key enzyme in the biosynthesis of patchouli oil.

In 2006, Deguerry et al[2] reported the successful recombinant production of PTS using *Escherichia coli* BL21 Star™ (DE3). In addition to (-)-patchoulol, 13 other sesquiterpenes were identified. PTS was purified using Ni^{2+}-immobilized metal affinity chromatography (IMAC). The quality of the purified enzyme was poor due to a nonoptimized purification protocol and no data concerning purification yield or recovery could be retrieved.

Subsequently, the first recombinant product for cosmetic industry (production in *Saccharomyces cerevisiae*[12,13]) called "Clearwood" was launched in 2014 by Firmenich SA, Swiss. This first commercially available patchouli oil, however, has a different odor,[14] without the earthy, leathery, and rubbery notes of the natural oil. Derivatives of the (-)-patchoulol (α-patchoulene, β-patchoulene, γ-patchoulene, and seychellene) are unlikely to be found in the biotechnological product as their formation is due to maturation in the plant after the sesquiterpene synthesis.[15] Further recombinant production was reported in 2014 and 2015 by Hartwig et al[16] and Frister et al.[17] Hartwig introduced a codon-optimized sequence of the PTS with a His-tag and thioredoxin as a solubility-tag using the BL21(DE3) strain. Afterwards, the recombinant protein was purified by Co^{2+}-IMAC. However, very few data concerning purification yield or enzyme characteristics were published. Frister et al in contrary purified the PTS using Ni^{2+}-IMAC and provided further kinetic data.

As the authors mentioned above focused on the establishment of the expression, the purification was not yet optimized. Therefore the recombinant PTS was rather unstable (instability index = 42.6) and tended to activity loss which correlates with the data of the natural enzyme published by Munck et al.[18]

In cosmetic industry, the quality of cosmetic ingredients is critical to assure the safety, quality, and efficacy of cosmetic products and related personal care products. Therefore, applying appropriate good manufacturing practice (GMP) principles to cosmetic ingredients is essential and should be considered during process development. Consumer safety on cosmetics products is regulated in the EU[19] and in the United States.[20] However, in contrast to pharmaceutical products, no legally binding GMP requirements are referenced in EU yet. Therefore, the European Federation for Cosmetic Ingredients has published a guidance[21] which is currently a GMP-standard for cosmetic ingredients and includes as well a certification scheme. The "Federal Food, Drug, and cosmetic act"[20] is the relevant regulation in United States and especially in chapter VI defines requirements for cosmetics.

To improve the purification of sensitive enzymes like PTS and therefore the product quality, disposable membrane chromatography can be applied. It offers many advantages compared to traditional column chromatography, for example, the high throughputs and therefore shorter cycle times because mass transport occurs mainly due to convection, high mechanical stability, and easy up- and downscaling.[22–25]

Membrane chromatography can be operated in continuous mode (continuous membrane chromatography) and has the potential to increase the quality of the product. Even the FDA advocates these continuous techniques.[26] Examples for the successful application of continuous chromatography can be found especially in the field of antibody purification[27–30] while there are still few examples for continuous membrane chromatography to be found.[31]

As industrial production of (-)-patchoulol is based on a whole-cell catalytic process, little attention has been paid to the purification and characterization of PTS. Hence, this study puts emphasis on the continuous purification of the bioactive enzyme with disposable membrane adsorbers. First, buffers are tested for the purification in regard to enzyme activity and (-)-patchoulol ratio. The batch method is then transferred to a continuous method to purify bioactive PTS. Continuous membrane chromatography is used to increase productivity and speed up the purification in one single unit operation which benefits the production process as the enzyme is rather unstable.

This study aims to depict an optimized continuous membrane production process for isolated native and active PTS which can be used for patchouli oil production for cosmetic industry.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Trans,trans-farnesyl pyrophosphate ammonium salt (FPP) and bulk chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2 | Bacterial strain

PTS (E.C. 4.2.3.70) was expressed in *E. coli* BL21 (DE3) containing pET16b::his-FX_aPTS. The sequence of PTS originated from *P. cablin* (GenBank: ABC87816.1) was codon-optimized.[17] The cloning was performed according to Gibson et al,[32] using the GENEART® Seamless Cloning and Assembly Kit. The bacteria was transformed by heat transformation.[33] The PTS has a theoretical size of 64.2 kDa and an isoelectric point of 5.2.

2.3 | Shake flask cultivation

The preculture (25 mL LB medium containing 100 mg/mL carbenicillin) was inoculated with 20 μL cryo stock and incubated over night at 37°C and 180 rpm. Main culture started at OD_{600} = 0.05–0.1 AU

(500 mL in 2 L shake flask) and was induced with 0.5 mM IPTG at OD_{600} = 0.8–1 AU and a temperature shift to 20°C for 24 hr. For harvesting, a centrifugation step at 4,700*g* for 1 hr was performed. The pellet containing the product was washed with PBS and a second centrifugation step was performed at 10,000*g* for 10 min. The pellet was resuspended in binding buffer for chromatography and cells were disrupted by sonification (4 times for 45 s on ice, amplitude 100%, 100 W, at time intervals of 0.6 s). A centrifugation (10,000*g* for 10 min) and sterile filtration step was carried out. After harvesting, the cooling chain at 4°C was maintained.

2.4 | Batch purification

Batch purification was optimized using the commercial FPLC System Äkta pure 25 L (GE Healthcare, Chicago, IL). An IMAC membrane adsorber (MA) Sartobind® IDA 75 (membrane area: 75 cm^2, column volume [CV]: 2.1 mL) was evaluated using different metal ions (Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}) at 5 mL/min and 4–10°C.

The membrane adsorber was equilibrated with 15 CV binding buffer (50 mM triethanolamine-NaOH pH 7, 0.5 M NaCl, 10% [vol/vol] glycerol, 5 mM $MgCl_2$). Then, the sample was loaded onto the membrane adsorber and a washing step with 10 CV followed. For elution, a step gradient was chosen at 10% elution buffer (50 mM triethanolamine-NaOH pH 7, 0.5 M NaCl, 10% [vol/vol] glycerol, 5 mM $MgCl_2$, 25 mM imidazole) for 5 CV to elute impurities and at 100% elution buffer (50 mM triethanolamine-NaOH pH 7, 0.5M NaCl, 10% [vol/vol] glycerol, 5 mM $MgCl_2$, 250 mM imidazole) for 5 CV to elute PTS. The membrane adsorber was further washed with 5 CV elution buffer and re-equilibrated with 5 CV binding buffer.

As imidazole showed a negative effect on enzyme activity, buffer exchange was performed immediately after purification using either a centrifugal concentrator with a cutoff of 30 kDa (Vivaspin® 20; Sartorius Stedim Biotech, Germany) or desalting column (HiPrep™ 26/10 Desalting; GE Healthcare, Sweden) at 4–10°C. After this buffer exchange, product was stored in 2-(N-morpholino)ethanesulfonic acid (MES) NaOH buffer (50 mM MES NaOH pH 6.4, 10% (vol/vol) glycerol, 5 mM $MgCl_2$) with DTT (1 mM) or without DTT at −20°C.

2.5 | Continuous purification

The developed method for batch purification was further transferred to the continuous chromatographic system described in Brämer et al[31] following the principle of periodic counter-current chromatography (PCCC).

An appropriate process control strategy is a key element of a continuous chromatography process design for industrialization as this is a significant prerequisite to efficiently remove impurities up to an acceptable level and to further assure the stability of the enzyme. The criticality of process parameters such as pH, UV/VIS, conductivity, temperature, time, back pressure, volume flow and so on need therefore careful consideration and are typically assessed during process characterization and finally monitored and controlled with PAT applications and qualified devices and software. Here, the chromatographic device was controlled by time instead of the UV signal as the product concentration of PTS was relatively low. Each step had a duration of 10 min with 5 mL/min, cooled reservoirs and fractionation. Process schedule was performed as shown in Figure 1.

Productivity (*P*) was calculated according to Kaltenbrunner et al[34] using the following equations to compare batch and continuous chromatography process:

$$P_{\text{Batch}} = \frac{m_{\text{Cycle}}}{V_{\text{Column}} \cdot (t_L + t_T)} \quad (1)$$

$$P_{\text{PCCC}} = \frac{m_{\text{Cycle}}}{V_{\text{Column}} \cdot N_{\text{Column}} \cdot t_L} \quad (2)$$

$$\text{Productivity increase} = \frac{P_{\text{PCCC}}}{P_{\text{Batch}}} \quad (3)$$

where m_{Cycle} is the mass of product bound to column or membrane adsorber, V_{Column} is the column or membrane volume, t_L is the time of loading, t_T is the turnaround duration and N_{Column} is the number of columns or membrane adsorbers.

2.6 | Analytical methods

2.6.1 | Protein quantification

Protein quantification was performed using the colorimetric Bradford method with BSA standards from 0.01 to 0.32 g/L. Purified PTS was further quantified using UV absorption at 280 nm under consideration of the molar extinction coefficient (90,190 L mol^{-1} cm^{-1}) and theoretical molecular weight (64.2 kDa).

2.6.2 | Qualitative protein analysis

For qualitative analysis, SDS-PAGE was carried out using a collecting gel with 6% and a separation gel with 12%. The samples were prepared with Laemmli buffer in the ratio 1:1 and boiled for 10 min at 95°C. The gel was loaded with 10 μL of the sample and 5 μL marker (Unstained Protein Molecular Weight Marker, Thermo Fisher Scientific). The SDS-PAGE was run for 15 min at 100 V and for 45–60 min at 150 V. For visualization Coomassie staining was used.

2.6.3 | Activity assay GC-FID

Activity assay was performed according to Frister et al[17] using 0.1–0.5 μM purified PTS/sample and 20–50 μM FPP in 1 mL reaction volume overlaid with 200 μL isooctane at 38°C.

3 | RESULTS

To establish the continuous purification process of PTS, two steps were carried out: optimization of the batch method (including buffer

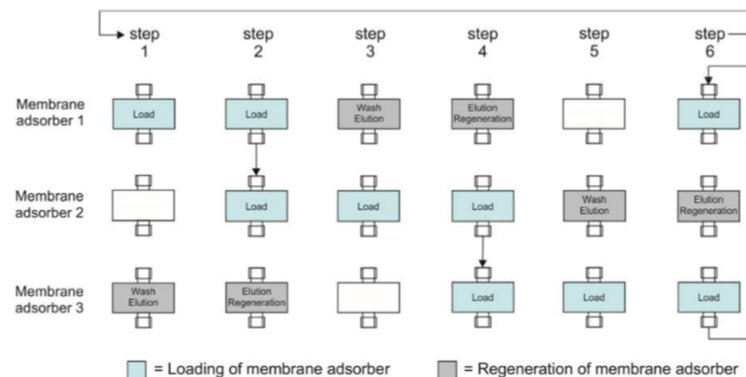


FIGURE 1 Schematic schedule of continuous chromatography for patchoulol synthase.[31] Three membrane adsorbers are loaded, washed, eluted, and regenerated in six steps. The principle is periodic counter-current chromatography. In step 1, the first membrane adsorber is loaded until a certain amount of product breaks through. In step 2, the second membrane adsorber is added to the loading (loading zone, light blue) until saturation of membrane adsorber 1. Membrane adsorber 2 and 3 are loaded according to the same principle in steps 3–6. The process runs continuously because of the regeneration (regeneration zone, gray) while another membrane adsorber is loaded

optimization to ensure a bioactive enzyme) and transfer of the batch to a continuous method.

PTS is relatively fragile (instability index 42.6, Expasy ProtParam), hence the recombinant production and purification should be performed under optimal conditions. The cell pellet is resuspended in the binding buffer for chromatography and then cell disruption is performed. The buffer system should guarantee highest enzyme activity and best conditions for the (-)-patchoulol formation as well as product binding in chromatographic purification. In recent studies, bis-TRIS propane-based buffer was used to find optimal buffer conditions (pH 6.5–9 and 0.001–100 mM Mg^{2+}). The optimal pH was evaluated to be pH 7–7.5 and it was assumed that the pH optimum could be transferred to other buffer systems.[2,18] These buffer tests were insufficient because buffer salts may have different effects on enzyme activity.[35]

Natural enzyme purification by Munck et al[18] already showed that enzyme activity (at least 90%) was lost during purification. Therefore, purification buffer should get optimized to reduce activity loss. For the overall process further optimization has to be carried out as the recovery was only 0.02% (purity >95%). Unfortunately, no detailed data regarding yield or recovery for the purification of the recombinant enzyme can yet be found in the literature.

3.1 | Optimization of batch chromatography

3.1.1 | Chromatography buffer

The recombinant PTS used in this study was fused with a His-tag so that the pH range of the chromatographic buffer was limited to pH 7–8 as binding of IMAC chromatography is performed under near-neutral pH buffer conditions.[36] Therefore, the following buffers in the pH range 7–8 were used for activity testing: 3-(N-morpholino) propane sulfonic acid (MOPS), Tris-aminomethane (TRIS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and triethanolamine (TEA). Phosphate buffer was not tested because phosphates sequester Mg^{2+} (natural cofactor). All buffers had a concentration of 50 mM buffer salt, 10 mM $MgCl_2$ and 10% (vol/vol) glycerol.

Figure 2 shows the product formation results for the three main products guaia-5,11-diene, α-bulnesene and (-)-patchoulol. TEA buffer was selected for further testing and optimization as overall activity as well as (-)-patchoulol ratio were the highest.

3.1.2 | Optimization of buffer additives

DoE screening (design: Frac Fac Res V+, model: interaction, fitted with multiple linear regression [MLR]) and optimization (design: central

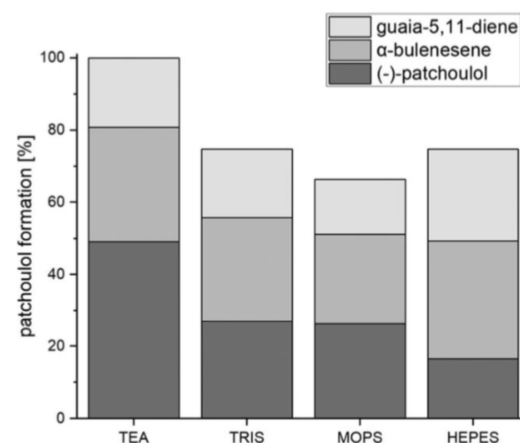


FIGURE 2 Results of activity testing with TEA, TRIS, MOPS, and HEPES buffer at pH 7–8 for the use as chromatography buffer. As TEA showed the best results, it is used for further optimization. HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(N-morpholino) propane sulfonic acid; TEA, triethanolamine; TRIS, tris-aminomethane

TABLE 1 Range of screening and optimization using DoE with Frac Fac Res V+ and CCF design

Component/condition	Range screening	Range optimization	Expected effect on enzyme stability/activity
$MgCl_2$	5–15 mM	5–15 mM	Stabilization, cofactor[18,37]
Glycerol	0–10% (vol/vol)	10%	Stabilization[38]
DTT	0–9 mM	0 M	Stabilize enzyme, reduce oxidation damage[39]
NaCl	0–1 M	0.5 M	Suppression of ionic interaction in IMAC chromatography[40]
pH	pH 7–8	pH 7–7.5	Binding of His-tag to chelate complex[41]

Abbreviations: CCF, central composite face-centered; DoE, design of experiments; IMAC, immobilized metal affinity chromatography.

composite face-centered, model: quadratic, fitted with MLR) were performed to find optimal buffer composition of TEA buffer in the pH range of 7–8. To maximize (-)-patchoulol formation, the additives listed in Table 1 were investigated. The concentration ranges of glycerol, DTT, and $MgCl_2$ were adapted to stated literature. So far, these factors have been separately optimized by one-factor-at-a-time (OFAT) method, resulting in different values. As the factors can influence each other, it is important to consider interaction, for example, through Design of Experiments (DoE).

NaCl, DTT, and pH showed a negative effect on the activity and thus on the (-)-patchoulol formation while $MgCl_2$ and glycerol showed a slightly positive effect (see Supporting Information). The pH value and salt concentration had the strongest impact on the activity of PTS. Therefore, it was concluded that the pH value and the NaCl concentration should be as low as possible while DTT was eliminated (but will be considered for storage in Section 3.2.1). Glycerol concentration was consequently set to the maximum value of 10%. Higher glycerol concentrations are possible but difficult to handle (viscosity increase). NaCl concentration was set to 0.5 M as this concentration is necessary to avoid ionic interactions on the membrane adsorber.

Considering these results, an optimization experiment (see Supporting Information) was performed, in which the optimal composition for binding buffer could be determined: 50 mM TEA-NaOH pH 7, 0.5M NaCl, 10% (vol/vol) glycerol, 5 mM $MgCl_2$. For elution 250 mM imidazole was added to the binding buffer.

The optimized chromatography buffer is compared to recently used buffers in Table 2. The optimized chromatography buffer is generally comparable to other buffers but the buffer salt and other components are optimized in regard to maximum enzyme activity. No positive effect of DTT was observed, thus it was not further used during the purification process. NaCl concentration was set to a minimum of 500 mM that is needed to avoid ionic interaction during IMAC and glycerol was set to a maximum of 10% (vol/vol). The proportion of pH and $MgCl_2$ was optimized (pH 7 and 5 mM $MgCl_2$).

3.1.3 | Optimization of activity buffer

Not only the pH range from pH 7–8 was monitored, but also the pH range from pH 3–10 to find the global maximum. It was found that the enzyme exhibits even higher activity and (-)-patchoulol ratio in MES pH 6.4 (data not shown). This buffer is therefore used as activity and storage buffer in the process. The result corresponds with the activity buffer of Munck et al but does not match the activity testing of Deguerry et al[2] as they observe a drastic activity decrease below pH 7 with bis-TRIS propane-based buffer.

