

INTEGRATED AUTOMATED FILTRATION FOR SEPARATION, WASHING AND DRYING
OF PEPTIDE CRYSTALS

FIELD OF INVENTION

5 Present invention relates to downstream processing of biologics, more specifically, to downstream processing of Insulin and its analogues. Present invention further relates to integration of crystal separation, followed by crystal washing and freeze-drying into a single continuous process for purification of insulin and its analogues.

10 **BACKGROUND OF INVENTION**

Traditionally, preparative protein crystallization involves a series of washing (of the settled crystals) and gravity settling steps. The end product of preparative protein crystallization is further processed by vacuum assisted freeze-drying to obtain the drug substance (DS) in dry powder form. The resultant drug substance must conform to purity by residual substance (RS) analysis, moisture content (LOD-Loss on Drying), sulphated ash content (ROI) and residual solvent limits as per universal quality and regulatory specifications. The entire process of preparative crystallization, settling of the crystals in the tank and washing to meet the desired quality requirements and freeze-drying requires a minimum of 7 days at commercial scale. Evidently, the discontinuous and modular nature of this process makes it energy, cost and time
20 intensive.

Methods of crystallization and purification of insulin or insulin analogs have been disclosed in WO2015084694, US8769841, WO1996040730, CN103512318 and US6408536.

25 WO2015084694 describes a method for crystallizing insulin or insulin analogues under alkaline conditions and purifying the crystals by filtering through a filter and drying the crystals captured on the filter to produce crystalline insulin or insulin analogue crystal compositions. After crystallization, the entire volume of decanted crystal suspension is then transferred to a filter apparatus. The filter apparatus described in WO2015084694 is an agitated Nutsche filter, which
30 have a stainless steel screen with a pore size about 5 μm .

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US8769841 discloses a process of freeze drying of an essentially aqueous solution comprising at least one first step having a first temperature and pressure level (i.e. primary drying phase) and at least one second step having a second temperature and pressure level following the first step (i.e. secondary drying phase).

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WO1996040730 disclose a method for recovering an acylated protein, those that resist recovery by precipitation or crystallization and subsequent filtration from aqueous solutions, as a powder. Acylated proteins are certain acylated proinsulins, insulins and insulin analogues. The method comprises of combination adjusting the aqueous solution to near the isoelectric pH of the protein and providing a suitable alcohol concentration to cause precipitation of the protein in the form of filterable particles at the adjusted pH.

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CN103512318 disclose a drug purification drying process that relates to a lyophilized processing of insulin wherein the process discloses a freeze-drying process for insulin. The impurity content is lower than conventional technology. This process avoids the use of organic solvents, eliminating the potential adverse effects on people and the environment, and saving the drying time of 2-3 days and improve industrial productivity.

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US6408536 disclose a process for drying protein crystals from an aqueous protein crystal suspension. The process comprises of filtering off the crystals from an aqueous suspension, washing the filter cake, spin-drying the same, drying the crystals in the fluidized bed with a stream of moistened nitrogen and emptying the dried crystals using a nitrogen pressure surge into a flanged container.

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Though several methods are available for crystallizing insulin and its analogues, there is a need to develop an alternative method to have simple and optimized process of crystallization, washing and separation of the crystal as well as drying the same which can be practised in the industry in a bigger scale equipment which works on the same principle.

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The present invention offers an integrated simple process for separation and drying of insulin and its analogues.

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OBJECT OF INVENTION

The object of present invention is to integrate the discrete processes of crystallization, washing of the crystals and freeze-drying into a single continuous process using pressure filtration while achieving comparable standards of critical quality attributes and process performance attributes.

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SUMMARY OF INVENTION

The present invention describes the integration of preparative Insulin and Insulin analogues crystallization, crystal separation and freeze-drying processes into single continuous process using pressure filtration. The process facilitates time reduction, and outlines the novel design of using multiple organic solvent washes and nitrogen gas purging for the removal of imbibed water and achieve final drug substance that meets the major quality specifications.

