POLITECNICO DI TORINO

Collegio di Ingegneria Chimica e dei Materiali

Corso di Laurea Magistrale
in Ingegneria Chimica e dei Processi Sostenibili

Tesi di Laurea Magistrale

Spray Freeze-Drying of a solution containing Lactate Dehydrogenase

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Sommario esteso

I biofarmaci

Negli ultimi anni l'industria farmaceutica si è concentrata sempre più sulla produzione di biofarmaci, ovvero farmaci costituiti da molecole proteiche complesse, prodotti in laboratorio all'interno di sistemi viventi.

I biofarmaci sono in genere costituiti da proteine che sono delle macromolecole biologicamente attive di vitale importanza non solo in ambito farmaceutico, ma anche all'interno del nostro organismo. Purtroppo, però, la loro importanza è direttamente proporzionale alla difficoltà nel maneggiarle. Quest'ultime, infatti, sono particolarmente sensibili a stress termici, a variazioni di pH e alla presenza di agenti esterni. A fronte di questi stress, le proteine possono cambiare la loro struttura secondaria, terziaria e quaternaria le quali responsabili della loro attività biologica. In particolare, presentano un grado di sensibilità elevato se disciolte in soluzione, motivo per il quale è preferibile conservarle in forma anidra in maniera tale da garantire una più prolungata conservabilità e stabilità.

Essendo sensibili agli stress termici, in particolare alle alte temperature, non è possibile ricorrere alle usuali tecniche di essiccazione a caldo. Al fine di ovviare a questo problema, è stata studiata una differente tecnica la quale permette l'allontanamento del solvente dalla soluzione sfruttando le basse temperature: la tecnica in questione prende il nome di liofilizzazione.

La liofilizzazione

La liofilizzazione consiste principalmente in due step: il congelamento e l'essiccamento. Il principio sulla quale si basa è quello della sublimazione, ovvero il passaggio diretto dell'acqua dallo stato solido allo stato vapore. Per fare ciò ci si avvale di temperature e pressioni molto basse. In Figura 1 sono riportate le fasi di tale processo sul diagramma di stato dell'acqua. Durante la fase di congelamento la temperatura viene progressivamente abbassata in maniera tale da trovarsi alla sinistra del punto triplo, favorendo la formazione di cristalli di ghiaccio. In seguito, la temperatura viene mantenuta costante e, abbassando la pressione, si ha una prima fase di essiccamento durante la quale avviene la sublimazione dei cristalli di ghiaccio. Al termine di ciò si mantiene costante la pressione e, con un lieve incremento di temperatura, la restante parte di acqua viene allontanata per desorbimento.

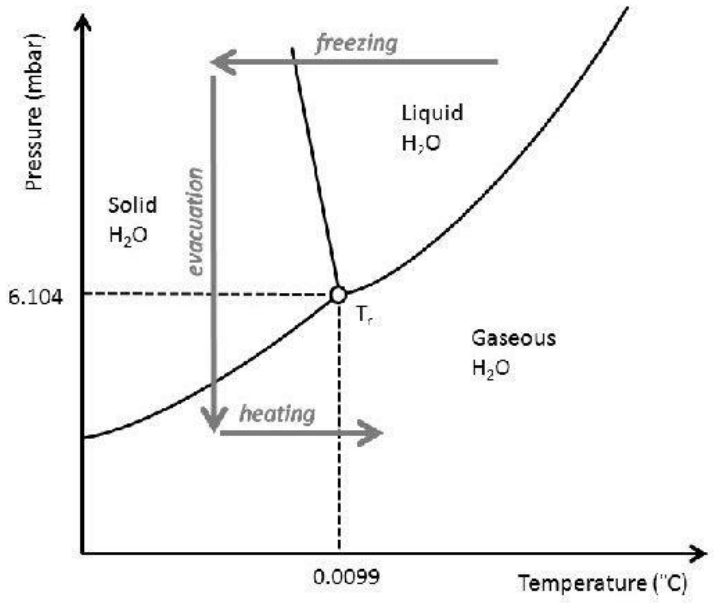

Figura 1: Steps del processo di liofilizzazione riportati sul diagramma di stato dell'acqua. Fonte: "https://www.researchgate.net/publication/283030918_Freeze-drying_of_Probiotics", con modifiche.

Le fasi della liofilizzazione

Come anticipato sopra, la liofilizzazione si compone principalmente di due fasi quali il congelamento e l'essiccamento, il quale a sua volta si divide in essiccamento primario ed essiccamento secondario. In Figura 2 vengono illustrate le varie fasi dal punto di vista della temperatura del prodotto.

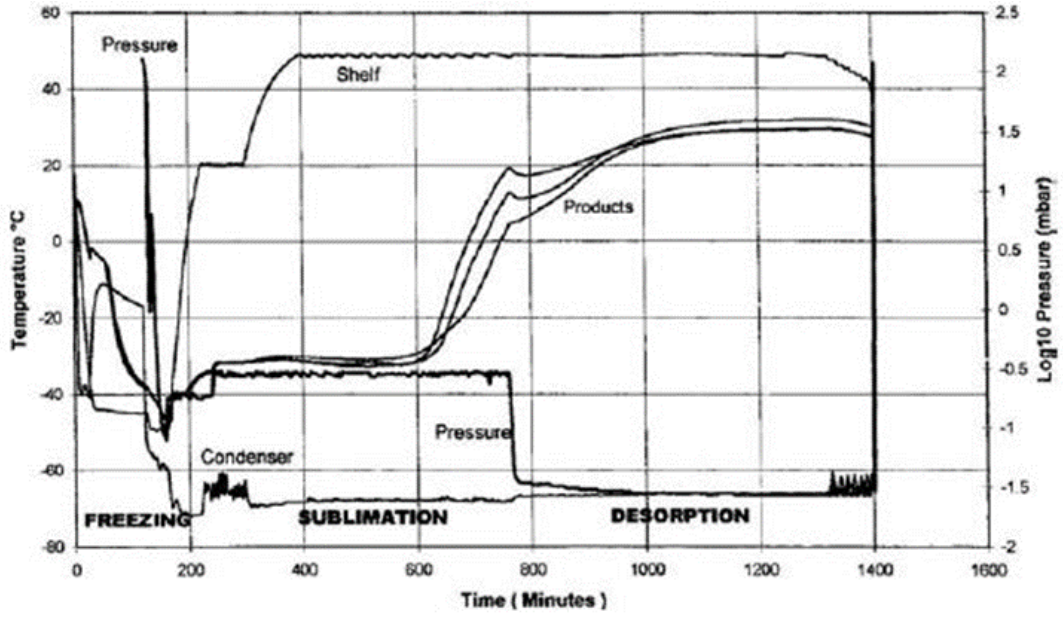

Figura 2: Andamento della temperatura del prodotto durante le fasi del ciclo di liofilizzazione. Fonte: "Freeze Drying/Lyophilization of Pharmaceutical and Biological Products"; L. Ray, J.C. May, con modifiche.

Il congelamento è la fase durante la quale avviene la separazione dell'acqua all'interno della soluzione con conseguente concentrazione della stessa. In questa fase la pressione in camera viene mantenuta al valore atmosferico e, grazie ad un graduale abbassamento delle temperature, si raggiunge una temperatura critica, al di sotto del punto di congelamento termodinamico, tale per cui si ha formazione di cluster di molecole. Da quel momento in poi, inglobando acqua dall'ambiente circostante, questi agglomerati di molecole aumentano progressivamente la loro dimensione formando dei cristalli di ghiaccio: questo fenomeno prende il nome di nucleazione. La dimensione e il numero di cristalli che si formano dipendono, principalmente, dalla velocità alla quale avviene questo processo: più veloce è il congelamento più i cristalli finali saranno di piccole dimensioni e in numero elevato.

La fase di congelamento è la fase più critica in quanto in questa fase si va a determinare quella che sarà la struttura finale del prodotto liofilizzato. I cristalli di ghiaccio che si formano verranno allontanati durante le successive fasi lasciando dei vuoti al loro posto: la struttura porosa finale sarà strettamente correlata alla dimensione dei cristalli ottenuta durante il congelamento. Purtroppo, però, è anche la fase più difficile da controllare, poiché influenzata dal fenomeno della nucleazione, un fenomeno stocastico puramente casuale.

Una volta terminata la fase di congelamento, la pressione in camera viene abbassata drasticamente mantenendo costante la temperatura e, grazie a un processo di sublimazione, l'acqua viene allontanata dal prodotto. La forza spingente dell'essiccamento primario è rappresentata dalla differenza tra la pressione parziale di acqua in camera e la tensione di vapore del ghiaccio.

La sublimazione è un processo esotermico, ciò vuol dire che, con il progredire dello stesso, la temperatura del prodotto aumenta. In vista di ciò, è necessario un rigoroso controllo di quest'ultima così da garantire che non superi mai il valore di collasso.

Per determinare la fine del processo di essiccamento primario possono essere usati vari metodi, uno fra questi è l'utilizzo di una sonda termoconduttiva detta sonda Pirani. Durante tutto il processo di essiccamento primario questo tipo di sonda segnerà un valore scorretto di pressione, in quanto tarata su un'atmosfera contenente azoto al 100%. Il valore di pressione rilevato con sonda Pirani sarà messo a rapporto, in continuo, con il valore di pressione ottenuto da una sonda capacitiva: quando questo rapporto sarà uguale a uno si avrà il punto di fine dell'essiccamento primario. Questo perché la corrente di azoto viene alimentata in camera in continuo in maniera tale da mantenere costante la pressione man mano che viene allontanato il vapore per sublimazione, ma finché non si arriva al termine del processo, ossia quando non vi è più vapore da allontanare, avrà una concentrazione diversa dal 100% falsando così le misure della sonda Pirani.

Durante l'essiccamento secondario, infine, la pressione viene mantenuta costante mentre la temperatura viene aumentata fino a circa la temperatura ambiente. In questo modo viene operata un'ultima fase di separazione, questa volta per desorbimento. In questa fase, quindi, viene allontanata quella che viene definita acqua "legata" alla superficie del prodotto.