The optimized activity buffer is compared to recently used buffers in Table 3. The optimized activity buffer is very similar to the buffer used by Munck et al for the natural enzyme purification, with the difference that no DTT was added. The other activity buffers have a slightly higher pH with high $MgCl_2$ concentration when DTT was added. Although Deguerry et al performed a pH screening, the pH

TABLE 2 Purification buffers for patchoulol synthase

Ref.	Buffer salt	NaCl (mM)	Glycerol (% vol/vol)	pH	DTT (mM)	$MgCl_2$ (mM)
Optimized buffer	TEA	500	10	7	-	5
Munck et al[18]	MES	-	10	6–7	1	15
	Phosphate	-	-	7–8	1	-
	TRIS	-	10	6.7	0.5	-
Deguerry et al[2]	TRIS	500	-	7.5	-	-
	HEPES	-	-	7.5	1	5
Hartwig et al[16]	Acetate	500	-	4.5	-	-
	Phosphate	500	-	8	-	-
	MOPSO	200	20	7.5	1	5
Frister et al[17]	MOPS	150	10	7.4	5	10

Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino) propane sulfonic acid; MOPSO, 3-morpholino-2-hydroxypropanesulfonic acid; TEA, triethanolamine; TRIS, tris-aminomethane.

TABLE 3 Assay buffers for patchoulol synthase

Ref.	Buffer salt	NaCl (mM)	Glycerol (% vol/vol)	pH	DTT (mM)	$MgCl_2$ (mM)
Optimized buffer	MES	-	10	6.4	-	5
Munck et al[18]	MES	-	10	6.5	1	25
Deguerry et al[2]	TRIS	-	-	7–7.5	-	1
Hartwig et al[16]	MOPSO	150	10	7.5	5	15
Frister et al[17]	MOPS	150	10	7.4	5	10

Abbreviations: MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino) propane sulfonic acid; MOPSO, 3-morpholino-2-hydroxypropanesulfonic acid; TRIS, tris-aminomethane.

optimum at pH 6.4 was not found as the tests were limited to one buffer system. These experiments show that a full buffer screening should not be neglected.

3.1.4 | Testing of metal ions

The optimized chromatography buffer from Section 3.1.2 was used for metal ion screening with membrane adsorber Sartobind® IDA 75. The membrane adsorber was chosen because of the mentioned advantages over columns. Monoliths are another alternative to columns, but monoliths have the major disadvantage that scale-up is a problem. Therefore, the membrane adsorber was selected.

About 20 mL of *E. coli* cell lysate (100 mg cell wet weight [CWW]/mL) were each applied to the membrane adsorber decorated with Ni^{2+}, Co^{2+}, Zn^{2+}, or Cu^{2+}. Impurities were washed away with 25 mM imidazole and product was eluted with 250 mM imidazole.

Figure 3 shows the chromatograms, SDS-PAGE and activity measurements of the four runs. For each run the breakthrough (FT), peak 1 (P1) and peak 2 (P2) were analyzed. The best result was achieved using Co^{2+} with a product purity of 90% after this single unit operation.

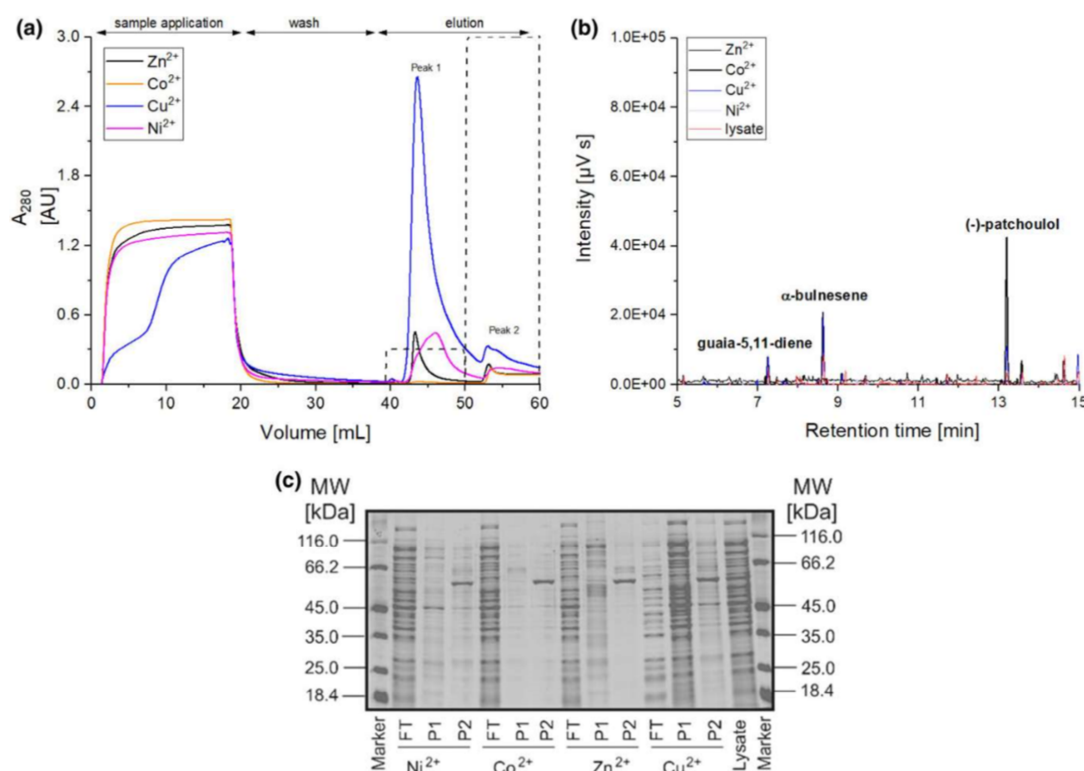


FIGURE 3 Testing of metal ions Ni^{2+}, Co^{2+}, Zn^{2+}, and Cu^{2+} for IMAC chromatography. Four chromatography runs (a) were performed. For analysis GC-FID activity measurement (b) and SDS-PAGE (c) were carried out. Co^{2+}-IMAC showed the best result as almost exclusively PTS was bound and could be eluted in high purity and bioactive. IMAC, immobilized metal affinity chromatography; PTS, patchoulol synthase

Activity could be confirmed using GC-FID activity assay (Figure 3b). Consistent with literature, the membrane adsorber decorated with Co^{2+} showed the highest specificity and lowest affinity, followed by Zn^{2+}, Ni^{2+}, and Cu^{2+} (see Figure 3c). Depending on the aim of purification, the purification using Zn^{2+}, Ni^{2+}, or Cu^{2+} could also be considered. In this study, the aim was to purify the enzyme as fast as possible to guarantee a stable and bioactive enzyme. Therefore, the membrane adsorber with cobalt ions is the best choice as only one unit operation was necessary to achieve high purity.

So far, column chromatography was the method of choice for PTS because 80% of the activity was lost using a membrane adsorber. Hartwig et al eluted PTS at pH 4.5 (sodium acetate buffer with 500 mM imidazole) which might have caused the activity loss. Using the optimized chromatography buffers, active and pure enzyme could be produced.

Although the capacity is much higher for a column, membrane chromatography is more suitable here, as the rather unstable PTS needs to be purified very fast and to a high purity. Another aspect is the relatively low product concentration of PTS in the lysate (see Figure 3c) which makes the MA even more suitable.

3.1.5 | Buffer exchange

After IMAC purification of the PTS, 250 mM imidazole was present in the elution buffer which had a negative effect on the enzyme activity.[42] Consequently, buffer exchange should be performed immediately. Vivaspin® 20 and HiPrep™ 26/10 Desalting were tested for this purpose. Two chromatography runs were performed using 35 mL *E. coli* lysate from the same batch. Buffer exchange using the desalting column resulted in a recovery higher than 90% but a dilution factor of 1.5, while recovery of Vivaspin was 81% with a concentration factor of 2. The activity measurement showed that Vivaspin led to a 2.6-fold activity compared to HiPrep™ desalting column and was therefore used to remove imidazole in the purification process.

3.2 | Continuous chromatography of PTS

The optimized membrane chromatography method of Section 3.1.4 was transferred to the continuous (3MA-PCCC) device to capture PTS. The Äkta system used for method development has a fivefold smaller path length than the PCCC system. Therefore, the lysate was

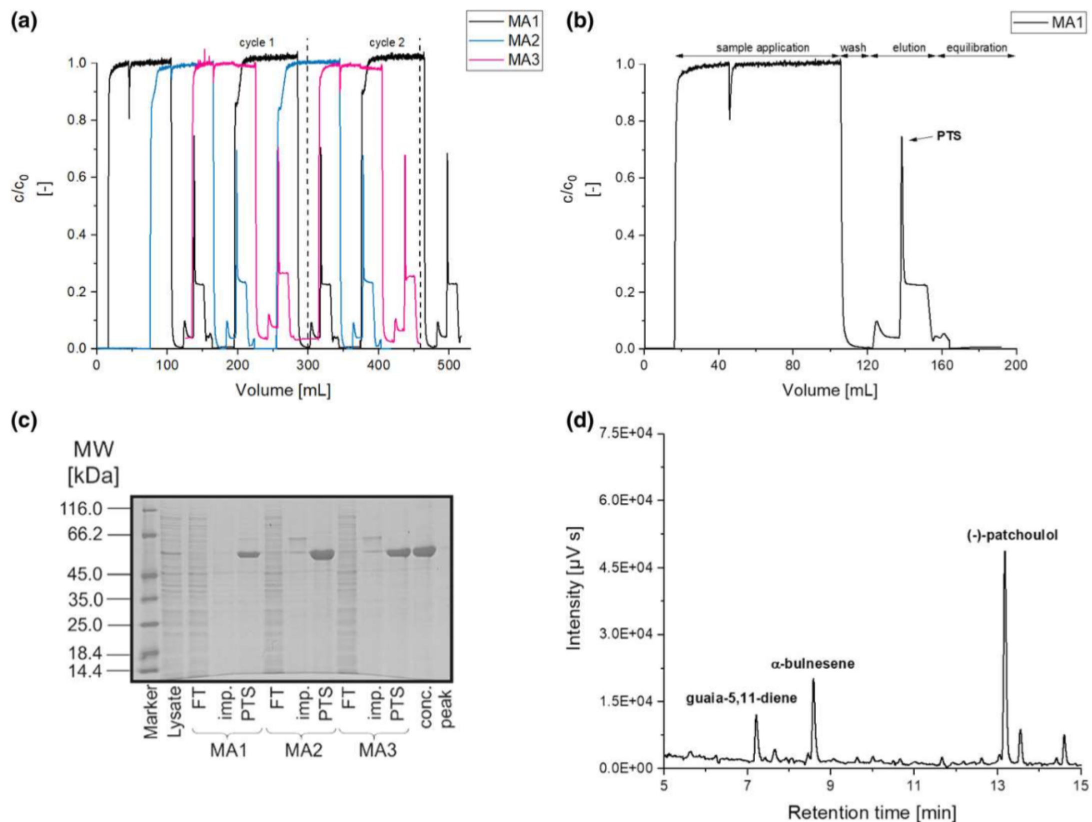


FIGURE 4 Results of the continuous purification of patchoulol synthase from *E. coli* lysate (a), zoom of the 1st cycle (b), SDS-PAGE of the 1st cycle (c), and GC-FID activity measurement (d). PTS was purified from 450 mL lysate within 1.5 hr and 2.33 PCCC cycles. PCCC, periodic counter-current chromatography; PTS, patchoulol synthase

diluted to 40 mg CWW/mL and 0.17 g/L total protein. As the control strategy of the PCCC is the UV-signal of the product breakthrough, a double breakthrough curve was recorded. However, the concentration of PTS in the lysate was too low (14.9 mg/L) and no product breakthrough could be detected. Consequently, the device was controlled by the time that could easily be programmed in the python™ script. The schedule from Figure 1 was used to purify PTS from 450 mL *E. coli* lysate within 1.5 hr.

PTS (4.55 mg) were achieved over 2.33 (2 cycles and one further loading of MA1) PCCC cycles with a three membrane adsorber setup without loss of performance. The elution was performed in two steps: at 10% buffer B to elute weakly bound impurities (imp.) and 100% buffer B (with 250 mM imidazole) to elute PTS (see Figure 4a). Figure 4b shows the zoom of the first loading of membrane adsorber 1 (MA1). During sample application, the flow through (FT) of nonbinding impurities was visible whereas no product was lost (see Figure 4c MA1, FT). PTS was successfully eluted at 250 mM imidazole with a concentration up to 0.33 g/L, considering the baseline shift caused by imidazole absorption.

All PTS peaks were pooled, and buffer was exchanged to remove imidazole with Vivaspin® 20 (conc. peak) so that 0.25 mg PTS/g CWW with 98% purity could be won. This corresponds to a recovery of 68%. Activity was confirmed by GC-FID measurement (Figure 4d), resulting in a (-)-patchoulol formation of 126 mg/L using 50 μM FPP and 1 μM of the purified enzyme. In the final product, the endotoxin level was below 10 EU/mL and therefore below the permitted limit[43,44] for example, if use as a fragrance is intended. Host cell proteins and DNA could not be detected. In case of contaminations with DNA or endotoxins, a further chromatography step with anion-exchange in flow through mode could be performed to meet the quality requirements of cosmetics.

The PTS loss of 32% could be due to the system setup (missing of interconnected washing step[28,31,45]) and the low product concentration in the lysate. Because of this, the usual control with the UV signal was not possible but could be performed with a temporal control. To enable the UV control of the PCCC and to achieve higher recovery values, the system should be completed to do the interconnected washing. To avoid dilution of the lysate and thus low concentration of the product, cuvettes with a path length of 2 mm should be added to the PCCC system.[31]

Nevertheless, continuous chromatography application increased the productivity of the process by 47%. Furthermore, 33% of the buffer could be saved. Productivity was calculated using the Equations (1) to (3) of Kaltenbrunner et al:

$$P_{Batch} = \frac{m_{Cycle}}{V_{Column} \cdot (t_L + t_T)} = \frac{0.76\,\text{mg}}{2.1\,\text{mL} \cdot (0.16\,\text{hr} + 0.32\,\text{hr})} = 0.75\,\frac{\text{mg}}{\text{mL} \cdot \text{h}} \tag{4}$$

$$P_{PCCC} = \frac{m_{Cycle}}{V_{Column} \cdot N_{Column} \cdot t_L} = \frac{2.28\,\text{mg}}{2.1\,\text{mL} \cdot 3 \cdot 0.33\,\text{hr}} = 1.10\,\frac{\text{mg}}{\text{mL} \cdot \text{h}} \tag{5}$$

$$\frac{P_{PCCC}}{P_{Batch}} = 1.47 \tag{6}$$

Patchoulol demand is expected to grow by 2.8% per year in the future. The biotechnological process could be used to handle the production of patchoulol. This process could easily be upscaled using a higher membrane area and a suitable chromatographic system for large-scale applications (e.g., BioProcess pcc by GE Healthcare). To cope with the increase, tons of enzyme would have to be produced per year.

3.2.1 | Stability testing

The activity (or (-)-patchoulol formation) of PTS was evaluated in lysate after cell disruption and in storage buffer after successful purification (see Supporting Information). The activity of PTS in the lysate was examined as activity loss was observed during overnight storage of the lysate. Additionally, it was important for PCCC purification to know the activity of PTS during processing over several hours.

Hence for activity in lysate, the cell pellet was resuspended in binding buffer, disrupted and stored for 14 days at −20°C, 4°C, and 20°C. For storage at 4°C and 20°C the activity was totally lost after these 14 days. The half-life of the PTS activity at 20°C was 2–3 days, at 4°C < 5 days and for −20°C 2 weeks.

(-)-Patchoulol formation of purified PTS (from Section 3.2) was tested in storage buffer at −20°C to verify the influence of long-term storage on the enzyme activity. At best, the enzyme activity should not decrease over the storage time. The storage buffer was either tested with DTT (1 mM) and without DTT. DTT is used for storage of cysteine-rich proteins because it can reduce oxidative damage, but in the experiments of Section 3.1.2 it showed a negative effect on PTS activity. Therefore, its necessity should be checked in the storage buffer.

After 4 months, no significant decrease in enzyme activity could be observed for both experimental series. However, the experimental series without DTT showed slight fluctuations, with the lowest activity value being 80 ± 8%. This can be attributed to systematic errors such as pipetting mistakes.

Lysate should be processed within 1 day and stored as cool as possible (4°C or −20°C) to avoid activity loss of the enzyme during purification. Although DTT was used for storage in recent studies,[2,16–18] no positive effect of DTT on PTS activity could be confirmed over the period of 4 months.

4 | CONCLUSIONS

This study depicts an optimized continuous purification process for isolated native and active PTS with membrane affinity chromatography. Buffers were optimized in terms of enzyme activity and (-)-patchoulol ratio because of the activity loss that occurred during PTS purification before. The results show that buffer screening and optimization is important for sensitive enzymes like PTS. Buffer salt, pH, and buffer additives have an effect on enzyme activity and selectivity. As PTS is a multiproduct sesquiterpene synthase, several products are produced. By using TEA buffer at pH 7 with 0.5 M NaCl, 10% (vol/vol) glycerol, 5 mM $MgCl_2$ (chromatography buffer) and MES buffer at pH 6.4 with 10% (vol/vol) glycerol, 5 mM $MgCl_2$ (activity buffer), PTS showed

highest activity and (-)-patchoulol ratio was maximized to 50%. It is further important to optimize the purification steps to maximize the yield and minimize process time. Here, continuous membrane chromatography was used as it is advantageous over columns in the case of low product titers and unstable enzyme and therefore short cycle times. The purified enzyme can be further characterized with respect to the production of patchouli oil. This is a step toward recombinant patchouli oil production to partially replace the volatile and expensive natural production of patchouli oil from plants.