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BRIEF DESCRIPTION OF DRAWINGS

Figure 1 represents flowchart of process of pressure filtration for insulin and insulin analogues purification

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Figure 2 represents representative image of lab scale pressure-filter

Figure 3 represents drug substance from conventional (left) and new (right) process

Figure 4 represents variability chart for Product loss/ recovery as Loss%

Figure 5 represents variability chart for residual moisture content in Drug Substance as LOD (loss on drying) %

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Figure 6 represents variability chart for High Molecular Weight Products (HMWP) %

Figure 7 represents variance chart for Purity %

DETAILED DESCRIPTION OF INVENTION**25 Definitions**

The term 'crystal suspension' refer to a volume of solution prepared by addition of Zinc Chloride (ZnCl_2) to insulin or insulin analogue followed by pH adjustment in the range of 4.5- 8.5 using acid/alkali. Addition of zinc improves the physical stability of insulin by multimerization.

30 The term 'filter' refer to a simple set up that is similar to a 'Buchner funnel' with the pore size of filter membrane used varying between '1-15 μm ' which does not involve any centrifugal force.

The term 'pressure filtration' refer to a solid-liquid filtration aided by controlled pressure that pushes the solution against a filter membrane so that the solid substance (crystalline insulin and insulin analogue) is retained as a cake while the (organic solvent) mother liquor passes through as permeate. This exogenous pressure results in increased effective flow rate, high pressure gradient across the membrane and use of inert gas ensures stability to the finished product. Pressure filtration is an instantaneous process with an operating time of between 2-5 minutes on a lab scale and 45-60 minutes on an industrial scale.

The term 'insulin' or 'insulin analogue' refer to 51 amino acid peptide hormone used for treatment of diabetes. It also refers to other insulin related forms such as insulin precursor or insulin analogue precursor, intermediate insulin or insulin analogue molecules obtained during downstream processes.

Description of invention

Insulin and insulin analogues were expressed extracellularly using a recombinant *Pichia* based fermentation process. Transformed *Pichia pastoris* yeast strain are fermented in culture medium. The secreted Insulin or Insulin analogues were recovered by method of chromatography.

Insulin and insulin analogue was crystallized from the chromatography elution pool. It was further made into crystal suspension in presence of Zinc Chloride (ZnCl_2) and at an appropriate pH (in the range of 4.5 to 8.5) by addition of acid or alkali. Profile of crystal suspension (percentage solids, purity) was determined. The resultant insulin or insulin analogue crystals had high purity. Entire volume of crystal suspension was washed with water to remove the free precipitant used for crystallization and was taken forward for further processing of freeze drying. The crystal suspension was first processed for separation through pressure filtration process to obtain the dried cake. This separation process is devoid of gravity and centrifugal force. The dried cake had imbibed water, which is removed by steps of washing by organic solvent and purging nitrogen gas through it.

The single continuous process can be depicted in following four steps.

1. Pressure Filtration of crystal suspension (initial separation of crystals)

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2. Drying: washing with organic solvents for displacement of imbibed water
 3. Drying: final purge of nitrogen to remove residual organic solvents
 4. Automatic dispensing of dried powder in a cGMP environment.
- 5 Similar integration of crystal separation, washing and drying can be performed at multiple preceding crystallization step during the downstream processing of insulin and insulin analogues. The raw material(s), reagent(s) and equipment(s) were used as per listed below in table 1, 2 and 3 respectively.

Table 1: list of raw material

Sr. No.	Raw Materials	Grade	Manufacturer
1.	Acetic acid	HPLC	Rankem
2.	Acetonitrile (ACN)	HPLC	Rankem
3.	Acetonitrile (ACN)	Commercial Grade	ASAHI KASEI
4.	L-Arginine	Reagent grade	Sigma-Aldrich
5.	1-butanol	Commercial Grade	Deepak fertilizers
6.	Ethanol	Commercial Grade	Changshu Yangyuan Chemicals
7.	Ethyl acetate	Commercial Grade	Lakshmi Organic Industries
8.	Filter membrane	Not Applicable	Sefar Filtration (India) Pvt. Ltd
9.	HCl	ACS Reagent	Sigma-Aldrich
10.	Methanol	Commercial Grade	Deepak fertilizers
11.	2-propanol	Commercial Grade	Deepak fertilizers
12.	Trifluoroacetic acid	Chromasolv	Sigma-Aldrich
13.	Water for injection	Not Applicable	Biocon