Lo spray freeze-drying

Un'alternativa al tradizionale ciclo di liofilizzazione è lo spray freeze-drying (SFD): questa tecnica unisce i vantaggi dell'essiccamento spray a quelli della liofilizzazione. Grazie all'atomizzazione della soluzione è possibile ottenere delle particelle molto piccole, riducendo notevolmente i tempi previsti per l'essiccamento; mentre grazie alla liofilizzazione si ha la possibilità di allontanare l'acqua da soluzioni estremamente sensibili al calore.

La prima fase del processo in questione è l'atomizzazione, la quale si attua ad opera di un atomizzatore ad ugello che può essere di più tipi: idraulico, pneumatico e a ultrasuoni. Nel caso di ugello a ultrasuoni, ad esempio, la dimensione delle particelle è influenzata prevalentemente dalla portata, alla quale viene atomizzata la soluzione, e dalla potenza.

Il congelamento non avviene in camera di essiccazione, bensì viene operato quasi simultaneamente all'atomizzazione. Il liquido, infatti, viene atomizzato direttamente in un liquido criogenico, solitamente azoto liquido, causando il congelamento immediato. Anche in questo caso sono possibili più metodologie, in particolare si può avere atomizzazione direttamente all'interno del liquido criogenico, oppure atomizzazione all'interno dei vapori del liquido criogenico (in questo modo quando la soluzione entrerà in contatto con l'azoto liquido sarà già congelata).

Una volta fatto ciò, quello che segue è il ciclo di liofilizzazione classico costituito da essiccamento primario e secondario, come descritto in precedenza. In figura 3 è rappresentato uno schema del processo studiato.

Lo scopo del lavoro in questione è quello di andare ad analizzare quali effetti questo processo ha sull'attività enzimatica, con uno sguardo finale alle proprietà morfologiche del prodotto finito.

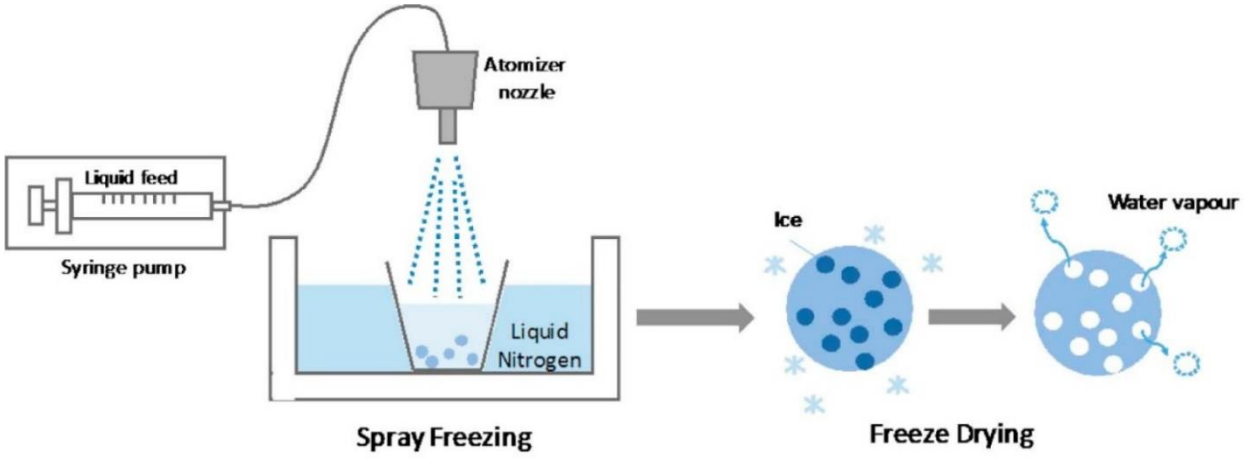

Figura 3: Rappresentazione schematica del processo di spray Freeze-Drying. Fonte: W. Liang, A. Y. L. Chan, M. Y. T. Chow, F. F. K. Lo, Y. Qiu, P. C. L. Kwok, J. K. W. Lama, 2018. Spray freeze drying of nucleic acids as inhaled powder for polmunary delivery. Asian Journal of Pharmaceutical Sciences. Volume 13, Pag. 163-172, con modifiche

Metodo sperimentale e principali risultati

L'enzima preso come campione per il lavoro in questione è il lattato deidrogenasi (LDH). In particolare, sono state analizzate sei differenti formulazioni al fine di studiare come la presenza di eccipienti possa andare a proteggere o meno l'attività enzimatica dello stesso. Le formulazioni scelte per l'analisi sono riassunte in tabella 1, con le relative concentrazioni.

Tabella 1: Formulazioni analizzate e rispettive concentrazioni

Formulazione	Concentrazione
LDH + soluzione tampone	10 mM soluzione tampone, 5µg/ml LDH
LDH + soluzione tampone + saccarosio	10 mM soluzione tampone, 5µg/ml LDH, 5 %w/w saccarosio
LDH + soluzione tampone + mannitolo	10 mM soluzione tampone, 5µg/ml LDH, 5 %w/w mannitolo
LDH + soluzione tampone + Tween 80	10 mM soluzione tampone, 5µg/ml LDH, 0,1 %w/w Tween 80
LDH + soluzione tampone + Tween 80 + saccarosio	10 mM soluzione tampone, 5µg/ml LDH, 0,1 %w/w Tween 80, 5 %w/w saccarosio
LDH + soluzione tampone + Tween 80 + mannitolo	10 mM soluzione tampone, 5µg/ml LDH, 0,1 %w/w Tween 80, 5 %w/w mannitolo

Come soluzione tampone è stata utilizzata una soluzione di acido citrico 10 mM a pH 6,5. Gli eccipienti scelti sono saccarosio e mannitolo, i quali fungono da crioprotettori. Al fine di valutare quanto i fenomeni all'interfaccia siano significati è stato scelto di aggiungere un tensioattivo alle soluzioni, la quale scelta è ricaduta sul Tween 80 (Polisorbato 80).

Per quanto riguarda l'attrezzatura utilizzata nel corso dell'analisi, partendo dalla fase di atomizzazione, è stato usato un atomizzatore ad ugello ultrasonico (Buchi, Switzerland): quello che, tra tutti, garantisce un controllo sulla granulometria migliore.

Per alimentare l'atomizzatore è stata usata una pompa realizzata dalla kdScientific (Modello KDS 200, KD Scientific, Holliston, MA), la quale permette di regolare la portata di alimentazione, il volume di soluzione che si vuole alimentare e la grandezza in termini di diametro della siringa utilizzata. Nel caso in esame è stato utilizzato come campione un volume di 1 ml e una siringa dal diametro di 15 cm. La portata è stata fatta variare scegliendo come target: 1 ml/min, 2,5 ml/min, 5 ml/min, 7,5 ml/min, 10 ml/min.

È stato scelto un atomizzatore della Buchi composto da due parti: l'atomizzatore stesso e il controller a ultrasuoni. Essendo la dimensione delle gocce dipendente principalmente dalla frequenza e dalla portata, nel caso in esame è stato scelto di far variare la portata come descritto sopra, mantenendo costanti la frequenza, pari a 60 kHz, e la potenza a 3 W.

L'ugello è stato posto ad un'altezza di circa 7-10 cm e la soluzione è stata atomizzata direttamente all'interno dei flaconi. In figura 4 è possibile vedere l'attrezzatura in questione, mentre in Figura 5 è riportato un esempio di soluzione atomizzata.

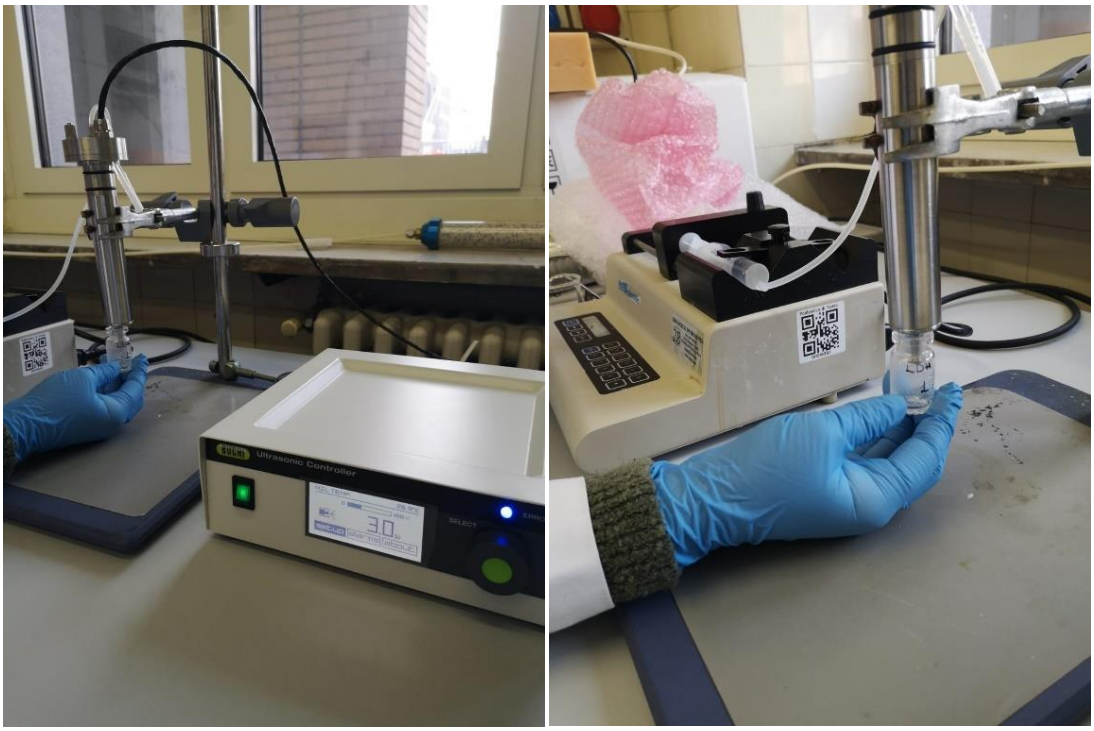

Figura 4: Attrezzatura utilizzata per l'atomizzazione. A sinistra, ugello e generatore di ultrasuoni. A destra, ugello e pompa con siringa.