ACKNOWLEDGMENT

We would like to thank the EU for financial support of the project "Refinement of plant raw materials" within the frame of the EFRE-program.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

ORCID

Chantal Brämer https://orcid.org/0000-0001-9202-2757

REFERENCES

1. Buchi G, Macleod WD. Synthesis of patchouli alcohol. *J Am Chem Soc*. 1962;84(16):3205-3206. https://doi.org/10.1021/ja00875a047.
2. Deguerry F, Pastore L, Wu S, Clark A, Chappell J, Schalk M. The diverse sesquiterpene profile of patchouli, *Pogostemon cablin*, is correlated with a limited number of sesquiterpene synthases. *Arch Biochem Biophys*. 2006;454(2):123-136. https://doi.org/10.1016/j.abb.2006.08.006.
3. Badan Standarisasi Nasional. Standar Nasional Indonesia, Minyak Nilam. SNI 06-2385-2006, Jakarta; 2006.
4. Organisation Internationale de Normalisation. Huile essentielle de patchouli (Pogostemon cablin [Blanco] Benth.); 2002.
5. Maxwell S, Fernando A. Cash crops in developing countries: the issues, the facts, the policies. *World Dev*. 1989;17(11):1677-1708.
6. Frister T, Beutel S. Moschusduft und Patchouliöl. *Chem Unserer Zeit*. 2015;49(5):294-301.
7. Insights FM. Patchouli Oil Sales Revenue to Surpass US$ 67 Million by 2019 – Future Market Insights. https://globenewswire.com/news-release/2018/12/10/1664400/0/en/Patchouli-Oil-Sales-Revenue-to-Surpass-US-67-Million-by-2019-Future-Market-Insights.html. Published 2018. Accessed February 27, 2019.
8. Henke NA, Wichmann J, Baier T, et al. Patchoulol production with metabolically engineered *Corynebacterium glutamicum*. *Genes (Basel)*. 2018;9(4):219.
9. Xu G-Q, Lin G-Q, Sun B-F. Concise asymmetric total synthesis of (−)-patchouli alcohol. *Org Chem Front*. 2017;4(10):2031-2033.
10. Kusuma HS, Mahfud M. The extraction of essential oils from patchouli leaves (*Pogostemon cablin* Benth) using a microwave air-hydrodistillation method as a new green technique. *RSC Adv*. 2017;7(3):1336-1347. https://doi.org/10.1039/C6RA25894H.
11. Bohlmann J, Keeling CI. Terpenoid biomaterials. *Plant J*. 2008;54(4):656-669.
12. Daviet L, Schalk M. Biotechnology in plant essential oil production: progress and perspective in metabolic engineering of the terpene pathway. *Flavour Fragr J*. 2010;25(3):123-127.
13. Asadollahi MA, Maury J, Møller K, et al. Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: effect of ERG9 repression on sesquiterpene biosynthesis. *Biotechnol Bioeng*. 2008;99(3):666-677.
14. Clearwood® 970953. http://www.firmenich.com/uploads/files/ingredients/marketing-sheet/perfumery/CLEARWOOD_970953.pdf. Accessed October 11, 2018.
15. Ohloff G, Pickenhagen W, Kraft P. *Scent and Chemistry*. Zürich: Verlag Helvetica Chimica Acta; 2011.
16. Hartwig S, Frister T, Alemdar S, et al. Expression, purification and activity assay of a patchoulol synthase cDNA variant fused to thioredoxin in *Escherichia coli*. *Protein Expr Purif*. 2014;97:61-71. https://doi.org/10.1016/j.pep.2014.02.003.
17. Frister T, Hartwig S, Alemdar S, et al. Characterisation of a recombinant patchoulol synthase variant for biocatalytic production of Terpenes. *Appl Biochem Biotechnol*. 2015;176(8):2185-2201. https://doi.org/10.1007/s12010-015-1707-y.
18. Munck SL, Croteau R. Purification and characterization of the sesquiterpene cyclase patchoulol synthase from *Pogostemon cablin*. *Arch Biochem Biophys*. 1990;282(1):58-64. https://doi.org/10.1016/0003-9861(90)90086-E.
19. Buzek J, Ask B. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. Off J Eur Union L. 2009;342.
20. Federal Food, Drug, and Cosmetic Act (FD&C Act). https://www.fda.gov/regulatoryinformation/lawsenforcedbyfda/federalfooddrugandcosmeticactfdcact/default.htm. Accessed January 9, 2019.
21. EFfCI GMP FOR COSMETIC INGREDIENTS Including the Certification Scheme for GMP for Cosmetic Ingredients Revision 2017 Prepared by the European Federation for Cosmetic Ingredients In Collaboration with; 2017.
22. Knudsen HL, Fahrner RL, Xu Y, Norling LA, Blank GS. Membrane ion-exchange chromatography for process-scale antibody purification. *J Chromatogr A*. 2001;907(1):145-154.
23. Wang J. Macroporous ion-exchange membrane adsorbers: correlation between membrane structure, separation conditions and performance in bioseparation; 2009.
24. Tennikova TB, Svec F. High-performance membrane chromatography: highly efficient separation method for proteins in ion-exchange, hydrophobic interaction and reversed-phase modes. *J Chromatogr A*. 1993;646(2):279-288.
25. Demmer W, Nussbaumer D. Large-scale membrane adsorbers. *J Chromatogr A*. 1999;852(1):73-81.
26. Chatterjee S. FDA perspective on continuous manufacturing. IFPAC Annual Meeting, Baltimore, MD; 2012.
27. Godawat R, Brower K, Jain S, Konstantinov K, Riske F, Warikoo V. Periodic counter-current chromatography–design and operational considerations for integrated and continuous purification of proteins. *Biotechnol J*. 2012;7(12):1496-1508.
28. Mahajan E, George A, Wolk B. Improving affinity chromatography resin efficiency using semi-continuous chromatography. *J Chromatogr A*. 2012;1227:154-162.
29. Castan A, Falkman T, Faldt E, Persson T, Blomqvist L, Forss A. Process intensification through integration of upstream perfusion cell culture with downstream continuous chromatography in monoclonal antibody production; 2016.
30. Gjoka X, Gantier R, Schofield M. Transfer of a three step mAb chromatography process from batch to continuous: optimizing productivity to minimize consumable requirements. *J Biotechnol*. 2017;242:11-18.
31. Brämer C, Schreiber S, Scheper T, Beutel S. Continuous purification of *Candida antarctica* lipase B using 3-membrane adsorber periodic counter-current chromatography. *Eng Life Sci*. 2018;18(7):414-424.

32. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods*. 2009;6(5):343-345. https://doi.org/10.1038/nmeth.1318.
33. Hartwig S. Biokatalytische Wege zur Darstellung funktioneller Sesquiterpene; 2016.
34. Kaltenbrunner O, Diaz L, Hu X, Shearer M. Continuous bind-and-elute protein A capture chromatography: optimization under process scale column constraints and comparison to batch operation. *Biotechnol Prog*. 2016;32(4):938-948. https://doi.org/10.1002/btpr.2291.
35. Ferreira CMH, Pinto ISS, Soares EV, Soares HMVM. (Un)suitability of the use of pH buffers in biological, biochemical and environmental studies and their interaction with metal ions - a review. *RSC Adv*. 2015;5(39):30989-31003. https://doi.org/10.1039/C4RA15453C.
36. GE Healthcare. *Affinity Chromatography, Vol. 2 Tagged Proteins*. Vol 2. Uppsala Sweden: GE Healthcare Bio-Sciences AB; 2017:1-284.
37. Christianson DW. Structural biology and chemistry of the terpenoid cyclases. *Chem Rev*. 2006;106(8):3412-3442. https://doi.org/10.1021/cr050286w.
38. Vagenende V, Yap MGS, Trout BL. Mechanisms of protein stabilization and prevention of protein aggregation by glycerol. *Biochemistry*. 2009;48(46):11084-11096. https://doi.org/10.1021/bi900649t.
39. Reed G, Nagodawithana TW. *Biotechnology: Enzymes, Biomass, Food and Feed*. Vol 9. Weinheim, VCH; 1995.
40. Janson J-C. *Protein Purification: Principles, High Resolution Methods, and Applications*. Vol 151. Somerset: John Wiley & Sons; 2012.
41. Cazes J. *Encyclopedia of Chromatography*. Vol 2. Boca Raton: CRC Press; 2005.
42. Frister THW. Herstellung, Charakterisierung und Anwendung einer rekombinanten Patchoulolsynthase zur biokatalytischen Herstellung von Sesquiterpenen; 2015.
43. Williams KL. *Endotoxins: Pyrogens, LAL Testing and Depyrogenation*. Boca Raton: CRC Press; 2007.
44. Affairs O of R. Inspection Technical Guides - Bacterial Endotoxins/Pyrogens. https://www.fda.gov/iceci/inspections/inspectionguides/inspectiontechnicalguides/ucm072918.htm. Accessed February 28, 2019.
45. Pollock J, Bolton G, Coffman J, Ho SV, Bracewell DG, Farid SS. Optimising the design and operation of semi-continuous affinity chromatography for clinical and commercial manufacture. *J Chromatogr A*. 2013;1284:17-27.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Brämer C, Ekramzadeh K, Lammers F, Scheper T, Beutel S. Optimization of continuous purification of recombinant patchoulol synthase from *Escherichia coli* with membrane adsorbers. *Biotechnol Progress*. 2019;e2812. https://doi.org/10.1002/btpr.2812

3.3 Kontinuierliche Aufreinigung eines monoklonalen Antikörpers aus Chinese Hamster Ovary-Zellüberstand

In diesem Kapitel wird die kontinuierliche Aufreinigung eines monoklonalen Antikörpers aus Chinese Hamster Ovary (CHO)-Zellüberstand beschrieben. Für die Abbildung der kontinuierlichen Chromatographie wurde die zuvor beschriebene, erweiterte PCCC-Anlage mit vier Membranadsorber-Einheiten verwendet. Durch die Integration einer weiteren Einheit konnte der sogenannte interconnected wash implementiert werden. Das System-Setup wurde detailliert in Kapitel 2.4 beschrieben. Besonders die Aufreinigung von Antikörpern eignet sich für den kontinuierlichen Betrieb, da die Kosteneffizienz durch die nahezu komplette Nutzung der Bindungskapazität des teuren chromatographischen Materials (Protein A) steigt.

Antikörper gehören zu den Immunglobulinen und machen ein Fünftel der menschlichen Plasmaproteine aus und werden von aktivierten B-Zellen als Immunreaktion auf fremde Moleküle (Antigene) gebildet. Sie werden in fünf Immunglobulinklassen (IgG, IgA, IgM, IgD und IgE) unterteilt, die ebenfalls in Untergruppen unterteilt sind (z. B. IgG_1, IgG_2, IgG_3, IgG_4). Antikörper bestehen. Abbildung 8 zeigt einen IgG-Antikörper aus vier Polypeptidketten: je zwei identischen leichten (23–25 kDa) und schweren Ketten (50–60 kDa).

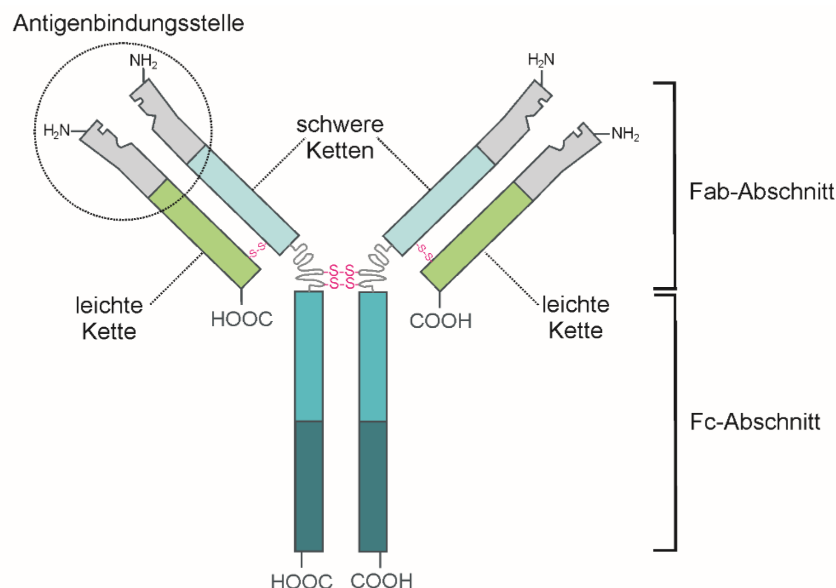


Abbildung 8: Schematischer Aufbau eines IgG-Antikörpers; in Anlehnung an [57]**.**

Die beiden identischen schweren Ketten sind durch zwei Disulfidbrücken verbunden und jeweils durch eine Disulfidbrücke mit einer der leichten Ketten verbunden. Die leichten

Ketten (LC) und die schweren Ketten (HC) weisen an einem Ende eine variable Region (V-Region, grau) auf, die als Bindungsstelle für das Antigen (Paratop) dient [57–59]. Monoklonale Antikörper (mAb) binden spezifisch an ein Epitop eines Antigens [58]. Das Anwendungsgebiet von monoklonalen Antikörpern in der Humanmedizin reicht von der Behandlung von Allergien, Asthma, Multipler Sklerose und der Bekämpfung verschiedener Krebsarten bis hin zu deren Einsatz bei Transplantationspatienten [60–62]. Im Jahr 2016 wurden sieben Medikamente auf Basis von monoklonalen Antikörpern in den USA und Europa zugelassen; Ende 2018 wurden 12 weitere in den USA und Europa zur Behandlung von beispielsweise Krebs, Behandlung von Transplantationspatienten, Autoimmunerkrankungen und anderen Krankheiten zugelassen [57, 63].

Die biotechnologische Produktion von monoklonalen Antikörpern erfolgt häufig in CHO-Zellen, wobei diese korrekt glykosyliert in das Medium abgegeben werden. Besonders die Downstream Processing Schritte sind für den Prozess entscheidend, da das Produkt hohe regulatorische Anforderungen erfüllen muss, damit die klinische Wirksamkeit gewährleistet ist [65]. Mit steigenden Produkttitern (vor allem in der Antikörper-Produktion) steigen die DSP Kosten proportional mit der Menge des aufzureinigenden Produkts, während die Kosten für das USP nicht signifikant ansteigen. Daher verlagern sich die Kosten mit 50–80 % [66] zum DSP [67–70]. Um dieses Problem zu adressieren, werden in diesem Kapitel Optimierungsansätze betrachtet; durch die Verwendung von kontinuierlicher Chromatographie sollen die Kosten durch eine verbesserte Kapazitätsnutzung des teuren Protein A Materials reduziert werden. Die Verwendung von Membranadsorbern soll die Raum-Zeit-Ausbeute und damit die Produktivität steigern.

In dieser Arbeit wurde der kritische Protein-A-Schritt mit Fokus auf der mAb Elution optimiert. Der Protein-A-Chromatographie-Schritt ist der Capture-Schritt in der Antikörper-Aufreinigung. In diesem Schritt soll der monoklonale Antikörper aus dem Überstand isoliert und konzentriert werden, gleichzeitig sollen Verunreinigungen wie DNA, host cell Proteine und Medienkomponenten entfernt werden. Anschließend werden die Polishing-Schritte durchgeführt, um letzte Verunreinigungen zu entfernen und die finale Reinheit des Produkts zu erreichen [71, 72]. Der Protein A-Schritt ist kritisch, da ein niedriger pH-Wert für die Produktelution und kombinierte Virusinaktivierung verwendet wird. Das kann zu Aggregation und damit zu Problemen in Qualität, Quantität, Weiterverarbeitung und vor allem in der Arzneimittelsicherheit führen [73, 74]. Im Rahmen dieser Arbeit wurde der Protein A-Schritt für einen therapeutischen humanen

monoklonalen Antikörper der Untergruppe IgG_1 gegen Tumornekrosefaktor-α optimiert. Verschiedene Elutionspuffer wurden unter Einsatz von Membranadsorbern (Sartobind® Protein A 2 mL) getestet und verglichen. Membranadsorber eignen sich hier besonders als Alternative zu Säulen aufgrund der kurzen Verweilzeiten und der Verwendung von „Disposables“ (Wegfallen von aufwendigen Reinigungsschritten und Validierung).

In dieser Arbeit wurden drei Puffersysteme für die monoklonale Antikörper-Elution getestet: Citratpuffer, Glycinpuffer und Acetatpuffer. Dabei wurde neben der Chromatographie-Leistung auch die Stabilität des Antikörpers bewertet. Die besten Ergebnisse in Bezug auf die Chromatographie-Leistung, Produktstabilität und Aggregation lieferte 0,1 M Citratpuffer pH 3,5 und 0,15 M NaCl mit einer Wiederfindung von > 95 % und einem Aggregatanteil von nur 0,26 % (vgl. Müller & Vajda [73] mit 1 %). Darüber hinaus konnte die Entwicklungszeit des Prozesses mit Membranadsorbern und unter Einsatz eines Design of Experiments (DoE)-basierten Ansatzes deutlich verkürzt werden. Das optimierte Chromatographie-Verfahren wurde erfolgreich auf das kontinuierliche Chromatographie-System mit vier Membranadsorbern (4MA-PCCC) übertragen. Der kontinuierliche Lauf wurde mit 160 mL Feed-Lösung mit 0,6 g/L mAb durchgeführt. Es wurden zwei Zyklen in 2 h durchgeführt. Die Elutionspeaks wiesen Konzentrationen zwischen 2,5 g/L und 5 g/L auf. Aufgrund der dynamischen Prozesskontrolle wurde jeder Membranadsorber bis zur Sättigung (70 % Produktdurchbruch, SC2) beladen. Eine konstante Leistung der MA war zu beobachten, wobei in jedem Zyklus ein Trend von abnehmenden Elutionspeaks (MA1 bis MA4) zu erkennen war. Dieser Trend lässt sich auf einen Rücksog von Puffer B im System zurückführen. Dennoch lag die Wiederfindung nach der Aufreinigung bei über 90 % (aufgrund des interconnected wash [8, 54]) und es wurden nur 600 mL Puffer verbraucht. Im Vergleich zum Batch-Prozess konnte die Kapazitätsnutzung der MA durch das Beladungsprinzip der PCCC um 20 % gesteigert werden.