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Table 2: list of buffer/reagents and preparation

Sr. No.	Buffers/Reagents	Preparation
1.	0.1% TFA in MilliQ water	Measure 1000mL of MilliQ water. Pipette out 1mL of water and add 1mL of HPLC grade TFA.
2.	1N HCl	Measure 70mL of water and then add 8.212mL of concentrated HCl (HPLC grade). Make up the final volume to

Sr. No.	Buffers/Reagents	Preparation
		100mL using MilliQ water in a volumetric flask.
3.	0.01N HCl	Measure 245mL of water. Add 2.5mL of 1N HCl (HPLC grade). Make up the volume to 250mL with MilliQ water.
4.	HMWP buffer (High Molecular Weight Transfer buffer)	Measure 600mL of milliQ water. Add 0.65g of Reagent grade L-Arginine while on stirring. Make up the volume to 650mL. Add 150mL of Acetic acid and 200mL of ACN. Filter the solution using a 0.45µm followed by 0.2µm filter and sonicate for 20 minutes.

Table 3: List of equipment/instruments

Sr. No.	Name	Model / Make
1.	Analytical HPLC	Agilent HPLC- 1200/Agilent technologies
		Agilent HPLC- 1100/Agilent technologies
		LC-2010CHT/Shimadzu
2.	Column	Advanced Chromatography Technologies: ACE 300-C18, 5µm; 4.6 × 250mm
		Waters: Insulin HMWP 7.8 x 300mm
3.	Pressure filter	Prototype from BHS Sonthofen
4.	Vacuum oven	Servewell
5.	Weighing balance	Mettler Toledo
6.	Water bath	Julabo
7.	Water bath	Equitron
8.	Sonicator	Servewell
9.	Vortexer	Shalom
10.	Nitrogen Gas cylinder	Biocon
11.	Cold room	Blue star
12.	Deep freezer	Vestfrost

The trials were performed in a lab scale pressure filter set up with an approximate filter cross-sectional area of 80cm² to 100cm². It also comprises of a pressure inlet for compressed inert gas (Nitrogen), a pressure gauge and safety release valve, sample inlet and outlet ports and a water jacket (figure 2).

Variable parameters were wash solvent, solvent volume, number of washes, time of nitrogen purging for each wash and an optional change of filter media, which is dependent on the size of crystals being processed. Process performance and quality attributes of the obtained drug substance were measured by analysing percentage of loss of crystals in permeate and loss on drying (LOD), purity and molecular weight (HMWP). The temperature of the set up was maintained in range of 25°C to 35°C.

The cake formed by process of pressure filtration of insulin and insulin analogue crystal suspension, had requisite thickness and texture. However, the goal was to dry the cake to achieve acceptable standards of LOD and very importantly to integrate the crystallization-freeze drying of the conventional process into a single continuous process. To achieve sufficient displacement of imbibed residual water from filter cake, single or multiple wash strategies with different organic solvents were studied. The wash steps were also designed to enable effective removal of precipitants, and residual solvents that are present in reverse phase (chromatography) elution pool and obtain a drug substance that complies with the universal quality and regulatory specifications.

The assembly of instrument(s) for achieving the dried powder in continuous and automated manner was as follows:

- i. The equipment was an SS cylindrical assemblage with jacketing to maintain required internal temperature as well as provision for passing an (inert) gas. Nitrogen gas cylinder and Julabo water chiller were used for these trials. The temperature was maintained in the range of 25-35°C.

- ii. A filter fabric of 80cm² to 100cm² area was placed on an SS mesh support and housed in the assemblage. Fabric filter was selected from PET 1703 (Poly ester fabric) and SK-011 (Poly propylene fabric).
- iii. The assemblage was sealed with a SS disc lined with silicon gasket, and clamps were fastened to make the compartment airtight, followed by pouring the homogenous neat suspension from the top of the vessel to commence pressure filtration.
- iv. Nitrogen gas at bar pressure in the range of 1 bar to 2.5 was applied via NRV/inlet port.
- v. Filtrate/permeate was collected via the sample outlet port at the bottom.
- vi. Steps 'iii' and 'iv' were repeated for single or multiple cake washes using organic solvent.
- vii. Drying was performed by addition of an organic solvent and further passing N₂ gas for about three minutes after which the assemblage was unfastened.
- viii. The fabric containing the cake was carefully removed and gently tapped to recover the cake which was then transferred into a sterile airtight amber colour container and stored at -20°C until further analysis.