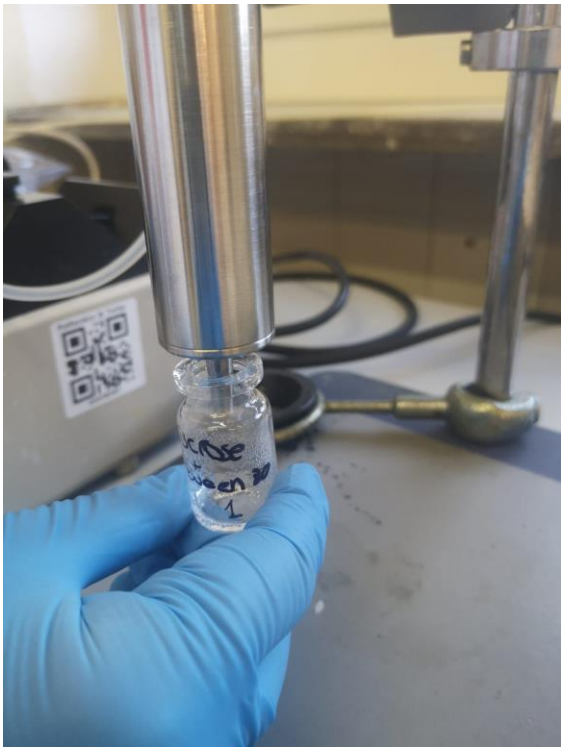

Figura 5: Soluzione atomizzata direttamente all'interno del flacone

Al fine di condurre l'analisi considerando la combinazione del processo di atomizzazione e congelamento, è stato inserito nel flacone azoto liquido. La soluzione è stata poi atomizzata all'interno dei vapori generati da quest'ultimo.

Per condurre l'analisi sull'attività enzimatica la soluzione congelata è stata fatta scongelare in un bagno d'acqua come riportata in figura 6.

Figura 6: Metodo utilizzato per far sciogliere il prodotto congelato

L'essiccamento è stato condotto all'interno del REVO della Millrock Technology, apparecchiatura costituita principalmente da: camera di essiccamento, pompa a vuoto e condensatore.

Prima di inserire i campioni all'interno del REVO è stata settata la temperatura interna a -50°C, temperatura alla quale, con la giusta pressione, avviene la sublimazione del ghiaccio. Le pressioni operative sono state differenti a seconda delle formulazioni analizzate: per il saccarosio si è arrivati a 10 Pa, mentre per il mannitolo a 20 Pa.

Una volta raggiunto il punto di fine dell'essiccamento primario, determinato mediante sonda Pirani, la temperatura è stata aumentata fino a 20°C mantenendo costante la pressione.

In figura 7 sono riportati due esempi di prodotto finale ottenuto dopo un processo di SFD completo.

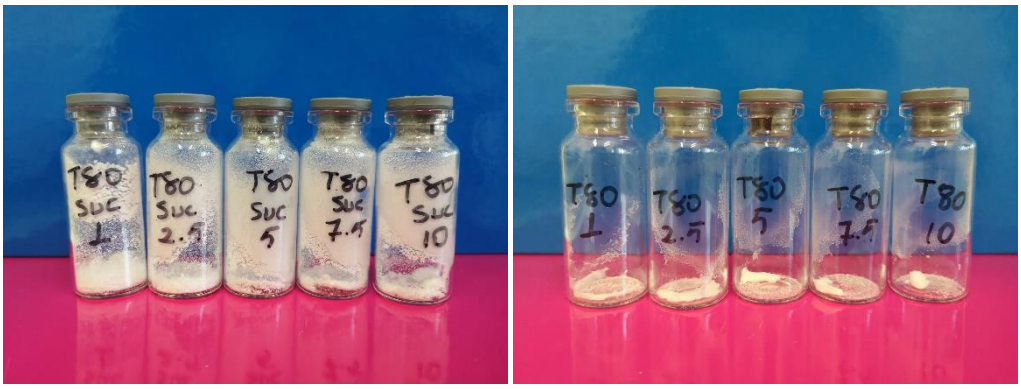

Figura 7: Formulazione contenente Tween 80 e saccarosio (sinistra) e formulazione contente Tween 80 (destra), in seguito a spray freeze-drying.

L'LDH presenta capacità riducenti, tali per cui è in grado di operare la reazione di conversione da NAD a NADH rilevabile tramite analisi colorimetrica mediante spettrofotometro a 450 nm. Alla luce di ciò, al fine di determinarne l'attività enzimatica, è stato utilizzato l'apposito kit "Lactate Dehydrogenase Activity Assay Kit" prodotto dalla Sigma-Aldrich, il quale consente di operare la reazione in questione per cui verrà studiata la variazione in assorbanza all'interno dello spettrofotometro.

Al fine di condurre questo tipo di analisi, è necessario avere una concentrazione minore di quella della soluzione di partenza: sono state operate, quindi, delle diluzioni in soluzione tampone per passare da una concentrazione di 5 µg/ml fino a 40 ng/ml.

Le analisi relative all'attività enzimatica sono state condotte all'interno dello spettrofotometro Multiskan Sky realizzato dalla Thermo Fisher Scientific. Le soluzioni sono state inserite in una piastra a fondo piatto da 96 pozzetti, come mostrato in Figura 8. Per ogni campione sono stati riempiti tre pozzetti utilizzando un volume di 50 µl per ognuno, ai quali sono stati aggiunti, rispettivamente, 50 µl del kit.

Figura 8: Spettrofotometro Multiskan Sky con piastra per analisi.

I risultati ottenuti in termini di attività enzimatica sono riassunti nelle seguenti figure 9, 10 e 11.

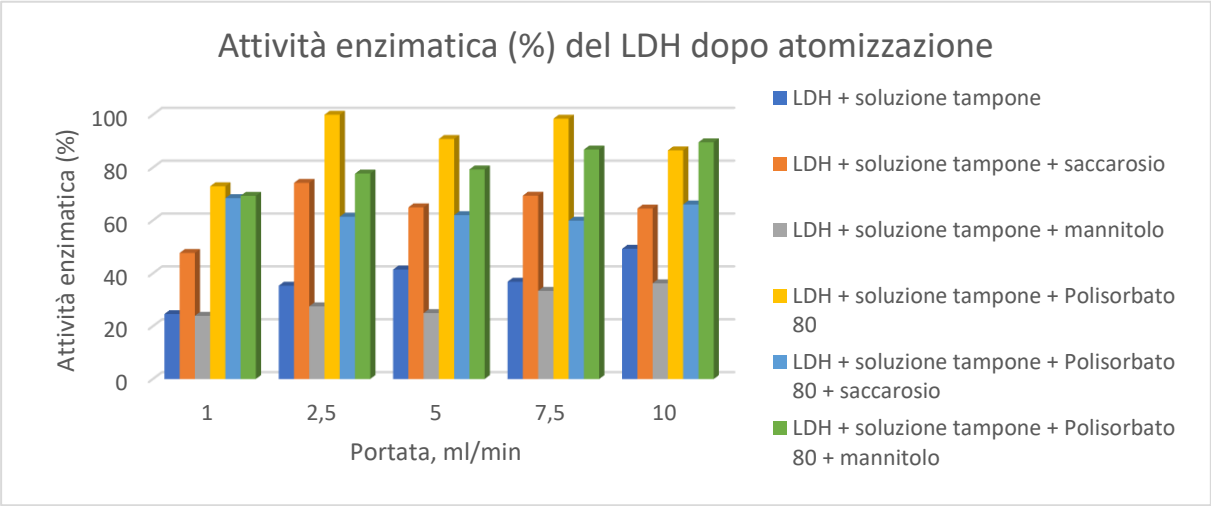

Figura 9: Attività enzimatica residua in seguito al processo di atomizzazione

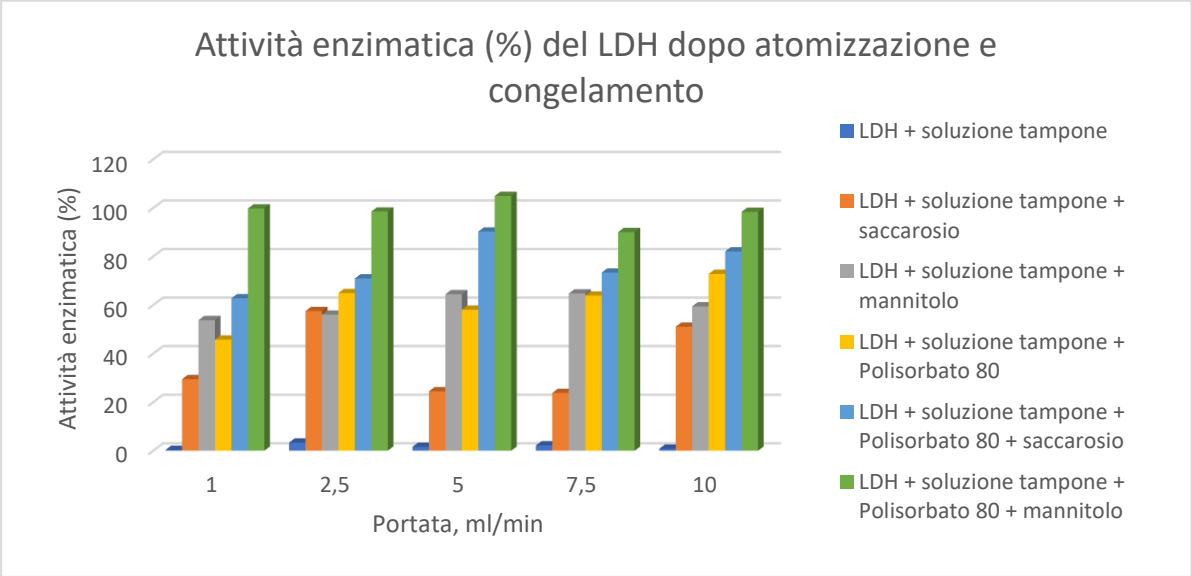

Figura 10: Attività enzimatica residua in seguito al processo di atomizzazione e congelamento