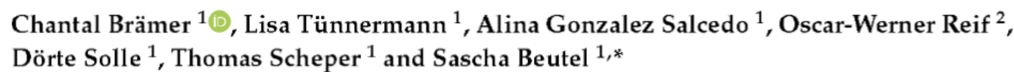
Das PCCC-Prinzip eignete sich besonders für die mAb-Aufreinigung, da die Produktkonzentration im Vergleich zu anderen Produkten (vgl. Kapitel 3.1 und 3.2) hoch genug war [56, 75]. Die Steuerung über das UV-Signal unter Nutzung der Switching Conditions konnte problemlos realisiert werden. Der Einsatz des PCCC-Prinzips ist insbesondere im Zusammenhang mit der von der FDA definierten Initiative Process Analytical Technology (PAT) sinnvoll [11]. Der Chromatographie-Prozess kann direkt überwacht werden und die Leistung ist direkt sichtbar. Der Prozess kann gestoppt werden,

bevor die Leistung nachlässt und die Qualität nicht mehr den gesetzlichen Anforderungen entspricht.

Im folgenden Artikel „*Membrane Adsorber for the Fast Purification of a Monoclonal Antibody Using Protein A Chromatography*" werden die Arbeiten ausführlich beschrieben und diskutiert.

Article

Membrane Adsorber for the Fast Purification of a Monoclonal Antibody Using Protein A Chromatography



Chantal Brämer [1], Lisa Tünnermann [1], Alina Gonzalez Salcedo [1], Oscar-Werner Reif [2], Dörte Solle [1], Thomas Scheper [1] and Sascha Beutel [1,*]

[1] Institute of Technical Chemistry, Callinstraße 5, 30167 Hannover, Germany; braemer@iftc.uni-hannover.de (C.B.); lisa.tuennermann@yahoo.com (L.T.); mehl@iftc.uni-hannover.de (A.G.S.); solle@iftc.uni-hannover.de (D.S.); scheper@iftc.uni-hannover.de (T.S.)

[2] Sartorius Stedim Biotech, August-Spindler-Straße 11, 37079 Göttingen, Germany; oscar.reif@sartorius-stedim.com

* Correspondence: beutel@iftc.uni-hannover.de; Tel.: +49-511-762-2868

Received: 24 September 2019; Accepted: 25 November 2019; Published: 27 November 2019

Abstract: Monoclonal antibodies are conquering the biopharmaceutical market because they can be used to treat a variety of diseases. Therefore, it is very important to establish robust and optimized processes for their production. In this article, the first step of chromatography (Protein A chromatography) in monoclonal antibody purification was optimized with a focus on the critical elution step. Therefore, different buffers (citrate, glycine, acetate) were tested for chromatographic performance and product quality. Membrane chromatography was evaluated because it promises high throughputs and short cycle times. The membrane adsorber Sartobind® Protein A 2 mL was used to accelerate the purification procedure and was further used to perform a continuous chromatographic run with a four-membrane adsorber-periodic counter-current chromatography (4MA-PCCC) system. It was found that citrate buffer at pH 3.5 and 0.15 M NaCl enabled the highest recovery of >95% and lowest total aggregate content of 0.26%. In the continuous process, the capacity utilization of the membrane adsorber was increased by 20%.

Keywords: monoclonal antibody; membrane adsorber; protein A chromatography; periodic counter-current chromatography

1. Introduction

Monoclonal antibodies (mAb) “deliver considerable medical benefits” [1]. This is reflected in the increase in the number of available drugs based on monoclonal antibodies. In 2016, seven mAbs were approved in the US or EU and by the end of 2018, a further 12 mAbs had been approved in the US or EU for the treatment of e.g., cancer, transplant patients, autoimmune diseases and others [2,3].

Antibodies are a central component of the human immune system and are produced by activated B-cells as an immune response to the intrusion of antigens. They belong to the immunoglobulin family and are divided into five immunoglobulin classes (IgG, IgA, IgM, IgD and IgE), which are also subdivided into subgroups (e.g., IgG1, IgG2, IgG3, IgG4). Antibodies are glycoproteins and consist of four polypeptide chains, two heavy chains (50–60 kDa) and two light chains (23–25 kDa). The two identical heavy chains are connected by two disulfide bridges and each is linked by a disulfide bridge to one of the light chains. The light chains (LC) and heavy chains (HC) have a variable region (V-region) at one end that serves as a binding site for the antigen [4–6]. Monoclonal antibodies bind specifically to a defined epitope of an antigen and thus trigger the immune defense system; this has attracted interest in their medicinal applications [4]. The application of monoclonal antibodies in human medicine

ranges from the treatment of allergies, asthma, multiple sclerosis, in the fight against various types of cancer and to their use in transplant patients [7–9].

The production process for monoclonal antibodies is divided into production (upstream processing) and purification (downstream processing). The efficiency of the upstream process has increased significantly over the past few decades. The hybridoma technique has provided the basis for the targeted and reproducible amplification of monoclonal antibodies, so that now it is possible to produce monoclonal antibodies in recombinant cells on a large scale [6,7,10]. Production is often performed in Chinese Hamster Ovary (CHO) cells, whereby the monoclonal antibody is correctly glycosylated and secreted into the medium. Not only is the cultivation crucial for the production, but the following steps in the process must also be carefully considered. Subsequent downstream processing (DSP) must be of a high standard to meet all regulatory requirements and ensure clinical efficacy [11]. With increasing product titers in cultivation, the upstream processing (USP) costs do not increase significantly, while process costs in downstream processing increase in proportion to the quantity of the product to be purified. Therefore, with improved production, production costs shift to the DSP and can account for 50–80% [12] of total process costs [13–16]. DSP can be divided into a number of steps (shown in Figure 1), which should lead to a highly purified and effective drug.

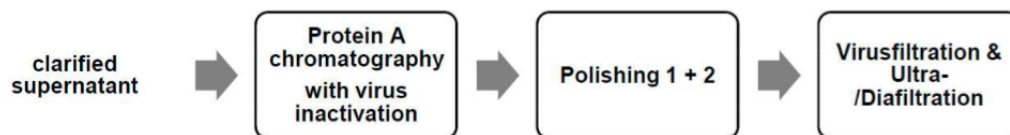


Figure 1. Process steps in the downstream processing of a monoclonal antibody (summarized and modified from [12]).

After cultivation, the cells are separated from the supernatant (by e.g., centrifugation or depth filtration [17]); this is the last operation in the USP. Obtaining the clarified supernatant legally divides the entire process into the cell-containing process steps (USP) and the subsequent cell-free process steps (DSP). The first step in the chromatography is the capture step using Protein A chromatography in combination with virus inactivation. In this step, the monoclonal antibody should be isolated and concentrated from the supernatant, and the contaminants or impurities (DNA, host cell proteins and cell culture medium components) should be eliminated. The polishing steps (e.g., cation exchange chromatography, hydrophobic interaction chromatography, anion exchange chromatography) are then performed to remove the last impurities and achieve the final purity of the product. Ultrafiltration and diafiltration are used to obtain suitable buffer and formulation conditions [18,19].

These steps should be optimized in order to reduce the process costs for downstream processing. The most expensive step is the Protein A chromatography step; thus it offers a good starting point. This affinity chromatography method is based on the interaction of the monoclonal antibody with immobilized Protein A. The binding is primarily formed by hydrophobic interactions, but also hydrogen bonds and ionic interactions have an influence on the interaction [20,21]. The Protein A ligand was originally derived from the bacterium *Staphylococcus aureus* and serves as a binding site for IgG class antibodies in the cell wall. Protein A binds the antibody at the fragment crystallizable (Fc) region of the heavy chain [20,22,23]. Depending on the subclass of the antibody, the binding between the antibody and Protein A takes place in a pH range of 6–9 and can be influenced by the salt concentration in the binding buffer [5,20,24]. To release the binding, an elution buffer with a low pH between pH 2.5 and pH 4.5 is selected, taking into account that a low pH may affect the functionality and stability of the antibody and it may also support aggregate formation, which can lead to problems in further processing or in drug safety [18,20,22,25,26]. Since the elution of the antibody takes place at a low pH value, this is also used for virus inactivation. The eluate should be incubated for 30–120 min at a pH value lower than pH 3.8 in order to inactivate retroviruses [22,27].

The selection of a suitable elution buffer and the parameters for elution is particularly important for effective Protein A chromatography and good product quality. The elution can be optimized by additives [20,28,29] or the use of salts to prevent e.g., ionic interaction and thus increase the pH value during elution [20,24]. The addition of small amounts of salt can also have a positive effect on the stability of the antibody. Different buffer systems have already been tested for the purification of IgG_1 antibodies with Protein A chromatography (citrate and acetate buffer) [30–32], whereas Müller and Vajda [32] observed better results with acetate buffer in regard to recovery. All authors observed that increasing pH had a negative effect on the recovery of the mAb. At pH 2.8–3.3, the recovery rate was higher than 90% whereas at pH 3.8 the recovery rate decreased to under 50%. Salt showed a negative effect on the elution in the work of Gagnon et al. [30]. Further, elution buffers were tested in regard to aggregate formation [32,33]: in the concentration range from 0–1.5 M NaCl, a negative effect of salt was observed and aggregation was induced while the pH value of the buffer also influenced the aggregate formation. Müller and Vajda [32] found about 1% aggregate in the range of pH 3–4. Singla et al. [34] investigated the aggregation kinetics, taking into account the pH, temperature, salt concentration (NaCl) and buffer species. They evaluated citrate, glycine and acetate buffer at pH 3.0 and found that these factors influenced aggregation in the following order with decreasing effect: pH, temperature, salt concentration and buffer species. At pH 3.0, citrate buffer induced the highest aggregation even without the addition of salt.

To address the issue that the process costs increase proportionally with the product titers in downstream processing, new optimization approaches were considered in this paper. One alternative is the use of other stationary phases such as disposable membrane adsorbers. They offer several advantages over conventional columns, e.g., higher throughputs and therefore shorter cycle times, an increase in productivity as well as easy up- and downscale of production, especially in the purification of low-concentrate products [35–39]. Some application examples of mAb purification with membrane adsorbers are summarized in Table 1.

Table 1. Application examples of membrane chromatography for monoclonal antibody (mAb) purification.

Field of Application	Ref.
Affinity chromatography	[40]
Ion exchange chromatography	[40–43]
Hydrophobic interaction chromatography	[41,42]
Accelerated, Seamless Antibody Purification (ASAP)-continuous method (Protein A chromatography, cation and anion exchange chromatography)	[44]

In addition, continuous chromatography processes promise a further increase in productivity [45] and are therefore increasingly used in mAb processing [46,47]. It has been shown that continuous chromatography overcomes the problems of batch chromatography, whereby the capacity utilization of the stationary phase is significantly increased. This reduces the required amount of stationary phase, and hence reduces the material costs in DSP. This is especially interesting for the costly Protein A chromatography step in mAb purification.

In this study, Protein A chromatography was performed with a design of experiments (DoE) based approach to optimize the critical elution step for mAb purification. Membrane adsorbers were utilized to evaluate the potential of alternatives to conventional column chromatography and to accelerate the purification process. The implementation of continuous membrane chromatography was performed to further increase the productivity of the process and to evaluate the use of continuous chromatography with membrane adsorbers.

2. Materials and Methods

2.1. Materials

In this study a monoclonal antibody of the IgG_1-type was used, which was produced with CHO cells in a serum-free medium. The antibody has a molecular weight of 148 kDa and a pI (isoelectric point) value at pH 8.25.

All chemicals were bought from Carl-Roth (Karlsruhe, Germany).

2.2. Methods

2.2.1. Cell Separation with Depth Filtration

The monoclonal antibody was produced in CHO cells and secreted into the cell culture medium. Since the antibody must be purified for later use, the cells and cell debris were first removed from the culture supernatant. For this first step, two-stage depth filtration (first step: Sartoclear® DL90, Göttingen, Germany; second step: Sartoclear® DL20, Göttingen, Germany) was performed. The clarified material contained 1.9 g/L monoclonal antibody.

2.2.2. Purification of the Monoclonal Antibody

The development of the Protein A chromatography method was performed using the Sartobind® Protein A 2 mL membrane adsorber from Sartorius Stedim Biotech (Göttingen, Germany) and the ÄKTA™ pure system with a fraction collector from GE Healthcare (Uppsala, Sweden).

A chromatography run consisted of different phases. First the membrane adsorber was equilibrated with buffer A (phosphate-buffered saline (PBS) buffer, pH 7.4), then the clarified supernatant was applied on to the membrane adsorber and all unbound substances were removed by a washing step with buffer A. Elution with buffer B (see Table 2) was then followed by a cleaning in place (CIP) step and regeneration of the membrane adsorber. The buffers were connected to the ÄKTA inlets as follows: Inlet A1: buffer A, Inlet A2: CIP buffer (50 mM NaOH, 1 M NaCl). The sample was introduced via the sample loop (maximum sample volume 10 mL), and the various elution buffers (buffer B) from the design of experiments (DoE) in Table 2 were connected via inlets B1 to B5. In this study, three buffers systems for the monoclonal antibody elution were tested: 0.1 M citrate buffer (pH 2.5–4 and 0–0.5 M NaCl), 0.1 M glycine buffer (pH 2.5–4 and 0–0.5 M NaCl) and 0.1 M acetate buffer (pH 3.5–4 and 0–0.5 M NaCl). In the chromatography experiments, recovery (in %) and peak height (in mAU) were defined as target parameters. The ÄKTA™ was cooled to 10 °C and operated at a flow rate of 5 mL/min during the experiments.

The DoE was performed with a 2-factor design (pH and NaCl concentration), whereas chromatography performance was evaluated by using the target parameters, peak height and recovery, and antibody stability was evaluated by the monomeric antibody amount and the aggregate content. The software, MODDE® (Umetrics, Version 12, Sweden) was used for planning and evaluation of the experiments. The models were selected according to the suggestions of the software.

2.2.3. Stability Experiments

In order to perform the stability experiments, 19.2 µL of the already purified mAb were pipetted into 0.25 mL of the various elution buffers to be incubated (for 1 h, 24 h and one week at 20 °C, 4 °C and −20 °C). A SEC-HPLC as described in Section 2.3.1, was then performed. The evaluation stability was investigated by determining the monomeric antibody amount (in %) and aggregate content (in µV*mL or %).

Membranes **2019**, *9*, 159

2.3. *Analytical Methods*

2.3.1. SEC-HPLC

Monoclonal antibody, fragment and aggregate concentration was determined using a commercial HPLC system operated with the column Yarra™ 3 µm SEC-3000 (Phenomenex, Torrance, CA, USA). The samples for the SEC-HPLC were diluted as required, so that a concentration of about 0.5 g/L was obtained. The samples were then filtered (0.2 µm) and cooled in an autosampler (10 °C). The system was operated with a flow rate of 1 mL/min and a pressure of 112 bar. A total of 5 µL from each sample was added to the column (oven temperature 25 °C) and the measurement was carried out for 20 min. A combination of 100 mM Na_3PO_4 buffer and 100 mM Na_2SO_4 at pH 6.6 was used as the mobile phase.

2.3.2. SDS-PAGE

For qualitative analysis of the monoclonal antibody, SDS-PAGE was carried out using a collecting gel with 6% acrylamide and a separation gel with 10% acrylamide. The samples were mixed with Lämmli buffer in the ratio 1:1 and boiled for 10 min at 95 °C. To run the gel, 1–10 µl of the sample as well as 5 µl of the Marker (PageRuler™ Unstained Protein Ladder, Thermo Scientific™, Waltham, MA, USA) were applied. For the separation, a voltage of 100 V was first applied to the loaded gel for 15 min, then the voltage was increased to 150 V for a further 60 min. For visualization, Coomassie staining [48] and silver staining [49] was used.

Table 2. Design of experiments (DoE) screening for 0.1 M citrate-, 0,1 M glycine-buffer and 0.1M acetate, N5–N7 are the center points.

Screening [1]								Optimization [2]			
Citrate/Glycine Buffer				Acetate Buffer				Citrate Buffer			
Exp.	**Order**	**pH**	**NaCl [M]**	**Exp.**	**Order**	**pH**	**NaCl [M]**	**Exp.**	**Order**	**pH**	**NaCl [M]**
N1	1	2.5	0	N1	1	3.5	0	N1	4	3	0
N2	7	4	0	N2	7	4	0	N2	7	3.8	0
N3	2	2.5	0.5	N3	2	3.5	0.5	N3	2	3	0.2
N4	4	4	0.5	N4	4	4	0.5	N4	6	3.8	0.2
N5	5	3.25	0.25	N5	5	3.75	0.25	N5	10	3	0.1
N6	6	3.25	0.25	N6	6	3.75	0.25	N6	1	3.8	0.1
N7	3	3.25	0.25	N7	3	3.75	0.25	N7	3	3.4	0
								N8	11	3.4	0.2
								N9	5	3.4	0.1
								N10	8	3.4	0.1
								N11	9	3.4	0.1

[1] Full Fac 2 level, interaction model, fitted with: PLS; [2] CCF, quadratic model, fitted with: PLS.

2.3.3. Intrinsic Protein Fluorescence

To study the folding of the monoclonal antibody, the intrinsic protein fluorescence was measured with Tycho NT.6 (NanoTemper Technologies, Munich, Germany). For the measurement, 10 µL sample were used. During the measurement, the samples are thermally denatured by a temperature gradient and the fluorescence is detected. Due to the progressive denaturation, a slow unfolding of the proteins takes place whereby the amino acids, tryptophan and tyrosine, which fluoresce when excited with UV light, are increasingly exposed to the medium. As a result, the intrinsic protein fluorescence increases. A high value of intrinsic fluorescence indicates increased unfolding. For a good evaluation of the results obtained, they should be compared with a reference in order to evaluate whether the structure of the sample also corresponds to the desired product. The ratio of light absorption at a wavelength of 350 nm and 330 nm provides information on the quality of the protein [50].