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Two trials were conducted. First, was preliminary screening (Trial I) and second (Trail II) was design of experiment (DOE).

Preliminary screening trials (trial 1) were performed in a lab scale prototype of industrial pressure filter (figure 2) with different organic solvents using the design described in Table 4. In preferable embodiment, acetonitrile was considered best working organic solvent. Filter fabrics were studied and screened for their pore size, i.e. retention of crystals, texture, workability on commercial scale and resilience/inertness to the pH and temperature conditions of the process. Two types of filter fabric were used viz. poly ester fabric and poly propylene fabric. Organic solvents were chosen based on their ability to displace water from the cake and their volatile nature (table 4). The FDA guidelines to acceptable solvents and their residual limits in final drug substance were also considered for the selection of solvents.

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Table 4: Solvent screening design

Trial number	Solvent	% Solvent volume based on crystal suspension volume	Washes
1.	Acetonitrile (ACN)	5	1
2.		5	2
3.		10	1
4.	Butanol	5	1
5.		5	2
6.		10	1
7.	Ethanol	5	1
8.		5	2
9.		10	1
10.	Ethyl acetate	5	1
11.		5	2
12.		10	1
13.	Isopropanol	5	1
14.		5	2
15.		10	1
16.	Water	5	1
17.		5	2
18.		10	1

Solvents and wash combinations yielding high percentage LOD (loss on drying) values in the above design were eliminated. Data set is presented in table 5 and table 6.

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The captured data set in table 5 and table 6 suggested that the resultant insulin and insulin analogue drug substance has comparable quality attributes to the drug substance made from traditional crystallization-freeze drying process with optimal recovery of product (figure 3).

Two washes of acetonitrile planned in Trail I has satisfactory outcome with respect to percentage LOD. However, to fall in-line with regulatory requirements a further reduction in LOD

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Table 5: Solvent Screening Result

Wash Solvent %	Crystallization 3 Neat suspension (NS)			Wash 1 (W1)		Wash 2 (W2)				
	NS conc. (g/L)	NS volume (mL)	NS permeate (mL)	W1 volume (mL)	W1 permeate (mL)	W2 volume (mL)	W2 permeate (mL)	Drying time (min)	DS weight (g)	LOD %
<u>16/01/2017</u>										
LP1-5%-W1	14.65	300	285	15	21	NAP	NAP	1.5	5.52	26
LP1-5%-W 1 & 2	14.65	300	290	15	13	15	17.5	1.5	5.26	20.46
LB1-5%-W1	14.65	300	280	15	18	NAP	NAP	1.5	6.33	35.14
LB1-5%-W 1 & 2	14.65	300	290	15	13.5	15	20	1.5	5.88	36.22
LA-5%-W1	14.65	300	290	15	14.3	NAP	NAP	1.5	4.96	15.99
LA-5%-W 1 & 2	14.65	300	295	15	12.5	15	13.5	1.5	4.88	9.34
LA-10%-W1	14.65	300	290	30	29	NAP	NAP	2	5.3	21.25
LP1-10%-W1	14.65	300	290	30	31.5	NAP	NAP	2	5.43	21.14
LB1-10%-W 1	14.65	290	275	30	30	NAP	NAP	2	5.5	25.1
LP1-5%-W1	14.65	200	190	10	9.7	NAP	NAP	1.5	3.81	NAV
<u>19/01/2017</u>										
LE-5%-W 1 & 2	14.65	300	290	15	9.8	15	18	2	5.66	22.35
LEA-5%-W1	14.65	300	290	15	21.5	NAP	NAP	2	7.07	37.84
LEA-5%-W 1 & 2	14.65	300	290	15	9	15	9.6	2	6.67	34.11
LEA 10% W1 & 2	14.65	300	280	30	25.5	30	28	2	6.87	36.03
LE-5%-W 1 & 2 T1	14.65	300	290	15	18.5	15	16	2	5.44	15.75
LE-5%-W 1 & 2 T2	14.65	300	285	15	13	15	16.5	2	6.06	16.43
LA-5%-W 1 & 2 T1	14.65	300	300	15	15	15	14	2	5.38	10.52
LA-5%-W 1 & 2 T2	14.65	300	305	15	10	15	9.5	2	5.7	11.53
LOD values in BOLD are actual values, rest are theoretical values based on neat suspension product concentration.										
LA: Acetonitrile, LP1: Isopropanol,										
LB1: Butanol, LEA: Ethyl acetate, NS: Neat suspension, W1/2: Wash 1 and Wash 2, T1/2: Trial 1 and Trial 2; LOD: Loss on drying; NAP: Not applicable, NAV: Not available										