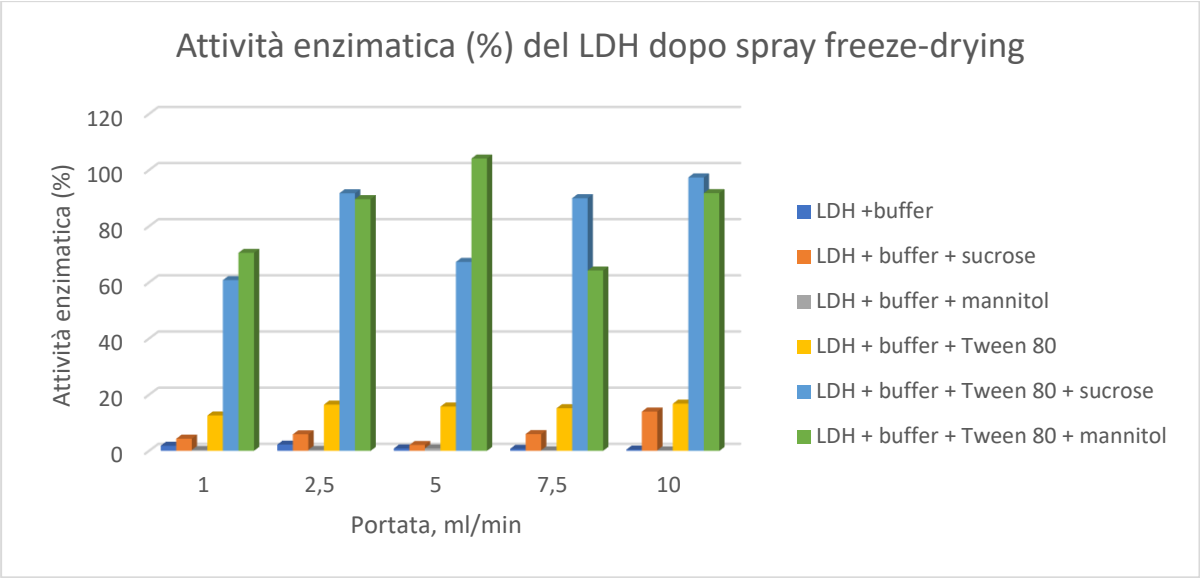

Figura 11: Attività enzimatica residua in seguito al processo di spray freeze-drying

La prima cosa da notare è che, sin dal principio, la formulazione contenente solo l'enzima in soluzione tampone è quella che restituisce i valori peggiori, non avendo nessun tipo di protezione per l'LDH.

Durante l'atomizzazione, a fornire un'elevata protezione all'enzima è il Tween 80. Gli stress maggiori che si hanno in questa fase sono concentrati in superficie, quindi il tensioattivo, grazie alle sue capacità in quanto tale, tende ad accumularsi all'interfaccia aria-liquido andando ad ostacolare la denaturazione della proteina.

In termini di zuccheri, invece, a mostrare un risultato migliore è il saccarosio in quanto tende a stabilizzare la soluzione.

Guardando al congelamento, invece, le cose cambiano poiché qui sono fondamentali saccarosio e mannitolo. Entrambi fungono da crioprotettori, evitando fenomeni di denaturazione a freddo grazie a meccanismi di "water replacement". Le formulazioni migliori sono quelle che presentano la combinazione di Tween 80 e saccarosio/mannitolo, garantendo così una buona protezione sia in fase di atomizzazione che di congelamento.

In seguito al processo di spray freeze-drying, l'enzima è completamente distrutto a meno che non si trovi in presenza di Tween 80. Anche qui, infatti, l'effetto del tensioattivo è determinante: avendo un segmento idrofobo, questo gli permette di legarsi alle regioni idrofobiche che le proteine espongono in superficie durante la denaturazione, proteggendole così dalla possibilità di ripiegamenti della struttura.

In seguito ai risultati ottenuti è possibile notare che gli step che maggiormente intaccano l'attività enzimatica sono il congelamento e l'essiccamento. A dare una riduzione più significativa è sicuramente l'essiccamento, ma già dopo il congelamento, se non sono presenti crioprotettori, l'attività enzimatica residua è quasi nulla.

Caratterizzazione del prodotto finito

Oltre ai test sull'attività enzimatica, sono state condotte varie analisi al fine di andare a caratterizzare il prodotto finito da un punto di vista morfologico e di residuo di umidità, analizzando le stesse formulazioni citate sopra senza l'aggiunta di LDH e in acqua. In questo caso il volume di soluzione atomizzata è passato da 1 ml a 30 ml.

La dimensione delle particelle del prodotto finito è stata determinata mediante analisi SEM (Scanning Electron Microscopy), ovvero un test tramite il quale un campione viene scansionato con un fascio di elettroni al fine produrre un'immagine ingrandita per valutare la morfologia del prodotto; in questo caso viene utilizzato per determinare la dimensione finale delle particelle dopo il processo.

La SEM è stata eseguita tramite un desktop SEM Phenom XL a una tensione di accelerazione di 15 kV e diversi ingrandimenti. Le particelle sono state rivestite con platino da 8 nm utilizzando un rivestimento a spruzzo. Al fine di determinare il diametro geometrico delle particelle, le immagini SEM sono state analizzate utilizzando il software ImageJ.

In figura 12, 13 e 14 sono riportate alcune delle immagini ottenute, in particolare quelle derivanti da formulazioni atomizzate a 5 ml/min contenenti rispettivamente: mannitolo e Tween 80, saccarorio, mannitolo.

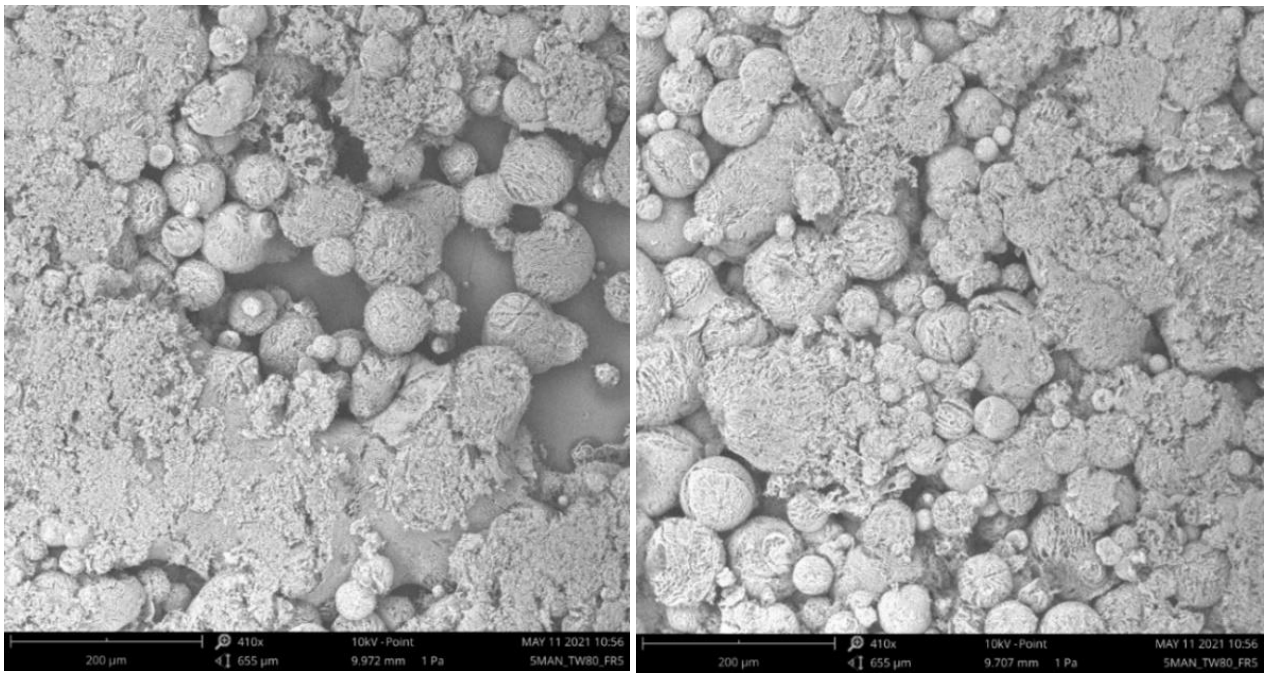

Figura 12: Immagini SEM per soluzione contente mannitolo al 5 %w/w e Tween 80 allo 0,1 %w/w, atomizzata alla portata di 5 ml/min

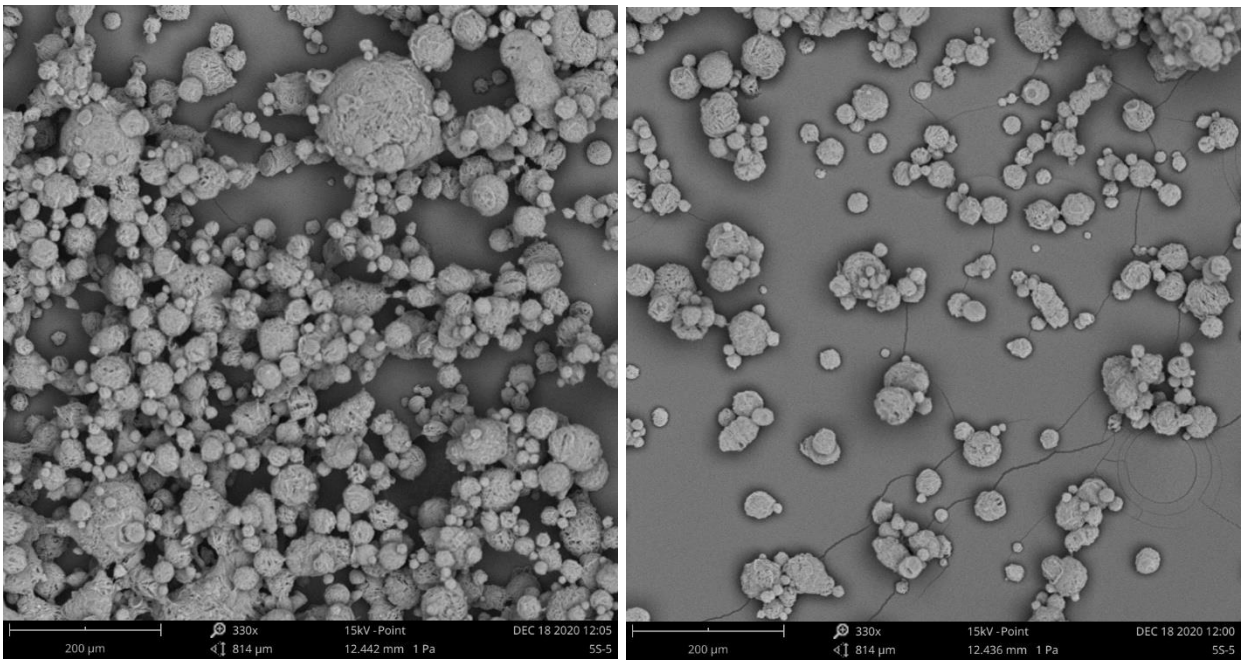

Figura 13: Immagini SEM per soluzione contente saccarosio al 5 %w/w atomizzata alla portata di 5 ml/min