2.4. Equipment

2.4.1. Membrane Adsorber

For this study the Sartobind® A membrane adsorber was used. The membrane adsorber consists of 20 layers (4 mm bed height) of stabilized reinforced cellulose (pore size 0.45 µm) and has a nominal adsorption area of 100 cm^2, a bed volume of 2 mL and a ligand density of 1.5 mg/mL recombinant Protein A. Information was taken from the manufacturer's user manual and the data sheet for the membrane adsorber. The dynamic binding capacity was determined as 5.9 mg/mL and the static binding capacity was 7.5 mg/mL.

2.4.2. Chromatographic System

The commercial system, ÄKTA™ pure (GE Healthcare) was used in this study. For continuous purification, a self-established chromatographic system with four membrane adsorbers (4MA-PCCC, Hanover, Germany) was used. The system was first described in [51] for use with three membrane adsorbers. A fourth chromatography unit with a measurement system was added to the system to provide more flexibility and functionality, and in particular, the implementation of the interconnected wash [51–53] to recover more product. The system is equipped with flow-through cuvettes with a 2 mm pathlength. A calibration curve was recorded with purified mAb and can be seen in Appendix A, Figure A1.

The continuous run was operated with a feed rate of 1.5 mL/min and a flow rate of 5 mL/min for buffer A and B. Switching conditions (SC1 and SC2) were set to 10 and 70% product breakthrough [54].

3. Results

3.1. Optimization of Chromatography Buffers

In order to achieve the best purification results, binding and elution buffers were tested and optimized for the membrane adsorber, Sartobind® Protein A 2 ml. PBS at a neutral pH is often used as a standard binding buffer. Therefore, PBS binding buffer was tested in the pH range of 6–8 (see Appendix A). Here, no significant difference in chromatography performance was observed, hence standard PBS with pH 7.4 was chosen for monoclonal antibody binding.

The elution in Protein A chromatography is much more critical as a low pH value may lead to aggregation and denaturation of the antibody [32,33]. Therefore, the elution buffer was investigated in more detail. Above all, a compromise must be found between the performance and stability of the antibody.

3.1.1. Screening of the Elution Buffers with Stability Testing

In the first experimental series, a DoE screening with seven experiments for each buffer variant was performed (see Table 2). The results are shown in Figure 2 and are a summary of the results of the chromatography and stability experiments (not all data is shown, see Appendix A, Figure A2 for citrate buffer results).

A clear trend can be seen in the chromatography experiments. Citrate buffer provided the best results regarding recovery and peak height. The results for glycine buffers were comparable with citrate, but the peaks are flatter. Acetate buffer gave the worst results, although it should be noted that a smaller pH range was tested. The same trend can be observed for all three buffers: the lower the pH value, the higher the recovery and the peak height. The salt concentration, on the other hand, had only a minor influence. These observed trends correspond to the results of Mazzer et al. [25] and Müller and Vajda. However, they contradict the high influence of the salt content described by Gangnon et al. [30] and Bickel et al., at least in the ranges tested here.

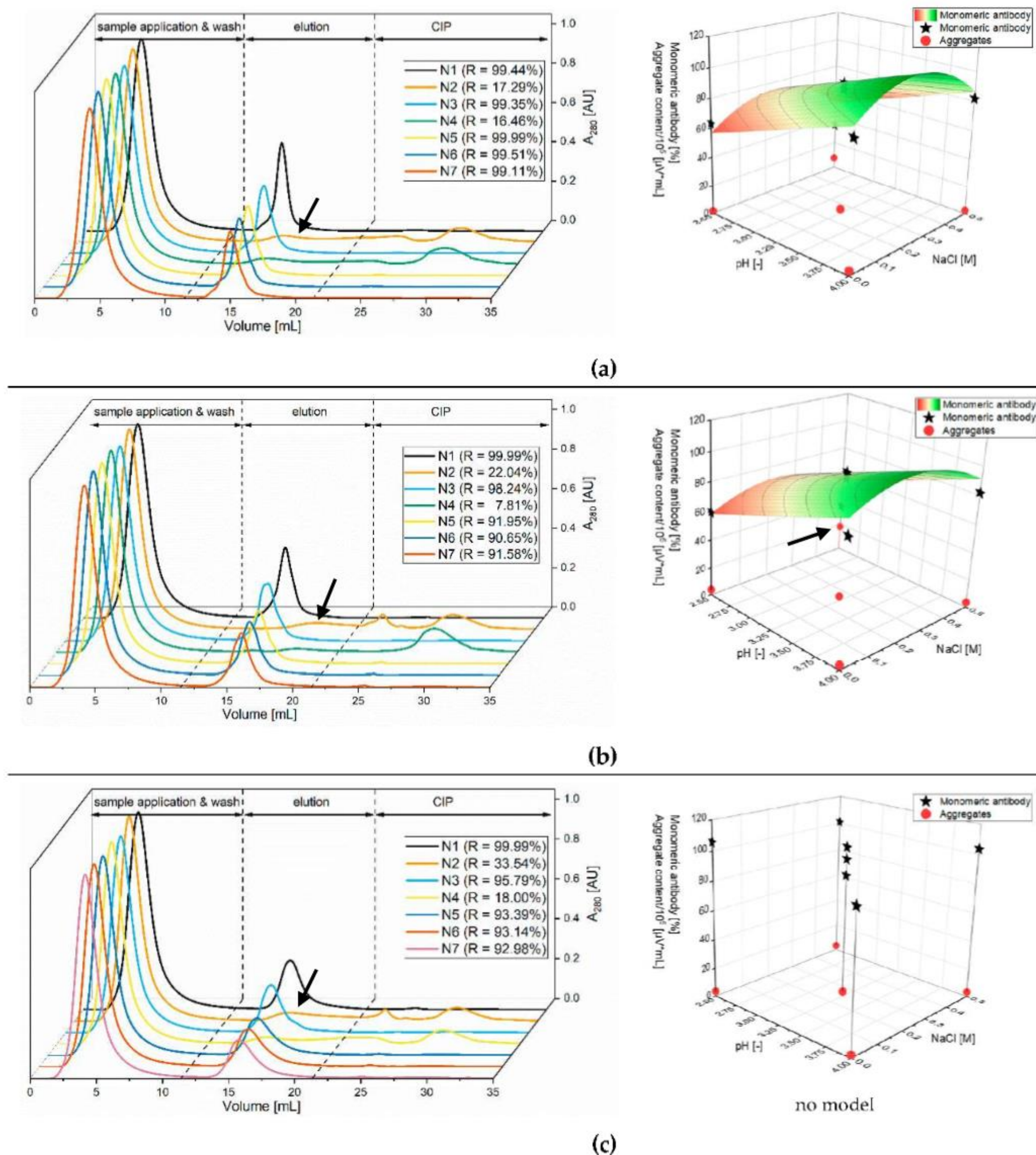


Figure 2. Chromatography (left), aggregation and stability (right) results of the DoE screening experiments with three elution buffers (**a**): citrate buffer pH 2.5–4, 0–0.5 M NaCl; (**b**): glycine buffer pH 2.5–4, 0–0.5M NaCl; (**c**): acetate buffer pH 3.5–4, 0–0.5 M NaCl). Aggregation and stability were measured after one day at 4 °C. Citrate buffer showed the best performance during chromatography runs as peaks were high and sharp (R = Recovery). The worst runs are marked with arrows. The aggregation was low for acetate buffer and increased at low pH and with salt (citrate and glycine buffer). The highest aggregation was measured with glycine buffer and is marked with an arrow. Stability experiments showed a clear trend: with increasing pH, the stability increases for citrate and glycine buffer. The models are shown with a surface diagram and experimental data was added.

In the stability tests (Figure 2, surface diagram) citrate and glycine buffer provided comparable results regarding the amount of intact monoclonal antibody. The effect was opposite to the chromatography results: the higher the pH value, the higher the proportion of intact antibody. The salt concentration had hardly any influence here but can have a non-linear effect depending on the

buffer used, in the investigated range of 0–0.5 M NaCl. For the acetate buffer, the results showed no influence of pH and salt in the tested range.

In addition to chromatographic performance and the amount of intact antibody after incubation in elution buffer, aggregate formation was investigated. Figure 2 shows the results after incubation for 1 d at 4 °C. The greatest aggregation effects can be seen with the glycine buffer at low pH and high salt concentration. Similar tendencies were observed for citrate buffers, however less aggregates were formed. The aggregate formation for acetate in the pH range was also comparable to the other buffers. Singla et al. [34] also compared these three buffer species at a fixed pH of 3.0. Their results showed that at pH 3.0 citrate buffer induced the highest aggregate formation. In this work, the NaCl concentration was higher and up to 0.5 M NaCl compared to the 0.1 M NaCl used by Singla et al. This corresponds to the previously described order of aggregate formation that pH and salt concentration have a higher influence than the buffer species. However, here acetate and citrate buffer gave better results in regard to aggregation than glycine. Considering that temperature also has a major effect on aggregation, it was measured after one week at −20 °C (freezing–thawing). Glycine aggregation increased by 30% and the acetate and citrate buffer was increased by 100%.

Taking into account the previous results, the citrate buffer was selected for further optimization. The citrate buffer provided the best results for chromatography with low aggregate formation and stable antibody in the experimental area. As storing at low pH values is not usual, the aggregation after one week is important but is not performed in a bioprocess.

3.1.2. Optimization of Citrate Buffer

To optimize the citrate buffer, the test range was limited to pH 3–3.8 and 0–0.2 M NaCl (see Appendix A, Figure A2). Eleven experiments (see Table 2) were carried out according to a DoE experiment plan. Chromatography runs were performed according to the experimental plan (Figure 3a). For the stability experiments the incubation time was set to 60 min to imitate virus inactivation conditions. For the chromatography experiments, the same tendencies were observed as in screening: the lower the pH value, the higher the recovery and the peak height. Almost exclusively the pH-value influences the performance. However, a contrary effect can again be seen in the stability results measured with SEC-HPLC and Tycho NT.6 (see Appendix A, Figure A3). A low concentration of salt had a positive effect on the stability and prevents aggregation. Therefore, pH 3.5 and 0.15 M NaCl were chosen for the purification and the optimization and robustness were checked (Figure 3b). Under the selected conditions, the recovery of the monoclonal antibody after the capture step was >95%. After purification with the membrane adsorber Sartobind® Protein A 2 mL, the sample contained 0.26% aggregates, which is comparable to the results of Müller and Vajda [32]. Taking into account the results after freezing and thawing for one week, the aggregate content might further increase, therefore, immediate buffer exchange is mandatory. These low values could be due to the experimental set up, which considers not only the pH but also the salt (NaCl) concentration. Further, the use of membrane adsorbers leads to decreased cycle times.

3.2. Continuous Membrane Purification of the Monoclonal Antibody

Continuous membrane chromatography was used to increase the productivity of the purification step further with the optimized method from Section 3.1.2. The continuous chromatography method is based on the principle of periodic counter-current chromatography (PCCC), which is controlled by the UV signal of the membrane adsorber outlet. Therefore, the feed was diluted 1:3 to be in the linear measuring range (A_{280} < ~1.5 AU) with a mAb concentration of 0.6 g/L.

3.2.1. Double Breakthrough Curve

In PCCC, up to two membrane adsorbers (MA) are loaded in series to capture the product breakthrough of the first MA on the second MA. The UV signal of the breakthrough curve serves as the dynamic control strategy for automation. This mode of operation allows loading of the MA close

to the static binding capacity (available capacity of the MA in equilibrium) and thus results in a higher capacity utilization of the MA. To design the process, different breakthrough curves were recorded with the 4MA-PCCC (see Figure 4) to find the optimal feed rate and to determine the switching conditions for the dynamic process control with the UV signal. The feed rate of 1.5 mL/min was found to be suitable because the time for loading was equal to the time for regeneration, which is a criterion for PCCC [55].

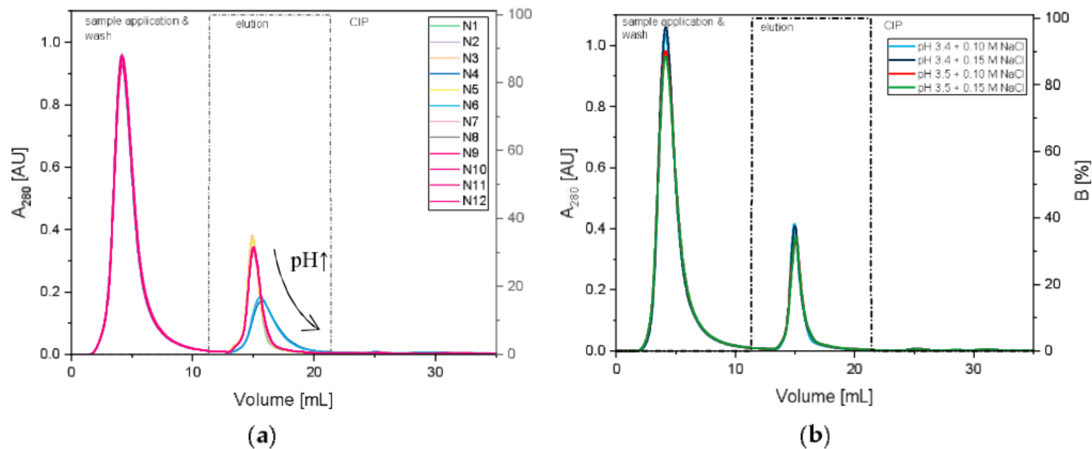


Figure 3. Optimization of Protein A chromatography elution with citrate buffer (**a**) and robustness testing (**b**). By increasing the pH, the performance of the chromatography decreased and the peaks became flatter. During the robustness testing, the result of the chromatography was not influenced by small fluctuations in the buffer system.

The switching conditions were calculated as follows:

$$SC1 = 10\%;\ \Delta UV + UV_{imp} = 1.28\ AU \quad (1)$$

$$SC2 = 70\%;\ \Delta UV + UV_{imp} = 1.43\ AU \quad (2)$$

where ΔUV is the difference between the UV signal of the feed (UV_{max}) and the UV signal of the impurity (here UV_{imp}).

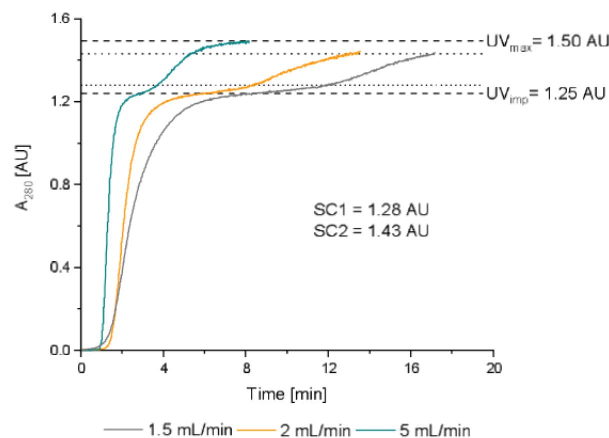


Figure 4. Determination of a suitable flow rate for loading of the Sartobind® Protein A membrane adsorber for periodic counter-current chromatography (PCCC) operation. By increasing the flow rate, the double breakthrough curve got steeper. 1.5 mL/min was chosen as suitable flow rate.

3.2.2. PCCC Application

The continuous run was performed with 160 mL feed containing 0.6 g/L mAb. The chromatogram can be seen in Figure 5. Two cycles are shown, which were performed in 2 h. The double breakthrough curves can be seen whereas the mAb elution is marked with arrows. The elution peaks depict concentrations between 2.5 and 5 g/L (see Appendix A, Figure A1). Due to the dynamic process control, each membrane adsorber was loaded until it was saturated. Slight differences occur due to the system setup. At the end of the loading a small peak can be seen, which was captured on a further membrane adsorber (interconnected wash [52,53]). A constant performance of the MA can be observed in the process, but a trend (MA1 to MA4) can be seen in each cycle. The elution peaks decrease because the self-built system has back suction from buffer B. In this system, the very complex circuit was realized with 37 valves and this resulted in a high dead volume, which causes the back suction. Nevertheless, the recovery after the purification was higher than 90% and only 600 mL buffer was consumed. Compared to the batch process, the capacity utilization of the MA was increased by 20% due to the loading principle of PCCC. Further, the optimized chromatography conditions showed a significant improvement in the PCCC compared to the non-optimized conditions (see Appendix A, Figure A3).

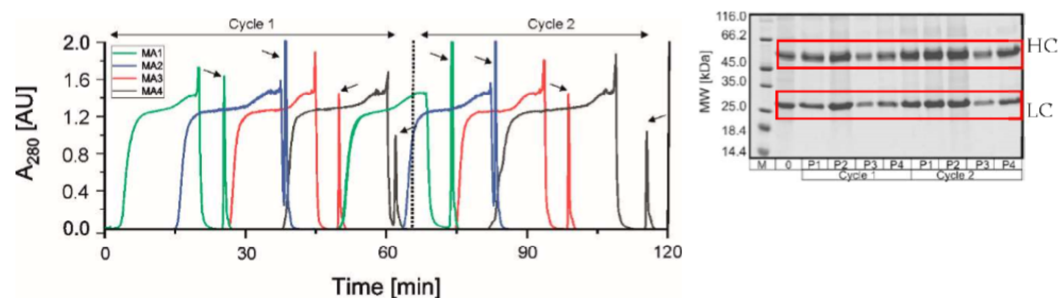


Figure 5. Chromatogram and reducing SDS-PAGE of two cycles with the 4MA-PCCC using Sartobind® Protein A for the purification of a monoclonal antibody. Two PCCC cycles are shown in the chromatogram. A cycle consists of the loading, elution and regeneration of the four membrane adsorbers. The product peaks are marked with black arrows. Throughout the PCCC run, a trend was observed in that the peaks decrease during the cycles. The reason for this is the complex system setup.