A full factorial experimental design along with the response data is presented in table 6. Fifty experiments were performed discretely as below (table 6).

Table 6: Experimental design with response data

Sr. No.	Wash I (% V/V)	Wash II (% V/V)	Wash III (% V/V)	Drying Temp (°C)	Drying Time (min)	% LOD	% Purity	% HMWP	% Loss
1	5	10	5	35	3	19.18	99.20	0.158	1.41
2	10	10	0	35	2	22.23	99.25	0.163	1.89
3	10	5	10	35	2	17.66	99.18	0.159	4.50
4	10	10	5	35	3	16.38	99.22	0.162	1.59
5	10	10	10	25	2	12.77	99.06	0.167	0.19
6	5	5	0	25	2	18.70	98.86	0.173	0.49
7	7.5	7.5	5	30	2.5	13.90	99.25	0.156	1.58
8	10	10	10	35	3	16.37	99.24	0.165	1.54
9	5	10	10	35	3	13.06	99.17	0.158	2.62
10	5	10	10	25	2	14.60	98.72	0.162	0.17
11	5	5	10	25	2	11.99	99.00	0.163	0.24
12	10	10	5	25	2	9.14	98.71	0.158	0.13
13	10	10	5	35	2	13.25	99.26	0.164	3.25
14	5	5	0	35	2	14.01	99.35	0.225	0.23
15	10	5	5	35	3	21.74	99.11	0.218	1.11
16	5	5	5	25	3	15.66	98.89	0.171	0.31
17	10	5	5	25	3	14.60	99.19	0.164	0.25
18	10	5	0	25	3	20.36	99.06	0.169	0.18
19	10	10	0	35	3	16.58	99.20	0.172	1.78
20	5	5	0	25	3	12.13	98.95	0.176	0.34
21	5	5	10	25	3	14.58	98.84	0.181	0.33
22	10	10	10	35	2	14.47	99.22	0.152	1.78

23	10	5	10	25	3	9.56	99.04	0.164	0.15
24	10	10	0	25	2	15.70	99.03	0.158	0.16
25	5	10	0	25	3	15.35	98.97	0.164	0.19
26	10	5	5	35	2	15.54	99.18	0.157	1.99
27	5	10	0	35	3	16.10	99.37	0.159	2.61
28	5	10	0	35	2	12.93	99.19	0.157	1.66
29	10	10	0	25	3	12.66	99.06	0.165	0.18
30	5	5	10	35	2	18.12	99.20	0.160	1.42
31	5	5	0	35	3	15.35	99.27	0.159	5.10
32	10	5	5	25	2	16.88	99.05	0.166	0.28
33	5	5	5	25	2	17.11	98.53	0.170	0.30
34	10	10	10	25	3	9.33	99.14	0.166	0.28
35	10	5	10	25	2	20.35	98.73	0.162	0.22
36	5	10	5	35	2	13.63	99.19	0.161	2.02
37	10	5	0	35	2	18.68	99.35	0.159	1.57
38	10	5	0	25	2	23.54	99.13	0.164	0.16
39	5	5	5	35	3	17.18	99.19	0.158	1.83
40	5	10	10	25	3	15.21	99.08	0.169	0.24
41	5	10	5	25	2	13.80	98.85	0.165	0.27
42	5	10	5	25	3	11.79	99.08	0.168	0.25
43	10	5	10	35	3	18.13	99.25	0.157	1.87
44	5	10	10	35	2	17.66	99.20	0.158	1.39
45	5	10	0	25	2	15.81	98.92	0.171	0.33
46	5	5	10	35	3	19.76	99.21	0.161	1.60
47	7.5	7.5	5	30	2.5	13.81	99.23	0.159	1.32
48	10	10	5	25	3	12.42	99.15	0.162	0.16
49	10	5	0	35	3	20.44	99.22	0.158	5.33
50	5	5	5	35	2	13.68	99.25	0.225	0.83