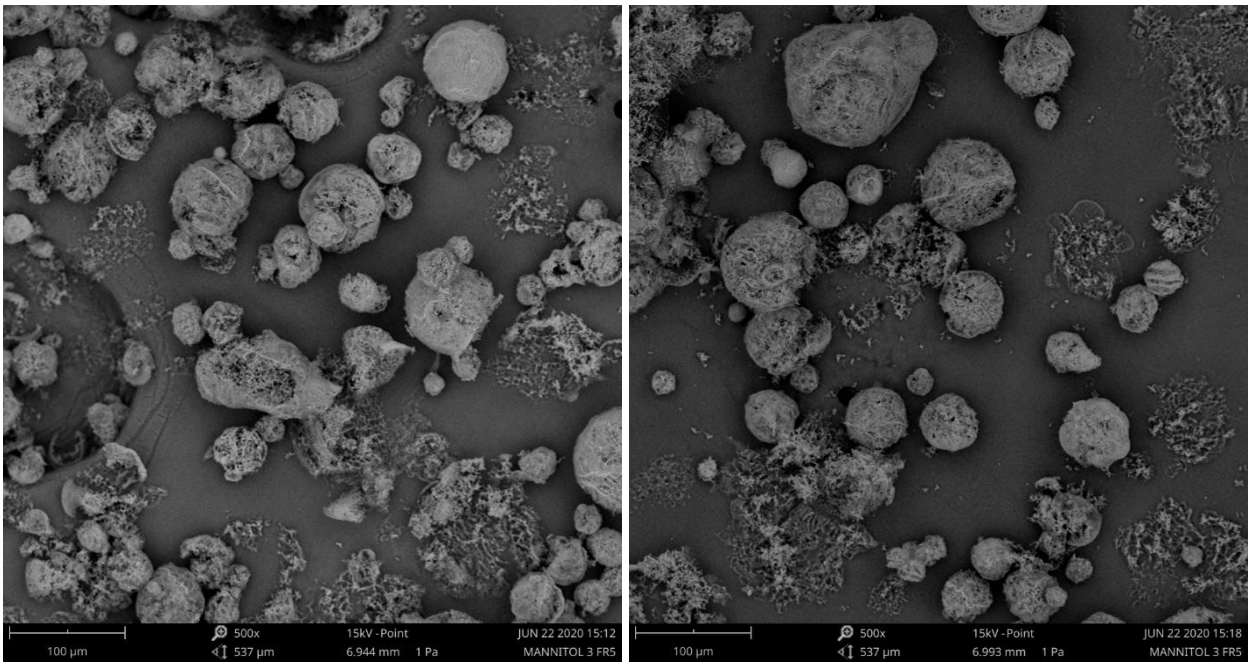

Figura 14: Immagini SEM per soluzione contente mannitolo al 5 %w/w atomizzata alla portata di 5 ml/min

È stato deciso di condurre l'analisi SEM solo su quelle formulazioni che hanno restituito i valori migliori in termini di attività enzimatica. Da analisi statistica è stato rilevato che, per quasi tutte le formulazioni in esame, i risultati migliori sono stati registrati per la portata di 5 ml/min: alla luce di ciò, l'analisi SEM è stata condotta solo su queste soluzioni.

A seguire (Figure 15, 16 e 17), sono riportate le distribuzioni ottenute in seguito a misurazioni su 200 particelle per ogni formulazione atomizzata al valore citato sopra. Per ogni particella sono state fatte sei misure.

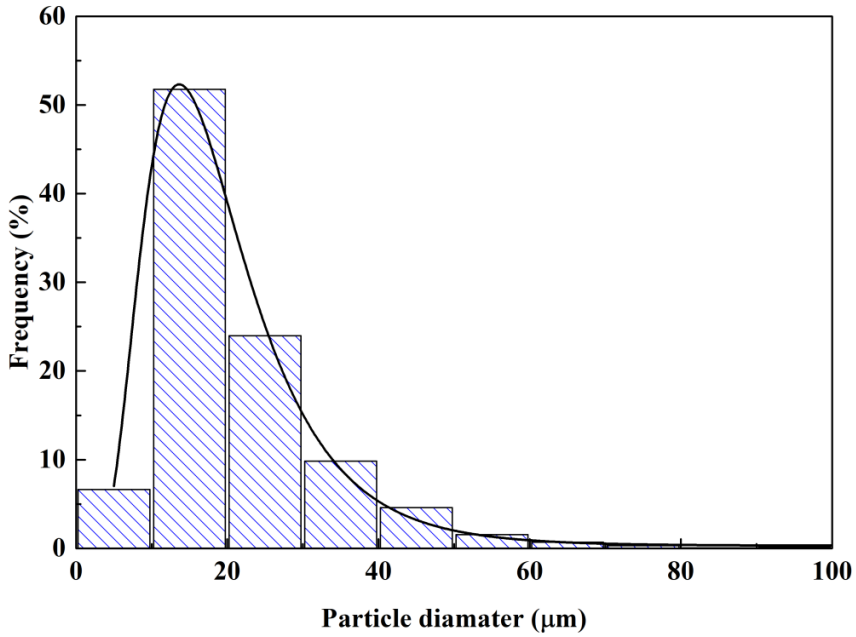

Figura 15: Distribuzione della dimensione delle particelle di una soluzione contenente saccarosio al 5%w/w.

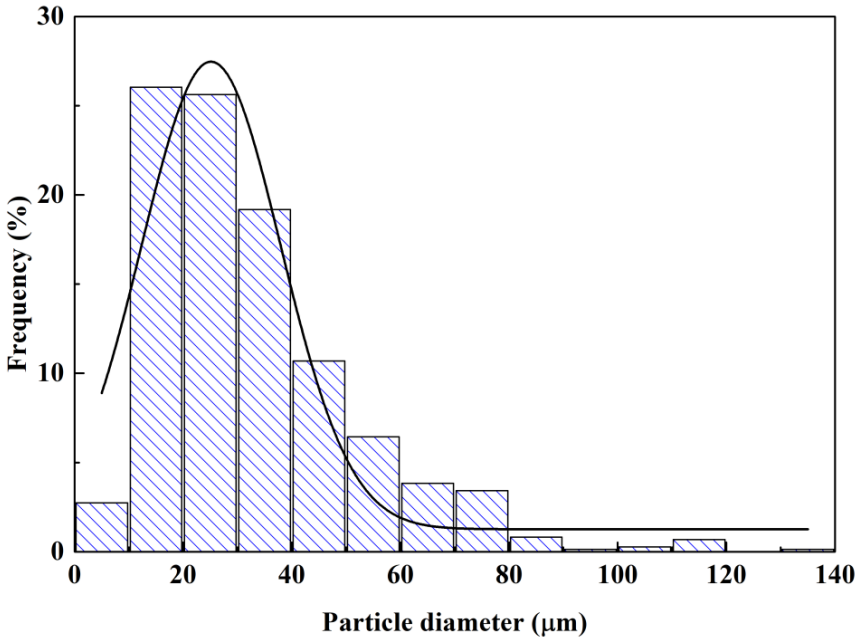

Figura 16: Distribuzione della dimensione delle particelle di una soluzione contenente mannitolo al 5%w/w.

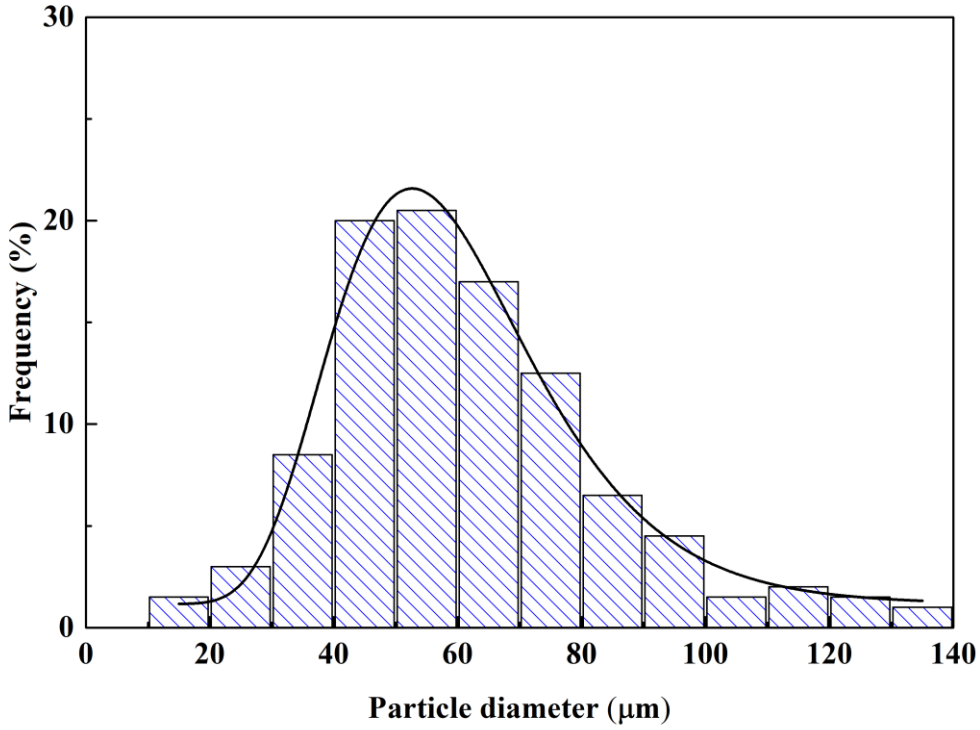

Figura 17: Distribuzione della dimensione delle particelle di una soluzione contenente mannitolo al 5%w/w e Tween 80 allo 0,1%w/w.