4. Discussion

This study presents the optimization of the critical Protein A purification step in monoclonal antibody downstream processing with a focus on the elution. Up until now, downstream processing has been a bottleneck in the production process, since high product titers must be processed in compliance with legal requirements. The Protein A step is critical because a low pH is used for product elution, which can lead to aggregation and thus to problems in quality, quantity, further processing and above all, in drug safety. It is therefore vital to optimize the downstream process. In this study, the elution was optimized with various buffers and the application of membrane adsorbers (Sartobind® Protein A 2 mL) as an alternative to conventional column chromatography. Although the capacities were still lower than in column chromatography, the throughputs were significantly higher and the residence times were shorter. This is particularly interesting for mAb elution. For the IgG_1 mAb, tested in this work, 0.1 M citrate buffer pH 3.5 + 0.15 M NaCl provided the best results in terms of MA chromatography performance, product stability and aggregation (tested with SEC-HPLC and Tycho NT.6). Furthermore, the development time of the process could be significantly reduced with membrane adsorbers. The optimized chromatography method was successfully transferred to a continuous chromatography system operated with four membrane adsorbers (4MA-PCCC).

The PCCC principle is very suitable for the purification of mAbs because the product concentration is quite high compared to other products [51,56]. The control via the UV signal using the switching conditions could be easily implemented. Due to increasing product titers > 5 g/L [57], this will improve

even further. This is particularly useful in relation to the process analytical technology (PAT) initiative defined by the FDA [58]. The process can be monitored directly and the performance is directly visible. The process can be stopped before the performance decreases and the product quality no longer meets the legal requirements. This could increase the economics of the DSP, since better utilization of the MA used would increase productivity.

In conclusion, this work shows the structured optimization of a monoclonal antibody purification step. It is very important to control the quality of the antibody throughout the entire optimization process in order to ensure high product quality and to identify the critical steps. By using DoE, the number of experiments could be reduced and thus the optimization was very fast. The use of membrane adsorbers has the advantage that the process is easily scalable and the disposable MA can be disposed of after use. Furthermore, the transfer of the batch to continuous operation mode was described, and this is very relevant to current developments in the biotechnological industry.

Author Contributions: Conceptualization, C.B. and S.B.; methodology, C.B.; formal analysis, S.B.; investigation, C.B., L.T., A.G.S.; resources, S.B., O.-W.R. and T.S.; writing—original draft preparation, C.B.; writing—review and editing, A.G.S., D.S., T.S., S.B.; visualization, C.B.; supervision, T.S. and S.B.; project administration, S.B.; funding acquisition, D.S.

Funding: We acknowledge financial support by the BMBF within the project Prozessallianz WiPro (031B0475I). No further external funding was received.

Acknowledgments: We would like to thank Florian Taft, Patrick Adametz and Katrin Töppner from Sartorius Stedim Biotech for their support in this project. The publication of this article was funded by the Open Access fund of Leibniz Universität Hannover.

Conflicts of Interest: The authors have declared no conflict of interest.

Appendix A

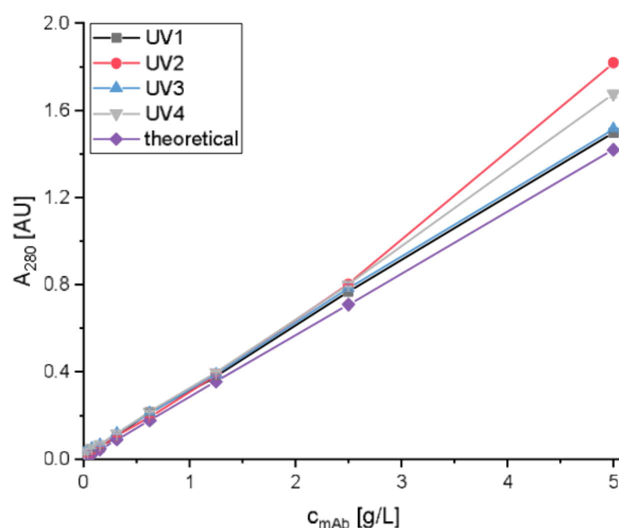


Figure A1. Calibration data of the mAb at the 4MA-PCCC system with a 2 mm flow through cuvette. Purified mAb solution was measured in the UV cuvettes. Small deviations occurred due to the use of optical fibers. Limit of detection (LOD) and limit of quantification (LOQ) were determined individually and the averages were calculated: LOD = 0.009 g/L and LOQ = 0.029 g/L.

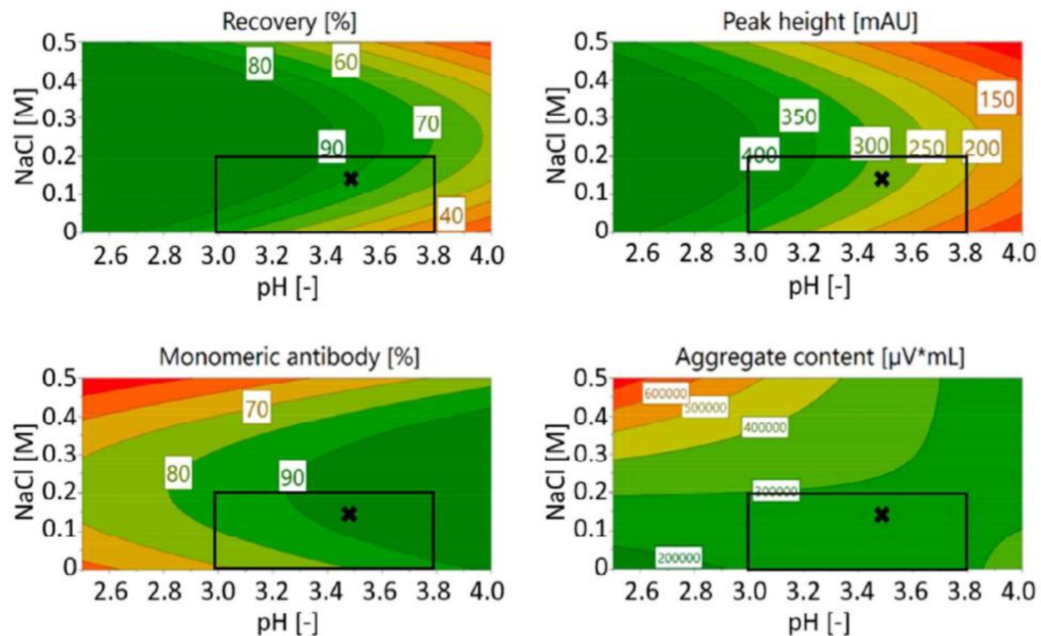


Figure A2. Results of the citrate buffer screening with chromatography experiments (Recovery and Peak height) and stability testing (Monomeric antibody and Aggregate content). The range for the next optimization step is marked with a box as well as the final operation point with a "×".

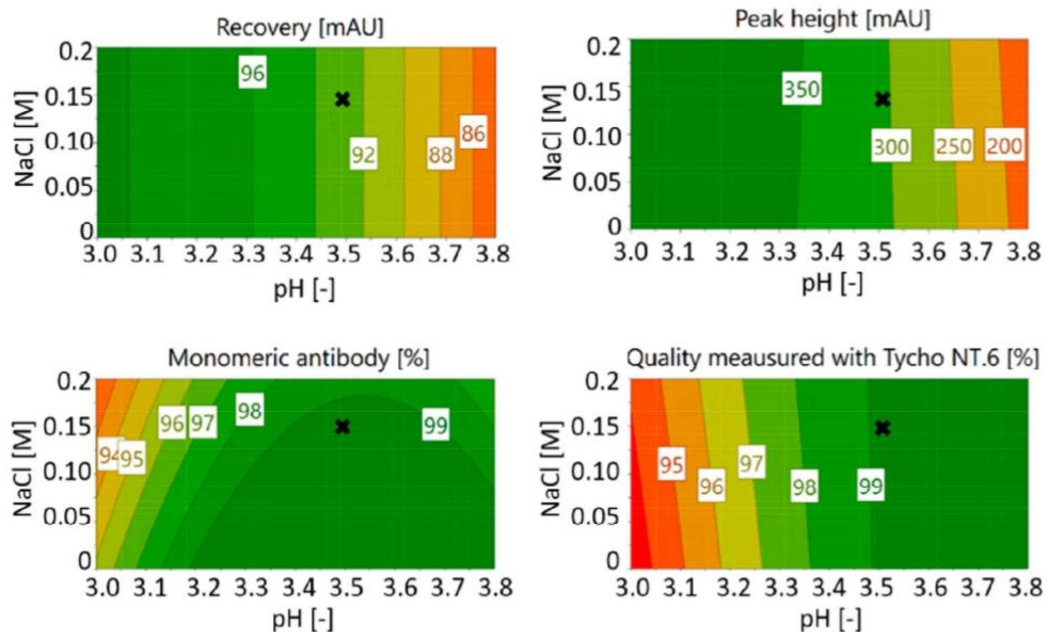


Figure A3. Results of the citrate buffer optimization with chromatography experiments (Recovery and Peak height) and stability testing (Monomeric antibody and Quality measured with Tycho NT.6). The final operation point is marked with a "×".

References

1. Chames, P.; Van Regenmortel, M.; Weiss, E.; Baty, D. Therapeutic antibodies: successes, limitations and hopes for the future. *Br. J. Pharmacol.* **2009**, *157*, 220–233. [CrossRef] [PubMed]
2. Reichert, J.M. Antibodies to watch in 2017. *MAbs* **2017**, *9*, 167–181. [CrossRef] [PubMed]

3. Kaplon, H.; Reichert, J.M. Antibodies to watch in 2019. *MAbs* **2019**, *11*, 219–238. [CrossRef] [PubMed]
4. Berg, J.M.; Stryer, L.; Tymoczko, J.L. *Stryer Biochemie*; Springer: Berlin/Heidelberg, Germany, 2007.
5. GE Healthcare. *Affinity Chromatography Vol. 1: Antibodies*; GE Healthcare: Uppsala, Sweden, 2014.
6. Herschel, T.; El-Armouche, A.; Weber, S. Monoklonale Antikörper. *DMW-Dtsch. Med. Wochenschr.* **2016**, *141*, 1390–1394. [CrossRef] [PubMed]
7. Sack, U.; Emmrich, F. Monoklonale Antikörper. *Internist* **2008**, *49*, 919–928. [CrossRef]
8. Schäffner, G.; Kabelitz, D. Monoklonale Antikörper zur Therapie. *DMW-Dtsch. Med. Wochenschr.* **2001**, *126*, 851–856. [CrossRef]
9. Blum, H.E. Therapeutische monoklonale Antikörper. *DMW-Dtsch. Med. Wochenschr.* **2000**, *125*, 1501–1504.
10. Ribatti, D. Edelman's view on the discovery of antibodies. *Immunol. Lett.* **2015**, *164*, 72–75. [CrossRef]
11. Kunert, R.; Reinhart, D. Advances in recombinant antibody manufacturing. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 3451–3461. [CrossRef]
12. Sommerfeld, S.; Strube, J. Challenges in biotechnology production—Generic processes and process optimization for monoclonal antibodies. *Chem. Eng. Process. Process Intensif.* **2005**, *44*, 1123–1137. [CrossRef]
13. Chon, J.H.; Zarbis-Papastoitsis, G. Advances in the production and downstream processing of antibodies. *New Biotechnol.* **2011**, *28*, 458–463. [CrossRef] [PubMed]
14. Kelley, B. Industrialization of mAb production technology: The bioprocessing industry at a crossroads. *MAbs* **2009**, *1*, 443–452. [CrossRef] [PubMed]
15. Strube, J.; Grote, F.; Ditz, R. Bioprocess design and production technology for the future. *Biopharm. Prod. Technol.* **2012**, *1*, 657–705.
16. Low, D.; O'Leary, R.; Pujar, N.S. Future of antibody purification. *J. Chromatogr. B* **2007**, *848*, 48–63. [CrossRef]
17. Seewoester, T. Cell separation and product capture. *Biotechnol. Bioprocess. Ser.* **2005**, *30*, 417.
18. Marichal-Gallardo, P.A.; Alvarez, M.M. State-of-the-art in downstream processing of monoclonal antibodies: process trends in design and validation. *Biotechnol. Prog.* **2012**, *28*, 899–916. [CrossRef]
19. Mendiratta, S.K.; Bandyopadhyay, S.; Singh, A.K. Purification Process for Monoclonal Antibodies. U.S. Patent No. 9,708,365, 18 July 2017.
20. Gottschalk, U. *Process Scale Purification of Antibodies*; no. 577.27 PRO; Wiley Online Library: Hoboken, NJ, USA, 2009.
21. Li, R.; Dowd, V.; Stewart, D.J.; Burton, S.J.; Lowe, C.R. Design, synthesis, and application of a protein A mimetic. *Nat. Biotechnol.* **1998**, *16*, 190. [CrossRef]
22. Ayyar, B.V.; Arora, S.; Murphy, C.; O'Kennedy, R. Affinity chromatography as a tool for antibody purification. *Methods* **2012**, *56*, 116–129. [CrossRef]
23. Deisenhofer, J. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-. ANG. resolution. *Biochemistry* **1981**, *20*, 2361–2370. [CrossRef]
24. Gagnon, P. *Purification Tools for Monoclonal Antibodies*; Validated Biosystems Inc., 1996; Volume 196.
25. Mazzer, A.R.; Perraud, X.; Halley, J.; O'Hara, J.; Bracewell, D.G. Protein A chromatography increases monoclonal antibody aggregation rate during subsequent low pH virus inactivation hold. *J. Chromatogr. A* **2015**, *1415*, 83–90. [CrossRef]
26. Huse, K.; Böhme, H.-J.; Scholz, G.H. Purification of antibodies by affinity chromatography. *J. Biochem. Biophys. Methods* **2002**, *51*, 217–231. [CrossRef]
27. Shukla, A.A.; Hubbard, B.; Tressel, T.; Guhan, S.; Low, D. Downstream processing of monoclonal antibodies—application of platform approaches. *J. Chromatogr. B* **2007**, *848*, 28–39. [CrossRef] [PubMed]
28. Shukla, A.A.; Hinckley, P.J.; Gupta, P.; Yigzaw, Y.; Hubbard, B. Strategies to address aggregation during protein A chromatography. *BioProcess. Int.* **2005**, *3*, 36–43.
29. Seed, B. Affinity Purification Methods Involving Imidazole Elution. U.S. Patent No. 5,726,293, 10 March 1998.
30. Gagnon, P.; Nian, R.; Leong, D.; Hoi, A. Transient conformational modification of immunoglobulin G during purification by protein A affinity chromatography. *J. Chromatogr. A* **2015**, *1395*, 136–142. [CrossRef]
31. Arakawa, T.; Philo, J.S.; Tsumoto, K.; Yumioka, R.; Ejima, D. Elution of antibodies from a Protein-A column by aqueous arginine solutions. *Protein Exp. Purif.* **2004**, *36*, 244–248. [CrossRef]
32. Müller, E.; Vajda, J. Routes to improve binding capacities of affinity resins demonstrated for protein A chromatography. *J. Chromatogr. B* **2016**, *1021*, 159–168. [CrossRef]

33. Bickel, F.; Herold, E.M.; Signes, A.; Romeijn, S.; Jiskoot, W.; Kiefer, H. Reversible NaCl-induced aggregation of a monoclonal antibody at low pH: characterization of aggregates and factors affecting aggregation. *Eur. J. Pharm. Biopharm.* **2016**, *107*, 310–320. [CrossRef]
34. Singla, A.; Bansal, R.; Joshi, V.; Rathore, A.S. Aggregation kinetics for IgG1-based monoclonal antibody therapeutics. *AAPS J.* **2016**, *18*, 689–702. [CrossRef]
35. Rathore, A.S.; Kateja, N.; Agarwal, H. Continuous Downstream Processing for Production of Biotech Therapeutics. In *Continuous Biomanufacturing: Innovative Technologies and Methods*; Subramanian, G., Ed.; Wiley-VCH: Weinheim, Germany, 2017; pp. 261–288.
36. Fraud, N.; Kuczewski, M.; Zarbis-Papastoitsis, G.; Hirai, M. Hydrophobic Membrane Adsorbers for Large-Scale Downstream Processing. *Biopharm. Int.* **2009**, *22*.
37. Yu, D.; Ghosh, R. Purification of PEGylated Protein Using Membrane Chromatography. *J. Pharm. Sci.* **2010**, *99*, 3326–3333. [CrossRef]
38. Zhou, J.X.; Tressel, T. Basic concepts in Q membrane chromatography for large-scale antibody production. *Biotechnol. Prog.* **2006**, *22*, 341–349. [CrossRef]
39. Vogel, J.H.; Nguyen, H.; Giovannini, R.; Ignowski, J.; Garger, S.; Salgotra, A.; Tom, J. A new large-scale manufacturing platform for complex biopharmaceuticals. *Biotechnol. Bioeng.* **2012**, *109*, 3049–3058. [CrossRef]
40. Boi, C. Membrane adsorbers as purification tools for monoclonal antibody purification. *J. Chromatogr. B* **2007**, *848*, 19–27. [CrossRef] [PubMed]
41. Liu, H.F.; Ma, J.; Winter, C.; Bayer, R. Recovery and purification process development for monoclonal antibody production. *MAbs* **2010**, *2*, 480–499. [CrossRef] [PubMed]
42. Kuczewski, M.; Schirmer, E.; Lain, B.; Zarbis-Papastoitsis, G. A single-use purification process for the production of a monoclonal antibody produced in a PER. C6 human cell line. *Biotechnol. J.* **2011**, *6*, 56–65. [CrossRef] [PubMed]
43. Knudsen, H.L.; Fahrner, R.L.; Xu, Y.; Norling, L.A.; Blank, G.S. Membrane ion-exchange chromatography for process-scale antibody purification. *J. Chromatogr. A* **2001**, *907*, 145–154. [CrossRef]
44. Mothes, B.; Pezzini, J.; Schroeder-Tittmann, K.; Villain, L. Accelerated, seamless antibody purification. *Bioprocess. Int.* **2016**, *14*, 5.
45. Jungbauer, A. Continuous downstream processing of biopharmaceuticals. *Trends Biotechnol.* **2013**, *31*, 479–492. [CrossRef]
46. Girard, V.; Hilbold, N.J.; Ng, C.K.; Pegon, L.; Chahim, W.; Rousset, F.; Monchois, V. Large-scale monoclonal antibody purification by continuous chromatography, from process design to scale-up. *J. Biotechnol.* **2015**, *213*, 65–73. [CrossRef]
47. Gjoka, X.; Gantier, R.; Schofield, M. Transfer of a three step mAb chromatography process from batch to continuous: Optimizing productivity to minimize consumable requirements. *J. Biotechnol.* **2017**, *242*, 11–18. [CrossRef]
48. De Fazekas, S.S.G.; Webster, R.G.; Datyner, A. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. *Biochim. Biophys. Acta* **1963**, *71*, 377–391.
49. Wray, W.; Boulikas, T.; Wray, V.P.; Hancock, R. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **1981**, *118*, 197–203. [CrossRef]
50. Breitsprecher, G.W.D.; Kern, B.; Gupta, A.J.; Knoll, K. Reproduzierbarkeit- Qualitätskontrolle von Proteinen durch thermische Entfaltungsprofile. *BIOspektrum* **2019**, 60–62. Available online: https://www.biospektrum.de/blatt/d_bs_pdf&_id=1539789 (accessed on 27 November 2019).
51. Brämer, C.; Schreiber, S.; Scheper, T.; Beutel, S. Continuous purification of Candida antarctica lipase B using 3-membrane adsorber periodic counter-current chromatography. *Eng. Life Sci.* **2018**. [CrossRef]
52. Mahajan, E.; George, A.; Wolk, B. Improving affinity chromatography resin efficiency using semi-continuous chromatography. *J. Chromatogr. A* **2012**, *1227*, 154–162. [CrossRef]
53. Pollock, J.; Bolton, G.; Coffman, J.; Ho, S.V.; Bracewell, D.G.; Farid, S.S. Optimising the design and operation of semi-continuous affinity chromatography for clinical and commercial manufacture. *J. Chromatogr. A* **2013**, *1284*, 17–27. [CrossRef]
54. Warikoo, V.; Godawat, R.; Brower, K.; Jain, S.; Cummings, D.; Simons, E.; Johnson, T.; Walther, J.; Yu, M.; Wright, B.; et al. Integrated continuous production of recombinant therapeutic proteins. *Biotechnol. Bioeng.* **2012**, *109*, 3018–3029. [CrossRef]