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The drug substance from Trials 12, 23 and 34 had the lowest LOD values i.e. of less than 10% (table 7), while drug substance from trials 11 and 42 had LOD values less than 12%. There were no significant changes in purity and HMWP profiles, and per-step losses were found to be well within range.

- 5 The results of variability are also represented in figure 4, 5, 6 and 7 for percentage loss of product, percentage LOD, percentage HMWP and percentage purity respectively.

Table 7: Summary of results of DOE (Trial II)

Trial number	WASHES (% V/V)			% LOD
	W I	W II	W III	
12	10	10	5	9.14
23	10	5	10	9.56
34	10	10	10	9.33

- From the multivariate studies, it was found out that more number of washes with organic solvent (3>2>1) resulted in ample displacement of imbibed water from filter cake. Three washes including wash 1 at 10%, wash 2 at 5% or 10% and wash 3 at 5% or 10%, were found to be most effective in reducing LOD. Volume of washes can be fine-tuned further as per process requirements. Increased drying time helped in forcing out some solvent imbibed in the cake post-washing. Increasing the temperature during filtration did not reduce the LOD of the drug substance, but it did not significantly affect the profile of the drug substance either. Thus, the temperature was maintained in the range of 25⁰ to 35⁰C. Based on this multivariate study further trials can be designed and executed to further reduce the LOD to <4%.

The results of this multivariate study were satisfactory and drew immediate attention to a novel process that is shorter, continuous, less resource intensive and automated.

CLAIMS

1. A single continuous process of insulin or insulin analogues crystal separation, washing and drying aided by pressure filtration comprising the below steps:
 - 5 a) Preparing crystal suspension of insulin and insulin analogues using ZnCl_2 and pH adjustment between 4.5 and 8.5;
 - b) Pouring the suspension obtained from step a) on filter fabric placed on area ranging between 80cm^2 to 100cm^2 of pressure filtration unit;
 - c) Purging nitrogen gas via inlet port of pressure filtration unit;
 - 10 d) Collecting filtrate via outlet port of pressure filtration unit;
 - e) Pouring 100% organic solvent onto the suspension of Step 'b' and repeating Step 'c', and further repeating Step 'e' for multiple cake washes;
 - f) Drying by passing nitrogen gas at a pressure range of 1 bar to 2.5 bar;
 - 15 g) Removal of dried cake in form of dried powder,wherein the 100% organic solvent is selected from acetonitrile, butanol, ethanol, ethyl acetate and isopropanol.
2. The single continuous process of claim 1, wherein insulin or insulin analogues have crystal size in the range of $1\text{-}40\mu\text{m}$.
3. The single continuous process of claim 1, wherein temperature is maintained in the range of 25° to 35°C .
- 20 4. The single continuous process of claim 1, wherein pressure filtration unit has filter membrane with pore size in the range of $1\text{-}15\mu\text{m}$.
5. The single continuous process of claim 1, wherein the single continuous pressure filtration process of crystallization of insulin or insulin analogues is completed within 5 to 10 minutes.
- 25 6. The single continuous process of claim 1, wherein the fabric filter is placed on SS mesh support of pressure filtration unit.

7. The single continuous process of claim 6, wherein the fabric filter is selected from poly ester fabric and poly propylene fabric.
8. The process of claim 1, wherein the volume of the 100% organic solvent is 5-10% of the volume of crystal suspension.
- 5 9. The process of claim 1, wherein the 100% organic solvent is acetonitrile.