In seguito alle misurazioni fatte è stato calcolato il diametro medio delle particelle per ogni formulazione analizzata: i risultati sono riassunti in Tabella 2. Non è stato possibile analizzare mediante SEM la soluzione contenente saccarosio e Tween 80, poiché quest'ultima, a contatto con l'aria, ha subito immediatamente un collasso ostacolando la fattibilità dell'analisi.

In particolare. vediamo come il diametro medio più basso è stato ottenuto per la soluzione di saccarosio al 5%w/w, a fronte delle formulazioni in cui è presente il mannitolo, le quali hanno restituito valori di diametro maggiori.

Tabella 2: Diametro medio delle particelle, ottenuto da analisi SEM

Formulazioni	Diametro medio (μm)
5%w/w saccarosio	21,88 ± 13,25
5%w/w mannitolo	32,48 ± 19,25
5%w/w saccarosio + 0,1%w/w Tween 80	-
5%w/w mannitolo + 0,1%w/w Tween 80	61,90 ± 22,36

Un altro tipo di analisi, condotta al fine di avere un quadro generale della morfologia del prodotto finito, è l'analisi BET (abbreviazione di Brunner-Emmett-Teller), tecnica utilizzata per misurare l'area superficiale dei materiali solidi o porosi. In particolare, l'analisi BET sfrutta l'adsorbimento di un gas non reattivo sulla superficie della polvere per determinarne l'area superficiale.

Anche quest'analisi è stata condotta solo su formulazioni atomizzate a 5 ml/min, le quali costituiscono la migliore risposta da analisi statistica. I risultati sono illustrati in Tabella 3.

Tabella 3: Risultati in termini di area superficiale ottenuti da analisi BET

Formulazioni	Area superficiale specifica (m^2/g)
5%w/w saccarosio	1,37 ± 0,02
5%w/w mannitolo	7,53 ± 0,05
5%w/w saccarosio + 0,1%w/w Tween 80	1.05± 0.02
5%w/w mannitolo + 0,1%w/w Tween 80	9.84 ± 0.03

L'area superficiale specifica è nettamente differente a seconda che si tratti di formulazioni contenenti saccarosio o mannitolo. Questo parametro rispecchia la porosità del prodotto finito, la quale è fortemente influenzata dal mannitolo. La cristallizzazione dello stesso mannitolo, infatti, va ad intaccare il riarrangiamento della struttura durante il processo di spray freeze-drying, aumentandone la porosità e quindi l'area superficiale specifica.

Un importante parametro al fine di caratterizzare la qualità del prodotto finito è il contenuto residuo di umidità. Per analizzare questo parametro è stata condotta un'analisi che prende il nome di titolazione di Karl-Fischer.

Circa 50-100 mg di polvere sono stati sciolti in 2-3 mL di reagente Karl Fischer, quindi sono stati iniettati nella cella del titolatore Karl Fischer al fine di reagire con il reagente di titolazione sotto agitazione magnetica. Grazie a questo processo la quantità finale di contenuto d'acqua è stata determinata sulla base del peso secco del campione. I risultati sono riportati in Tabella 4.

Tabella 4: Contenuto residuo di umidità

Formulazioni	Contenuto di umidità (%)
5%w/w saccarosio	6,08 ± 0,24
5%w/w mannitolo	1,81 ± 0,24
5%w/w saccarosio + 0,1%w/w Tween 80	5,83 ± 0,14
5%w/w mannitolo + 0,1%w/w Tween 80	2,52 ± 1,79

Dai risultati ottenuti in termini di contenuto residuo di umidità si evince come a dar i risultati migliori siano le formulazioni contenenti mannitolo, questo può essere dovuto al fatto che gli eccipienti hanno una maggiore capacità di trattenere l'acqua se allo stato amorfo, meno in caso di struttura cristallina.

La diminuzione del contenuto di umidità con il Tween 80 riscontrata in caso di formulazioni con saccarosio, invece, può essere attribuita alle interazioni idrofobiche del tensioattivo. Nel caso in cui sia presente mannitolo l'effetto predominante deriva dal comportamento di quest'ultimo.

Infine, sul mannitolo è stata effettuata un'ultima analisi, la XRD (X-ray powder diffraction).

Il mannitolo tende a cristallizzare durante la liofilizzazione e la forma cristallina fornisce supporto strutturale ed eleganza al prodotto risultante. Inoltre, un'altra peculiarità da attribuire al mannitolo cristallino è quella di ridurre il tempo di ricostituzione delle formulazioni proteiche liofilizzate ad alta concentrazione. Se tenuto amorfo, invece, il mannitolo agisce come crioprotettore. In generale, il mannitolo cristallizza come una miscela di polimorfi anidri a seconda della formulazione e dei parametri di lavorazione.

La diffrazione a raggi X su polvere (XRD) è una tecnica utilizzata per la caratterizzazione dei materiali cristallini, identificando la struttura atomica e molecolare di un cristallo.

Durante l'analisi XRD, un fascio di raggi X viene diretto verso un campione e l'intensità diffusa viene misurata in funzione della direzione in uscita. Il principio di funzionamento di un diffrattometro a raggi X si basa sulla riflessione o diffrazione di Bragg.

I modelli di diffrazione a raggi X (XRD) sono stati caratterizzati utilizzando la radiazione Cu-Kα con una lunghezza d'onda di 1,54054 a 40 kV e 40 mA da un diffrattometro a raggi X. La polvere campione è stata posta in un supporto per campioni e scansionata da 5 a 65° (2θ) ogni 0,026°.

In Figura 18 sono riportati gli spettri ottenuti per le formulazioni contenenti mannitolo al 5%w/w e mannitolo al 5%w/w più Tween 80 allo 0,1%w/w. Per entrambe le formulazioni l'analisi è stata condotta su quelle atomizzate a 5ml/min.

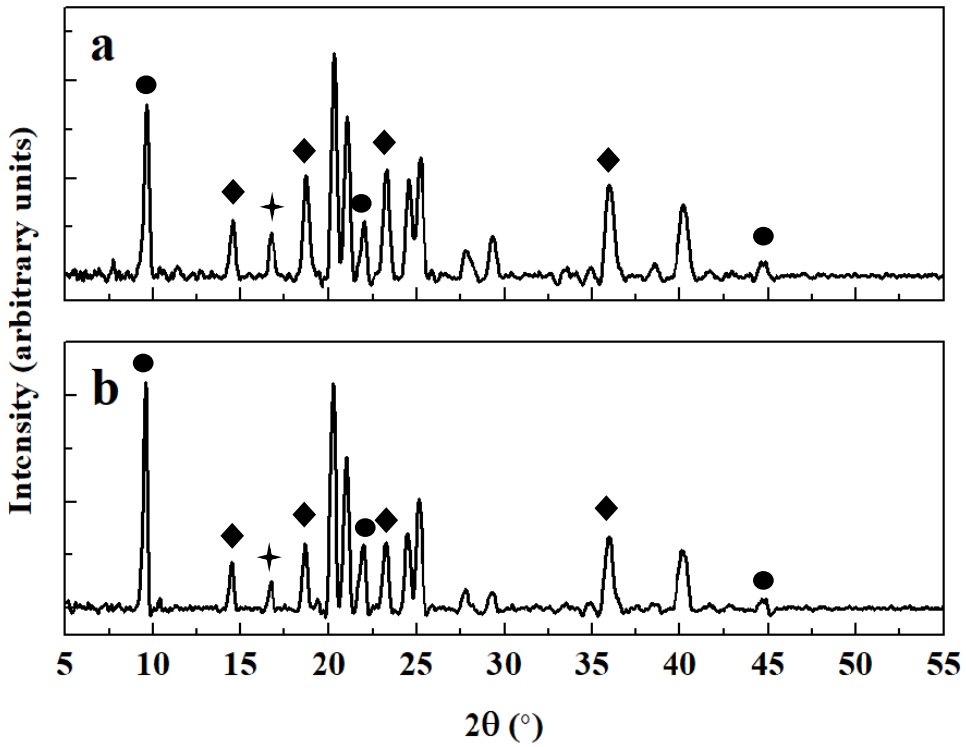

Figura 18: Risultati ottenuti da analisi XRD per: a) formulazione contenente mannitolo al 5%w/w e b) formulazione contenente mannitolo al 5%w/w e Tween 80 allo 0,1%w/w.

Il simbolo ● e il simbolo ◆ rappresentano, rispettivamente, le forme δ e β polimorfe, mentre il simbolo ✦ rappresenta la forma emiidrata.

Dallo spettro in questione è possibile notare come la presenza o meno di Tween 80 non influenzi la cristallizzazione del mannitolo, in quanto, in entrambi i casi sono stati rilevati gli stessi picchi. In particolare, si evince che il mannitolo cristallizza in forma δ per valori circa pari a 10, 22 e 45 °, mentre presenta dei picchi a circa 15, 19, 27 e 35° i quali rappresentano una cristallizzazione in forma β. Per valori intorno ai 14° si ha la forma emiidrata di mannitolo.

In conclusione, da questo studio si evince come il processo di Spray Freeze-drying sia possibile su soluzioni contenti l'enzima lattato deidrogenasi, purché quest'ultimo sia in formulazioni contenenti dei protettori.

Dalle analisi condotte, si è visto come le formulazioni contenenti Tween 80, quindi il tensioattivo, presentino i risultati migliori: grazie alla presenza di quest'ultimo, infatti, la maggior parte dei fenomeni di denaturazione all'interfaccia vengono ostacolati.