55. Godawat, R.; Brower, K.; Jain, S.; Konstantinov, K.; Riske, F.; Warikoo, V. Periodic counter-current chromatography–design and operational considerations for integrated and continuous purification of proteins. *Biotechnol. J.* **2012**, *7*, 1496–1508. [CrossRef]
56. Brämer, C.; Ekramzadeh, K.; Lammers, F.; Scheper, T.; Beutel, S. Optimization of continuous purification of recombinant patchoulol synthase from Escherichia coli with membrane adsorbers. *Biotechnol. Prog.* **2019**, *35*, e2812. [CrossRef]
57. Shukla, A.A.; Wolfe, L.S.; Mostafa, S.S.; Norman, C. Evolving trends in mAb production processes. *Bioeng. Transl. Med.* **2017**, *2*, 58–69. [CrossRef]
58. Skoglar, H.; Blom, H.; Mathiasson, L.; Akerblom, A.; Łącki, K. The use of dynamic control in periodic counter-current chromatography. *Bioprocess. Int.* **2015**, *13*, 29150261.

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

4 Zusammenfassung und Ausblick

Im Rahmen dieser Arbeit wurde ein kontinuierliches Chromatographie-System entwickelt und dessen Anwendung anhand von drei Beispielen gezeigt. Das System wurde zunächst mit drei Chromatographie-Einheiten nach dem Prinzip der Periodic-Counter-Current-Chromatographie (PCCC) aufgebaut. Anders als bei den kommerziell erhältlichen Anlagen wurde das System auf (Einweg-)Membranadsorber (MA) ausgelegt, da diese im Vergleich zu konventionell-verwendeten Säulen einige Vorteile bieten. Das sind vor allem die gesteigerten Durchsätze, die aufgrund der Eliminierung von Porendiffusion möglich sind.

Mit einem Modellproteingemisch aus BSA und Lysozym wurde die PCCC-Anlage erfolgreich in Betrieb genommen und getestet. Anschließend wurde die Anlage für die Aufreinigung von drei Proteinen bzw. Enzymen eingesetzt: der Aufreinigung von *Candida antarctica* Lipase B (CalB) aus *E. coli*-Lysat, der Aufreinigung von Patchoulolsynthase (PTS) aus *E. coli*-Lysat und der Aufreinigung eines monoklonalen Antikörpers (mAb) aus Chinese Hamster Ovary (CHO)-Zellüberstand.

Für die Aufreinigung von CalB wurde eine AEX-Methode (Sartobind® Q75) mit einer zwei-stufigen Elution unter Verwendung von 20 mM Tris-HCl-Puffer pH 8,5 entwickelt. CalB wurde in der ersten Elutionsstufe bei 0,2 M NaCl und die Verunreinigungen bei 1 M NaCl eluiert. Die kontinuierliche Aufreinigung mit der 3MA-PCCC wurde über fünf Zyklen durchgeführt. Aus 0,9 L *E. coli*-Lysat wurden 0,22 g CalB mit einer Wiederfindung von 80 % isoliert. Im Vergleich zum Batch-Prozess, wurde die Produktivität des Prozesses um 36 % erhöht. Generell hat sich die PCCC zur Aufreinigung der CalB als praktikabel erweisen, jedoch wurden neben der CalB auch Verunreinigungen (vor allem *E. coli* host cell Proteine und DNA) an den MA gebunden. Daher hat im Laufe des Prozesses die Leistung der Chromatographie abgenommen.

Aus den Versuchen wurde geschlussfolgert, dass die PCCC-Anlage sich daher eher für die Aufreinigung von spezifisch bindenden Produkten mittels Affinitätschromatographie eignet. Mögliche Beispiele sind die Aufreinigung von Zielproteinen, die einen His-Tag besitzen oder die Aufreinigung von einem mAb. In der Literatur ist fast ausschließlich die Aufreinigung von monoklonalen Antikörpern mit der PCCC zu finden [8, 76–78].

Daher handelt es sich bei dem zweiten Anwendungsbeispiel um die Aufreinigung der Patchoulolsynthase (PTS). Diese wurde mit einem His-Tag versehen und konnte daher

mittels Co^{2+}-Immobilisierter-Metallionen-Affinitätschromatographie (IMAC) mit dem Membranadsorber Sartobind® IDA 75 aufgereinigt werden. Zur kontinuierlichen Aufreinigung der PTS mittels 3MA-PCCC wurde 50 mM Triethanolamin-NaOH-Puffer pH 7 unter Zugabe verschiedener Additive eingesetzt. Zur Elution der PTS wurden 250 mM Imidazol zugesetzt. Im Rahmen der 3MA-PCCC wurden 0,45 L *E. coli*-Lysat über 2,33 Zyklen aufgereinigt und die Wiederfindung lag bei knapp 70 %. Obwohl die dynamische Prozessteuerung aufgrund der niedrigen Produktkonzentration nicht möglich war, konnte die Produktivität dennoch um 47 % im Vergleich zum Batch-Prozess gesteigert werden.

Bei den oben beschriebenen Anwendungsbeispielen kam es zu Produktverlusten von ca. 20–30 %. Diese sind auf den Systemaufbau der 3MA-PCCC zurückzuführen, sodass beim Waschen des Membranadsorbers nach einer Beladungsphase Produkt verloren geht. Daher und weiterhin zur Optimierung des Systems wurde die PCCC-Anlage auf eine vierte Membranadsorber-Einheit (4MA-PCCC) sowie um weitere Ventile erweitert. Damit wurde neben der flexibleren Anwendung mit vier MA auch der sogenannte interconnected wash implementiert. Nach Beladung eines MA wird die Waschfraktion auf einen weiteren MA umgeleitet, sodass die Produktausbeute gesteigert wird.

Mit dem erweiterten 4MA-PCCC-Setup wurde die Aufreinigung eines monoklonalen Antikörpers mittels Protein-A-Chromatographie (Sartobind® Protein A 2 mL) etabliert. Im Vorfeld wurden die Chromatographie-Puffer optimiert, um die gewünschte Produktqualität und Prozessleitung zu gewährleisten. In 2 h wurden 160 mL CHO-Zellüberstand mit 0,6 g/L mAb über zwei PCCC-Zyklen aufgereinigt. In den PCCC-Zyklen war ein negativer Trend der Höhe der Elutionspeaks und damit der Produktausbeute zu erkennen, dennoch lag die mAb-Wiederfindung bei über 90 %. Bei gleichmäßiger Prozessleistung ist eine Produktivitäts- und Kapazitätssteigerung von jeweils 20 % zu erwarten.

In dieser Arbeit wurden damit für die kontinuierliche Chromatographie Kapazitätssteigerungen von etwa 20 % und Produktivitätssteigerungen von 20 bis etwa 50 % (berechnet nach Kaltenbrunner *et al.* [55]) festgestellt. Damit kann keine pauschale Aussage darüber getroffen werden, inwieweit sich die Produktivität eines Prozesses unter Verwendung kontinuierlicher Methoden steigern lässt. Dies muss für jeden Prozess individuell bestimmt werden. In der Literatur sind simulierte Werte für die kontinuierliche Chromatographie mit Membranadsorbern zu finden. Im Vergleich zum

Batch-Prozess sind Produktivitätssteigerungen von etwa 50 % und Kapazitätssteigerungen von etwa 20 % pro Einheit zu erwarten [79].

Aufgrund des diffusiven Massentransports in Säulen sind die Durchbruchskurven vergleichsweise flacher und die Kapazitätsnutzung mit etwa 50 % [33, 80] im kontinuierlichen Prozess höher. Die durchschnittliche Produktivitätssteigerung liegt bei etwa 40 % [80]. Durch den Einsatz von Membranadsorbern ist damit vor allem mit einer Steigerung der Produktivität im kontinuierlichen Prozess zu rechnen, wobei die Kapazität etwas schlechter als bei Säulen genutzt wird. Damit eignet sich die kontinuierliche Membranchromatographie vor allem für Prozesse, die in der Produktivität gesteigert und in der Prozesszeit reduziert werden sollen. Das ist besonders interessant für teure, eher instabile Produkte.

Die Bindungskapazität von Membranadsorbern ist unabhängig von der Flussrate [30], somit konnten die Flussraten von Feed und Puffern entsprechend des PCCC-Prinzips angepasst werden. Das ist ein großer Vorteil gegenüber Säulen, da hier die Flussrate die Kapazität aufgrund der Porendiffusion stark beeinflusst. Daher ist die Auslegung des PCCC-Prozesses mit Membranadsorbern flexibler, wobei beachtet werden muss, dass auch bei Membranadsorbern bei zu niedrigen Flussraten diffusive Effekte auftreten können.

Generell sollte das PCCC-Prinzip für spezifisch bindenden Proteine wie Antikörper oder His-getaggte Proteine (Affinitätschromatographie) verwendet werden. Produkte, die extrazellular vorliegen, können grundsätzlich leichter mit der PCCC aufgereinigt werden, da kaum Verunreinigungen (Medienbestandteile, DNA oder host cell Proteine) vorliegen, welche die Chromatographie beeinflussen. In jedem Fall sollte das Produkt in ausreichender Menge vorliegen, damit die intelligente, dynamische Prozessteuerung und damit das Prinzip der PCCC erfüllt wird. Trotzdem eignet sich das vorgestellte Anlagen-Setup der 4MA-PCCC ebenso für die Aufreinigung anderer Proteine, wie in der vorliegenden Arbeit am Beispiel der CalB gezeigt wurde. Die Steigerung der Produktivität und Kapazitätsnutzung fällt lediglich geringer aus. Langfristig ist das Ziel, das System in einen kontinuierlichen Produktionsprozess aus USP und DSP zu integrieren. Dafür würde sich besonders der Antikörper-Prozess aus CHO-Zellen eignen.

5 Literaturverzeichnis

[1] J. Puetz and F. M. Wurm, "Recombinant Proteins for Industrial versus Pharmaceutical Purposes: A Review of Process and Pricing," *Processes*, vol. 7, no. 8, p. 476, 2019.

[2] A. S. Rathore, H. Agarwal, A. K. Sharma, M. Pathak, and S. Muthukumar, "Continuous processing for production of biopharmaceuticals," *Prep. Biochem. Biotechnol.*, vol. 45, no. 8, pp. 836–849, 2015.

[3] A. Jungbauer, "Continuous downstream processing of biopharmaceuticals," *Trends Biotechnol.*, vol. 31, no. 8, pp. 479–492, 2013.

[4] J.-C. Janson and J. Å. Jönsson, "Introduction to Chromatography," *Protein Purification*. pp. 23–50, 07-Mar-2011.

[5] S. Chatterjee, "FDA perspective on continuous manufacturing," in *IFPAC Annual Meeting, Baltimore, MD*, 2012.

[6] F. Steinebach, T. Müller-Späth, and M. Morbidelli, "Continuous counter-current chromatography for capture and polishing steps in biopharmaceutical production," *Biotechnol. J.*, vol. 11, no. 9, pp. 1126–1141, 2016.

[7] M. Hamende, "10 Case study in production-scale multicolumn continuous chromatography," *Prep. Enantioselective Chromatogr.*, p. 253, 2005.

[8] E. Mahajan, A. George, and B. Wolk, "Improving affinity chromatography resin efficiency using semi-continuous chromatography," *J. Chromatogr. A*, vol. 1227, pp. 154–162, 2012.

[9] H. A. Anurag S. Rathore, Nikhil Kateja, "Continuous Downstream Processing for Production o fBiotech Therapeutics."

[10] F. Single-use, C. Bio-manufacturing, and C. Corporation, "Press release 200," no. 215, pp. 214–215, 2017.

[11] H. Skoglar, H. Blom, L. Mathiasson, A. Akerblom, and K. Łącki, "The use of dynamic control in periodic counter-current chromatography," *Bioprocess Int.*, vol. 13, p. 29150261, 2015.

[12] J. Wolfgang and A. Prior, "Continuous Annular Chromatography," vol. 76, 2002.

[13] E. Karlsson and I. Hirsh, "Ion Exchange Chromatography," in *Protein Purification*, John Wiley & Sons, Inc., 2011, pp. 93–133.

[14] P. M. Cummins, O. Dowling, and B. F. O'Connor, "Ion-exchange chromatography: basic principles and application to the partial purification of soluble mammalian prolyl oligopeptidase," *Protein Chromatogr. Methods Protoc.*, pp. 215–228, 2011.

[15] GE Healthcare, "Ion exchange chromatography & chromatofocusing: Principles and methods," *Ed. AA, Amersham Biosci.*, p. 7, 2010.

[16] G. Karp, *Molekulare Zellbiologie*. Springer, 2005.

[17] B. V. Ayyar, S. Arora, C. Murphy, and R. O'Kennedy, "Affinity chromatography as a tool for antibody purification," *Methods*, vol. 56, no. 2, pp. 116–129, 2012.

[18] P. Neubauer and M. N. Cruz-Bournazou, "Continuous Bioprocess Development: Methods for Control and Characterization of the Biological System," *Contin. Biomanufacturing Innov. Technol. Methods; John Wiley Sons Hoboken, NJ, USA*, 2017.

[19] K. B. Konstantinov and C. L. Cooney, "White paper on continuous bioprocessing. May 20–21, 2014 Continuous Manufacturing Symposium," *J. Pharm. Sci.*, vol. 104, no. 3, pp. 813–820, 2015.

[20] M. Bisschops, "Bio SMB ™ Technology : Continuous Countercurrent Chromatography Enabling a Fully Disposable Process," pp. 769–791, 2012.

[21] S. S. Ozturk, "Opportunities and Challenges for the Implementation of Continuous Processing in Biomanufacturing," *Contin. Process. Pharm. Manuf.*, 2014.

[22] T. Müller-Späth and M. Morbidelli, "Multicolumn Countercurrent Gradient Chromatography for the Purification of Biopharmaceuticals," *Contin. Process. Pharm. Manuf.*, pp. 227–254, 2015.

[23] A. Rajendran, G. Paredes, and M. Mazzotti, "Simulated moving bed chromatography for the separation of enantiomers," *J. Chromatogr. A*, vol. 1216, no. 4, pp. 709–738, 2009.

[24] S. Imamoglu, "Simulated moving bed chromatography (SMB) for application in bioseparation," in *Modern Advances in Chromatography*, Springer, 2002, pp. 211–231.

[25] Z. Molnár *et al.*, "Separation of amino acids with simulated moving bed chromatography," *J. Chromatogr. A*, vol. 1075, no. 1–2, pp. 77–86, 2005.

[26] H. S. Traub, "Preparative chromatography," 2005.

[27] H. L. Knudsen, R. L. Fahrner, Y. Xu, L. A. Norling, and G. S. Blank, "Membrane ion-exchange chromatography for process-scale antibody purification," *J. Chromatogr. A*, vol. 907, no. 1, pp. 145–154, 2001.

[28] S. Fischer-Frühholz, "Membranadsrober Chromatographische Aufreinigung in neuen Dimensionen," *GIT Labor-Fachzeitschrift*, vol. 2, pp. 2–4, 2004.

[29] J. Wang, "Macroporous ion-exchange membrane adsorbers: correlation between membrane structure, separation conditions and performance in bioseparation." Universität Duisburg-Essen, Fakultät für Chemie» Technische Chemie, 2009.