La presenza di eccipienti come saccarosio e mannitolo, invece, può essere intercambiabile a seconda delle proprietà che si vogliono ottenere. In generale entrambi proteggono bene l'enzima durante il processo di congelamento. Nel caso in cui sia richiesta una distribuzione delle particelle con diametro minore si opterà per formulazioni contenenti saccarosio mentre, nel caso in cui il parametro determinante sia la porosità, quindi un'area superficiale maggiore, sicuramente il mannitolo restituirà i risultati migliori.

1. Introduction

1.1 How to preserve biopharmaceuticals

Proteins are a group of biologically active macromolecules of fundamental importance in various fields. They cover a large number of roles within our body, in particular, they are seen as the "building material" of our cells. Their importance is not only due to their vital functions, but in fact, they have also assumed a key role in the pharmaceutical industry, thanks to the growing interest in biopharmaceuticals.

Unfortunately, their great importance is directly proportional to the difficulty of managing them. When proteins are in the liquid phase, they are extremely sensitive to thermal stress, changes in solution pH, environmental conditions, and the presence of interfaces (Arsiccio, Giorsello, Marenco, Pisano, 2020). The stability of proteins strictly depends on their structure. In general proteins in the liquid phase show a strong tendency to refold and change their structure through a process called denaturation. After denaturation, proteins change their activity and can make some groups reactive, thereby forming intramolecular bonds that can cause the aggregation of multiple protein molecules.

Due to their instability, it is preferable to dry them and store them in an anhydrous form to ensure the preservation of their biological activity.

Drying is the oldest technique used to remove the liquid part from a product. For example, it is widely used in the food industry as it ensures the stability of food for long shelf life. In bioproducts, the liquid part is usually water, which exists in two different forms: unbound and bound. Unbound water is found as free crystals in the structure, while bound water is adsorbed on the product surface Thanks to drying, it is possible to reduce the amount of water to the desired level.

Drying techniques can be varied, classified according to the methods of providing heat and removing water vapor; usually, the most common drying technique involves passing a stream of hot air in direct contact with the product to be dried (Padma Ishwarya, Anandharamakrishnan, Stapley, 2014). Unfortunately, this process, which causes an increase in product temperature, involves significant chemical, physical and structural changes that adversely affect the quality of biological macromolecules such as proteins.

At temperatures around 60°C, the peptide bonds responsible for the 3D structure of proteins are destroyed and proteins tend to rearrange, which changes their biological activity.

For this reason, other techniques were studied until the discovery of freeze-drying.

1.2 Lyophilization

Lyophilization, or Freeze-Drying (FD), is a process used to remove water from heat-sensitive products. It is a technique that exploits low temperatures and removes water by first sublimation and then desorption at low temperature and pressure.

In lyophilized products, most of the reactions that could cause degradation are blocked or drastically slowed down, ensuring long-term stability with easier storage. However, it is an expensive and time-consuming process.

In Figure 1.1 the three main steps of a freeze-drying cycle are reported: freezing, primary drying and secondary drying. It takes place in a chamber where the pressure initially is kept at the atmospheric value and the product is frozen to separate water in form of ice crystals, lowering the temperature in the chamber by cooling the shelves on which the product is placed. Once the freezing phase is over, primary drying occurs where the pressure in the chamber is reduced and the shelves are slightly heated to have sublimation of the ice crystals and water vapor is collected in a condenser. Finally, in the secondary drying phase, the pressure is always maintained at the values of the previous phase, but there is a slight increase in temperature, which favours the desorption of the water-bound on the surface of the product.

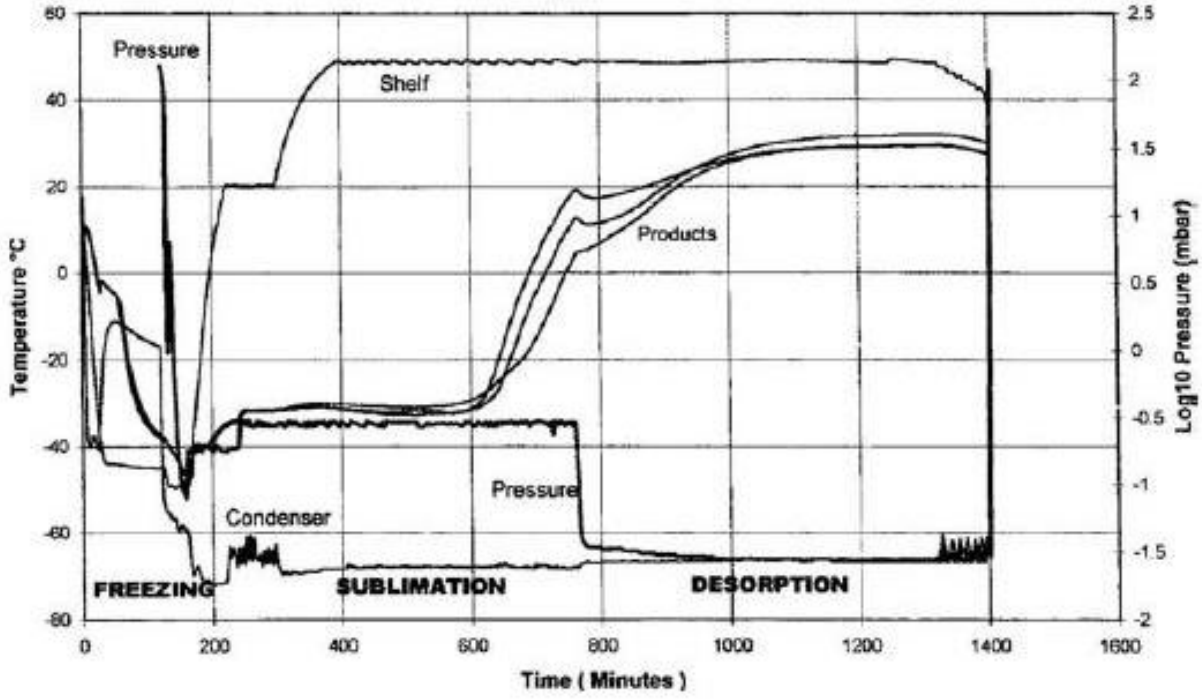

Figure 1.1: A typical Freeze-Drying cycle. Figure reprinted from "Freeze Drying/Lyophilization of Pharmaceutical and Biological Products"; L. Ray, J.C. May, with modifications.

1.2.1 Freezing

For a long time, this stage was neglected or little studied, with more emphasis on primary drying instead. However, after various studies, it was concluded that the freezing stage is the most critical and should be investigated with deep attention.

Despite the numerous advantages that can attribute to the FD technique, the problems in this case of FD are due to low temperatures rather than high temperatures. In fact, during freezing the solution is exposed to stress factors such as: pH variation deriving from preferential crystallization of buffer species, freeze concentration of solutes in the remaining unfrozen solution, structural degradation of proteins at ice-water interfaces, and phase separation mainly due to the formation of multiple amorphs phases or crystallization of solutes (Minatovicz, Sun, Foran, Chaudhuri, Tang, Shameem, 2020).

As a consequence of these stress phenomena, changes in the native protein structure may occur, resulting in impaired enzymatic activity and induction phenomena of immune response when the drug is administered. For these reasons, it is very important to know the

freezing process very well and manage the freezing process as best as possible to minimize any denaturation phenomena.

Freezing is the basic dehydration step, in fact ice crystals are formed in this step, thus allowing the water to separate and increasing the solute concentration of the solution. It determines the product morphology and the final properties of the freeze-dried product (Kasper, Friess, 2011). Product morphology is closely linked to ice size which determines the drying time, while stability and activity of lyophilized products are determined by the conditions under which crystallization of buffer components and co-solute occurs. Following the observations made, it is clear how important it is to control freezing, but, unfortunately, the presence of the stochastic nucleation phenomenon makes it challenging.

When it comes to freezing, it is necessary to pay attention to concepts such as cooling rate, freezing rate and "supercooling". The cooling rate is the rate at which the solution is cooled, while the freezing rate is defined as the rate of ice crystal growth after nucleation. About "supercooling", instead, it is important to know that when a solution is supercooled, it does not solidify spontaneously at its thermodynamics freezing point, but must be further cooled until the first nucleus is formed: the difference between the equilibrium freezing point and the nucleation temperature is defined as "supercooling" and is usually around 10-15°C (Kasper, Friess, 2011). In other words, it is possible to see "supercooling" as an energetic barrier that nuclei must overcome in order to become stable.

During freezing, nucleation occurs, but what is nucleation? And how does it work? Nucleation is the process by which a certain number of molecules reach a critical size after the collision and form a stable cluster that will later become a crystal incorporating material from the surrounding environment. The formation of the first stable nucleus is energetically unfavourable, but the probability of this happening increases as the temperature decreases. Once the first stable nucleus is formed, growth occurs, an exothermic phenomenon that allows the temperature of the product to increase up to the thermodynamic equilibrium temperature.

Ice size is strictly dependent on nucleation state and is the parameter that most influences the primary drying time: this is one reason why it is important to be able to control nucleation. The size of the ice crystals depends on the degree of supercooling: the higher the degree of supercooling, the greater the number of stable nuclei formed. Consequently, the more nuclei formed, the smaller their size will be and vice versa. In order to control nucleation, many techniques have been studied that limit the degree of supercooling by adding heterogeneity or by regulating the cooling rate. Moreover, the nucleation rate is essential for the structure of the excipients, such as mannitol, which may be in crystalline or amorphous form depending on the nucleation conditions. Controlled nucleation is also an advantage in terms of vial-to-vial heterogeneity within the batch and is not always ensured given the random nature of this phenomenon. In the end, the crystal size influences also the secondary drying performances and, consequently, the residual moisture content of the final product.

In general, to optimize the freeze-drying cycle, the size of the crystals should be large enough to guarantee a short primary drying time, but small enough to have a large specific surface area of the dried matrix to simplify desorption.

1.2.2 Primary drying

Primary drying is the longest phase of the freeze-drying cycle. At this phase, water is removed by sublimation under vacuum conditions.