[30] T. B. Tennikova and F. Svec, "High-performance membrane chromatography: highly efficient separation method for proteins in ion-exchange, hydrophobic interaction and reversed-phase modes," *J. Chromatogr. A*, vol. 646, no. 2, pp. 279–288, 1993.

[31] K. H. Gebauer, J. Thömmes, and M. R. Kula, "Breakthrough performance of high-capacity membrane adsorbers in protein chromatography," *Chem. Eng. Sci.*, vol. 52, no. 3, pp. 405–419, 1997.

[32] W. Demmer and D. Nussbaumer, “Large-scale membrane adsorbers,” *J. Chromatogr. A*, vol. 852, no. 1, pp. 73–81, 1999.

[33] V. Warikoo *et al.*, “Integrated continuous production of recombinant therapeutic proteins,” *Biotechnol. Bioeng.*, vol. 109, no. 12, pp. 3018–3029, 2012.

[34] P. Trodler, J. Nieveler, M. Rusnak, R. D. Schmid, and J. Pleiss, “Rational design of a new one-step purification strategy for Candida antarctica lipase B by ion-exchange chromatography,” *J. Chromatogr. A*, vol. 1179, no. 2, pp. 161–167, 2008.

[35] H. Yao, T. Zhang, H. Xue, K. Tang, and R. Li, “Biomimetic affinity purification of Candida antarctica lipase B,” *J. Chromatogr. B*, vol. 879, no. 32, pp. 3896–3900, 2011.

[36] C. R. Llerena-Suster, L. E. Briand, and S. R. Morcelle, “Analytical characterization and purification of a commercial extract of enzymes: A case study,” *Colloids Surfaces B Biointerfaces*, vol. 121, pp. 11–20, 2014.

[37] A. Ujiie, H. Nakano, and Y. Iwasaki, “Extracellular production of Pseudozyma (Candida) antarctica lipase B with genuine primary sequence in recombinant Escherichia coli,” *J. Biosci. Bioeng.*, vol. 121, no. 3, pp. 303–309, 2016.

[38] J. Uppenberg, “The three-dimensional structure of lipase B from Candida antarctica,” 1994.

[39] M. S. Albrich, *Lipase B from Candida Antarctica in Bicontinuous Microemulsions: A Structural Study*. Shaker Verlag Gmbh, 2014.

[40] P. Trodler, “Untersuchung von Lipasen-Elektrostatik, Selektivität und Einfluss von Lösungsmitteln auf Struktur und Dynamik,” 2008.

[41] S. Ferreira-Dias, G. Sandoval, F. Plou, and F. Valero, “The potential use of lipases in the production of fatty acid derivatives for the food and nutraceutical industries,” *Electron. J. Biotechnol.*, vol. 16, no. 3, p. 12, 2013.

[42] Z. Jin, J. Ntwali, S.-Y. Han, S.-P. Zheng, and Y. Lin, “Production of flavor esters catalyzed by CALB-displaying Pichia pastoris whole-cells in a batch reactor,” *J. Biotechnol.*, vol. 159, no. 1, pp. 108–114, 2012.

[43] R. G. Berger, *Flavours and fragrances: chemistry, bioprocessing and sustainability*. Springer Science & Business Media, 2007.

[44] M. C. R. Franssen, L. Alessandrini, and G. Terraneo, “Biocatalytic production of flavors and fragrances,” *Pure Appl. Chem.*, vol. 77, no. 1, pp. 273–279, 2005.

[45] G. Buchi and W. D. Macleod, “Synthesis of Patchouli Alcohol,” *J. Am. Chem. Soc.*, vol. 84, no. 16, pp. 3205–3206, 1962.

[46] F. Deguerry, L. Pastore, S. Wu, A. Clark, J. Chappell, and M. Schalk, “The diverse sesquiterpene profile of patchouli, Pogostemon cablin, is correlated with a limited number of sesquiterpene synthases,” *Arch. Biochem. Biophys.*, vol. 454, no. 2, pp. 123–136, 2006.

[47] Badan Standarisasi Nasional, “Standar Nasional Indonesia, Minyak Nilam,” SNI

06-2385-2006, Jakarta, 2006.

[48] Organisation Internationale de Normalisation, “Huile essentielle de patchouli (Pogostemon cablin (Blanco) Benth.),” 2002.

[49] S. Maxwell and A. Fernando, “Cash crops in developing countries: the issues, the facts, the policies,” *World Dev.*, vol. 17, no. 11, pp. 1677–1708, 1989.

[50] T. Frister and S. Beutel, “Moschusduft und Patchouliöl,” *Chemie unserer Zeit*, vol. 49, no. 5, pp. 294–301, 2015.

[51] S. L. Munck and R. Croteau, “Purification and characterization of the sesquiterpene cyclase patchoulol synthase from Pogostemon cablin,” *Arch. Biochem. Biophys.*, vol. 282, no. 1, pp. 58–64, 1990.

[52] S. Hartwig *et al.*, “Expression, purification and activity assay of a patchoulol synthase cDNA variant fused to thioredoxin in Escherichia coli,” *Protein Expr. Purif.*, vol. 97, pp. 61–71, 2014.

[53] T. Frister *et al.*, “Characterisation of a Recombinant Patchoulol Synthase Variant for Biocatalytic Production of Terpenes,” *Appl. Biochem. Biotechnol.*, vol. 176, no. 8, pp. 2185–2201, 2015.

[54] J. Pollock, G. Bolton, J. Coffman, S. V Ho, D. G. Bracewell, and S. S. Farid, “Optimising the design and operation of semi-continuous affinity chromatography for clinical and commercial manufacture,” *J. Chromatogr. A*, vol. 1284, pp. 17–27, 2013.

[55] O. Kaltenbrunner, L. Diaz, X. Hu, and M. Shearer, “Continuous bind-and-elute protein A capture chromatography: Optimization under process scale column constraints and comparison to batch operation,” *Biotechnol. Prog.*, vol. 32, no. 4, pp. 938–948, 2016.

[56] C. Brämer, K. Ekramzadeh, F. Lammers, T. Scheper, and S. Beutel, “Optimization of continuous purification of recombinant patchoulol synthase from Escherichia coli with membrane adsorbers,” *Biotechnol. Prog.*, no. January, pp. 1–10, 2019.

[57] T. Herschel, A. El-Armouche, and S. Weber, “Monoklonale Antikörper,” *DMW-Deutsche Medizinische Wochenschrift*, vol. 141, no. 19, pp. 1390–1394, 2016.

[58] J. M. Berg, L. Stryer, and J. L. Tymoczko, *Stryer Biochemie*. Springer-Verlag, 2007.

[59] *Affinity Chromatography Vol. 1: Antibodies*. GE Healthcare.

[60] U. Sack and F. Emmrich, “Monoklonale Antikörper,” *Internist (Berl).*, vol. 49, no. 8, pp. 919–928, 2008.

[61] G. Schäffner and D. Kabelitz, “Monoklonale Antikörper zur Therapie,” *DMW-Deutsche Medizinische Wochenschrift*, vol. 126, no. 30, pp. 851–856, 2001.

[62] H. E. Blum, “Therapeutische monoklonale Antikörper,” *DMW-Deutsche Medizinische Wochenschrift*, vol. 125, no. 49, pp. 1501–1504, 2000.

[63] J. M. Reichert, “Antibodies to watch in 2017,” in *MAbs*, 2017, vol. 9, no. 2, pp.

167–181.

[64] H. Kaplon and J. M. Reichert, “Antibodies to watch in 2019,” in *MAbs*, 2019, vol. 11, no. 2, pp. 219–238.

[65] R. Kunert and D. Reinhart, “Advances in recombinant antibody manufacturing,” *Appl. Microbiol. Biotechnol.*, vol. 100, no. 8, pp. 3451–3461, Apr. 2016.

[66] S. Sommerfeld and J. Strube, “Challenges in biotechnology production—generic processes and process optimization for monoclonal antibodies,” *Chem. Eng. Process. Process Intensif.*, vol. 44, no. 10, pp. 1123–1137, 2005.

[67] J. H. Chon and G. Zarbis-Papastoitsis, “Advances in the production and downstream processing of antibodies,” *N. Biotechnol.*, vol. 28, no. 5, pp. 458–463, 2011.

[68] B. Kelley, “Industrialization of mAb production technology: the bioprocessing industry at a crossroads,” in *MAbs*, 2009, vol. 1, no. 5, pp. 443–452.

[69] J. Strube, F. Grote, and R. Ditz, “Bioprocess design and production technology for the future,” *Biopharm. Prod. Technol.*, vol. 1, pp. 657–705, 2012.

[70] D. Low, R. O’Leary, and N. S. Pujar, “Future of antibody purification,” *J. Chromatogr. B*, vol. 848, no. 1, pp. 48–63, 2007.

[71] P. A. Marichal-Gallardo and M. M. Alvarez, “State-of-the-art in downstream processing of monoclonal antibodies: process trends in design and validation,” *Biotechnol. Prog.*, vol. 28, no. 4, pp. 899–916, 2012.

[72] S. K. Mendiratta, S. BANDYOPADHYAY, and A. K. Singh, “Purification process for monoclonal antibodies.” Google Patents, Apr-2016.

[73] E. Müller and J. Vajda, “Routes to improve binding capacities of affinity resins demonstrated for protein A chromatography,” *J. Chromatogr. B*, vol. 1021, pp. 159–168, 2016.

[74] F. Bickel, E. M. Herold, A. Signes, S. Romeijn, W. Jiskoot, and H. Kiefer, “Reversible NaCl-induced aggregation of a monoclonal antibody at low pH: characterization of aggregates and factors affecting aggregation,” *Eur. J. Pharm. Biopharm.*, vol. 107, pp. 310–320, 2016.

[75] C. Brämer, S. Schreiber, T. Scheper, and S. Beutel, “Continuous purification of Candida antarctica lipase B using 3-membrane adsorber periodic counter-current chromatography,” *Eng. Life Sci.*, 2018.

[76] A. Castan, T. Falkman, E. Faldt, T. Persson, L. Blomqvist, and A. Forss, “Process intensification through integration of upstream perfusion cell culture with downstream continuous chromatography in monoclonal antibody production,” 2016.

[77] R. Godawat, K. Brower, S. Jain, K. Konstantinov, F. Riske, and V. Warikoo, “Periodic counter-current chromatography–design and operational considerations for integrated and continuous purification of proteins,” *Biotechnol. J.*, vol. 7, no. 12, pp. 1496–1508, 2012.

[78] X. Gjoka, R. Gantier, and M. Schofield, "Transfer of a three step mAb chromatography process from batch to continuous: Optimizing productivity to minimize consumable requirements," *J. Biotechnol.*, vol. 242, pp. 11–18, 2017.

[79] J. Zobel-Roos, Steffen; Stein, Dominik; Strube, "Evaluation of Continuous Membrane Chromatography Concepts with an Enhanced Process Simulation Approach," *Antibodies*, vol. 7, no. 1, p. 13, 2018.

[80] D. Baur, M. Angarita, T. Müller-Späth, F. Steinebach, and M. Morbidelli, "Comparison of batch and continuous multi-column protein A capture processes by optimal design," *Biotechnol. J.*, 2016.

6 Anhang

6.1 Betreute Abschlussarbeiten

Bachelorarbeit: Lisa Sophie Tünnermann – Aufreinigung eines monoklonalen Antikörpers mit Membranadsorbern, 2019 (Teile der Arbeit wurden in Kapitel 3.3 verwendet)

6.2 Veröffentlichungen und Konferenzbeiträge

Veröffentlichungen (chronologisch, peer-reviewed)

Brämer, C. (80 %), Schreiber, S. (15 %), Scheper, T., Beutel, S. (5 %) (2018). Continuous purification of *Candida antarctica* lipase B using 3-membrane adsorber periodic counter-current chromatography. *Engineering in Life Sciences*, *18*(7), 414-424.

Brämer, C. (80 %), Ekramzadeh, K. (15 %), Lammers, F., Scheper, T., Beutel, S. (5 %) (2019). Optimization of continuous purification of recombinant patchoulol synthase from *E. coli* with membrane adsorbers. *Biotechnology progress*, e2812.

Ekramzadeh, K. (80 %), **Brämer, C. (15 %)**, Frister, T., Fohrer, J., Kirschning, A., Scheper, T., Beutel, S. (5 %) (2019) Optimization of factors influencing enzyme activity and product selectivity and the role of proton transfer in the catalytic mechanism of patchoulol synthase. *Biotechnology progress.*

Brämer, C. (95 %), Lammers, F., Scheper, T., Beutel, S. (5 %) (2019) Development and Testing of a 4-Columns Periodic Counter-Current Chromatography System Based on Membrane Adsorbers. *Separations*, *6*(4), 55.

Brämer, C. (80 %), Tünnermann, L. (5 %), Gonzalez Salcedo, A. (10 %), Reif, O.-W., Solle, D., Scheper, T., Beutel, S. (5 %) (2019) Membrane adsorber for the fast purification of a monoclonal antibody using Protein A chromatography. *Membranes*, *9*(12), 159.

Tabelle A1: Verantwortungsbereiche der Autoren der vier Publikationen dieser kumulativen Dissertation.

	Verantwortungsbereiche an der Publikation							
Autoren	**Konzept**	**Experimentelles Arbeiten**	**Methodik**	**Abbildungen und Tabellen**	**Verfassen des Manuskripts**	**Korrekturen der Revisionen und Edition**	**Projekt-administration**	**Finanzierung**
Brämer, Chantal	**a, b, c, d**	**a, b, c, d**	**a, b, c, d**	**a, b, c, d**	**a, b, c, d**	**a, b, c, d**		
Beutel, Sascha						a, b, c, d	a, b, c, d	a, b, c, d
Ekramzadeh, Kimia		c				c		
Gonzalez Salcedo, Alina		d	d			d		
Lammers, Frank						a, c		
Reif, Oscar-Werner						d		
Scheper, Thomas						a, b, c, d		a, b, c, d
Schreiber, Sarah	b	b						
Solle, Dörte						d	d	d
Tünnermann, Lisa		d						

a Development and Testing of a 4-Columns Periodic Counter-Current Chromatography System Based on Membrane Adsorbers

b Continuous purification of *Candida antarctica* lipase B using 3-membrane adsorber periodic counter-current chromatography

c Optimization of continuous purification of recombinant patchoulol synthase from *E. coli* with membrane adsorbers

d Membrane Adsorber for the Fast Purification of a Monoclonal Antibody Using Protein A Chromatography

Vorträge (chronologisch)

Schreiber, S., **Brämer**, **C.,** Scheper, T., Beutel, S. Integrated continuous purification of model proteins with three-column periodic counter-current chromatography, *13th International PhD Seminar on Chromatographic Separation Science*, Trifels, Deutschland, **2017**

Schreiber, S., **Brämer**, **C.,** Scheper, T., Beutel, S. Three-column periodic counter-current chromatography – Purification of model proteins, *Himmelfahrtstagung 2017: Models for Developing and Optimising Biotech Production*, Neu-Ulm, Deutschland, **2017**

Brämer, **C.,** Scheper, T., Beutel, S. Establishment of a periodic counter-current chromatography device for the continuous purification of industrial relevant proteins, *14th International PhD Seminar on Chromatographic Separation Science*, Burghausen, Deutschland, **2018**

Brämer, **C.,** Scheper, T., Beutel, S. Application of periodic counter-current chromatography for the purification of industrial relevant proteins, *9th International Congress on Biocatalysis*, Hamburg, Deutschland, **2018**

Brämer, **C.,** Scheper, T., Beutel, S. Continuous purification of industrial relevant proteins with a self-established periodic counter-current chromatography device, *38th International Symposium on the Purification of Proteins, Peptides and Polynucleotides*, Berlin, Deutschland, **2018**

Brämer, **C.,** Scheper, T., Beutel, S. Monoclonal antibody purification using a self-established periodic counter-current chromatography device, *15th International PhD Seminar on Chromatographic Separation Science*, Quedlinburg, Deutschland, **2019**

Poster (chronologisch)

Niemeyer, L., Porr, M., **Brämer**, **C.,** Austerjost, J., Lange, F., Marquard, D., Scheper, T., Beutel, S., Lindner, P. Digital integration of a periodic counter-current chromatography system for continuous downstream processing, Labvolution 2019, Hannover, Deutschland, **2019**

Niemeyer, L., Porr, M., **Brämer**, **C.,** Austerjost, J., Lange, F., Marquard, D., Scheper, T., Beutel, S., Lindner, P. Digital integration of a periodic counter-current chromatography system for continuous downstream processing, Himmelfahrtstagung 2019: Intensification and digitalisation for integral bioprocessing, Hamburg, Deutschland, **2019**

Habib, T., **Brämer**, **C.,** Ebbecke, J., Enders, A., Beutel, S., Bahnemann, J. 3D-printed microfluidic chip for preparative chromatography, 2nd International EUROMBR Training Course - Innovative microbioreactor applications in bioprocess development, Braunschweig, Deutschland, **2019**

6.3 Lebenslauf

Persönliche Angaben

Name	Chantal Marie Brämer
Geburtsdatum	31.07.1992
Geburtsort	Hamburg
Nationalität	deutsch

Ausbildung

2017-2019	Promotionsstudium im Fach Chemie an der Leibniz Universität Hannover
2015-2017	Masterstudium *Pharmaceutical Biotechnology* an der HAW Hamburg
2011-2015	Bachelorstudium Biotechnologie an der HAW Hamburg
2011	Allgemeine Hochschulreife am Lichtenberg Gymnasium Cuxhaven

Beruflicher Werdegang

2017-2020	Wissenschaftliche Mitarbeiterin am Institut für Technische Chemie der Leibniz Universität Hannover
2015-2016 & 2017	Werkstudentin in der Qualitätssicherung bei Richter-Helm BioLogics GmbH & Co. KG
2014-2015	Tutorin für Mess- und Regelungstechnik an der HAW Hamburg
2014	Tutorin für Elektronik an der HAW Hamburg