Sublimation is a phenomenon in which water avoids the liquid state by going directly from the solid-state to the vapor state. Looking at Figure 1.2, the phase diagram of water is reported. The prerequisite for sublimation is that the vapour pressure and the temperature are below 0.6 kPa and 0,01°C, respectively, that is the triple point of water (Padma Ishwarya, Anandharamakrishnan, Stapley, 2014). The driving force of this process is the difference between the vapour pressure of ice and pressure chamber. It is an endothermic process: its evolution, therefore, involves a constant supply of heat to compensate for what is removed by the process itself: this makes heat transfer particularly important.

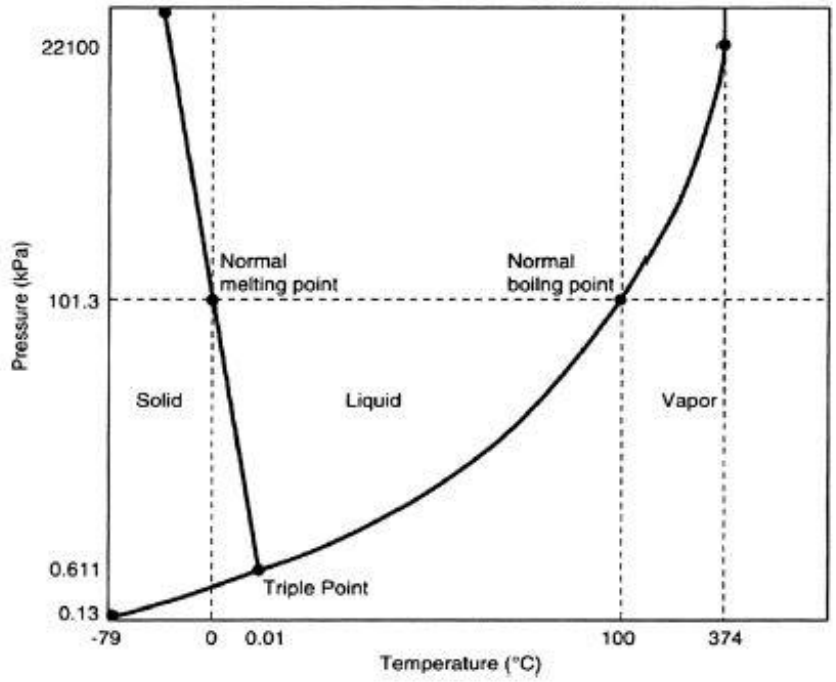

Figure 1.2: Phase diagram of water. Figure reprinted from "Spary Freeze-Drying: A novel process for the drying of food and bioproducts"; Padma Ishwarya, Anandharamakrishnan, Stapley, 2014, with modifications.

Generally, the mechanisms of heat transport are three: radiation, direct contact, and conduction through the gas held at the bottom of the vial. Radiation takes place through the walls of the chamber and the shelf, which are suitably heated, and it is the most important mechanism. Direct contact occurs between the bottom of the vial, which is in direct contact with the shelf on which it is placed. The least is the conduction mechanism which is caused by the heat exchange between the vial and the gas trapped between the shelf and its concavity.

In addition to heat transfer, the transfer of substance also plays an important role, creating a real drying front that advances as the process progresses. The sublimation front starts from the surface of the product and leaves a porous layer as it progresses. The passage of the sublimated vapour through this dried porous layer takes place by diffusion and convective flow. At this stage, the pore size of ice crystals achieved by freezing plays a key role in the substance diffusivity.

The most critical parameter during primary drying is the temperature of the product, in which case it must never exceed the maximum acceptable value as the product would collapse. To control the latter, shelf temperature and pressure chamber are regulated.

After freezing, the pressure in the chamber is reduced until it reaches a lower value than the vapor pressure of the ice at the drying front of the product, at this point sublimation occurs. There is an optimal pressure value in the chamber: at low pressure values there is low efficiency in the transport of heat and the process proceeds slowly, while at high pressure values the driving force for the transport of matter is reduced. For these reasons, it is recommended to have a chamber pressure around one-third of the vapor pressure of the ice at the desired product temperature. The pressure chamber can be controlled in two different ways: sending into the camber a stream of dry inert gas (for example nitrogen) with a needle valve or by regulating the valve between the condenser and the vacuum pump that opens when the pressure in the condenser exceeds.

What is fundamentally important is the evaluation of the end point of primary drying at which there is a high residual moisture content. It is important to increase the temperature of the shelf gradually to avoid collapse. Also, it is fundamental not to exceed the glass transition temperature, which would give the product mobility and cause it to collapse.

1.2.3 Secondary drying

Secondary drying is the final step of the lyophilization cycle. It allows removing bound water in the product by desorption.

The driving force is the difference between the concentration of residual water in the product and its equilibrium concentration. Unlike primary drying, here the temperature is not constant throughout the process but increases with the progress of desorption.

It is important to make a distinction between crystalline materials and amorphous materials. In the case of crystalline state, it is possible to operate at a high temperature, while in amorphous materials a lower temperature must be maintained so that the glass transition temperature of the product is not exceeded.

The amount of water to be removed is not high, but, in any case requires about 30% of the time required for primary drying (Bhushani, Anandharamakrishnan, 2017). Drying time depends on the specific surface area of the product and therefore strictly dependent on the morphology achieved by freezing.

1.3 Spray Freeze-Drying as an alternative to conventional freeze-drying

Spray Freeze-Drying (SFD) is a technique that combines two different methods: spray drying and freeze-drying. This technology was first studied by Benson and Ellis in 1940, but it took until the 90s to see the first real application (Adali, Barresi, Boccardo, Pisano, 2020). Thanks to spray freeze-drying it is possible to combine the advantage of obtaining small dispersed particles thanks to spray drying, with the possibility of drying heat-sensitive materials thanks to lyophilization.

As can be seen in Figure 1.3, spray freeze-drying is developed in three stages:

1. Dispersion of a solution into droplets thanks to an atomizer
2. Freezing of the droplets, atomizing them directly in a cryogenic liquid
3. Production of powder by sublimation of water from the frozen droplets

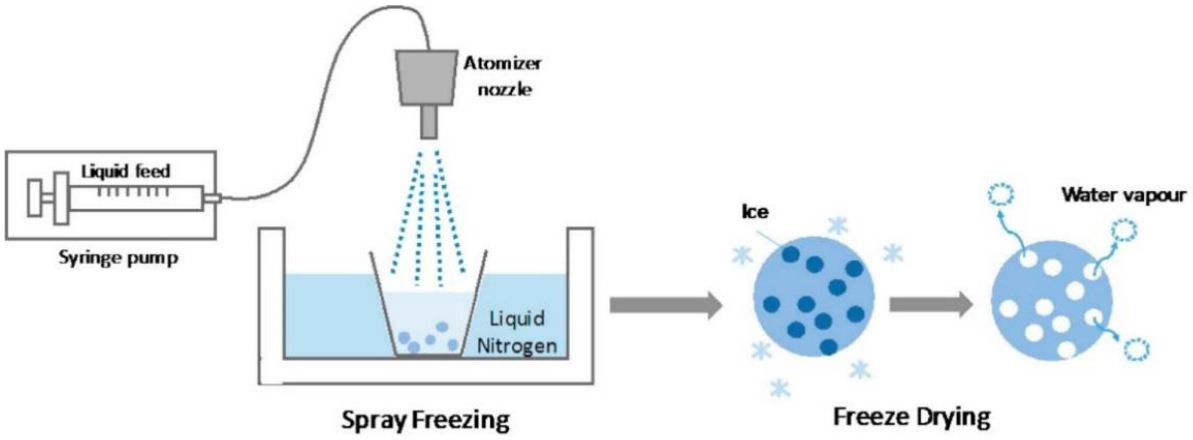

Figure 1.3: Spray Freeze-Drying steps. Figure reprinted from https://www.sciencedirect.com/science/article/pii/S1818087617303902, with modifications

Considering the various advantages of this technology, firstly, it is possible to obtain a powder with controlled particle-size distribution with spray freeze-drying, which will promote drying and thus overcome one of the limitations of freeze-drying. Another advantage over freeze-drying is that it saves both time and energy consumption.

The use of SFD methods allows to increase the solubility of poorly water-soluble drugs. Furthermore, the rapid freezing causes the proteins to be embedded in the excipients, preventing phase separation between them and guaranteeing a molecular distribution of drug into excipient material (Wanning, Suverkrup, Lamprecht,2015). Also, with the spray freeze drying technique it is possible to achieve a good level of control over residual moisture content, particle size and mass density (Adali, Barresi, Boccardo, Pisano, 2020).

1.3.1 Spray freezing into vapor over a liquid

In the case in exam, in particular, the focus is pointed on the technique of spray freezing into vapor over a liquid: the nozzle is putted inside the vial which contains a cryogenic liquid inside, in this way the atomized droplets enter first in contact with the vapour of the liquid and immediately frozen.

1.3.1.1 Atomization

The atomization can be carried out through different types of nozzles: hydraulic, pneumatic and ultrasonic nozzles. The use of hydraulic nozzles provides spraying of the liquid by passing it through an orifice, exploiting the conversion of pressure into kinetic energy. Pneumatic nozzles use a compressed gas flow which, interacting with the liquid, produces a shear field which causes the solution to be dispersed in droplets. Finally, ultrasonic nozzles convert a high-frequency electrical signal into mechanical energy which disperses the liquid in droplets. This step directly determines the particle size distribution of the final product, which will be more or less the same as the size distribution of the atomized droplets. The droplets' size is in turn dependent on the conditions in which the atomization takes place, with particular regard to the pressure at which it occurs, the flow rate and the surface tension. For example, at constant atomization pressure, an increase in liquid flow causes an

increase in droplet size due to the high amount of solution to be atomized (Padma Ishwarya, Anandharamakrishnan, Stapley, 2014). Surface tension also plays a particularly important role in the atomization process as it is a sort of energy barrier to be overcome to create new surfaces (Adali, Barresi, Boccardo, Pisano, 2020).

1.3.1.2 Droplet freezing

Thanks to spray freezing it is possible to make nucleation occur within milliseconds, avoiding crystallization phenomena of excipients, fragmentation of solutes and pH variations. Spray freezing is mainly classified based on the technique used: the solution can be atomized in a cryogenic liquid (SFL) or vapours over a boiling cryogenic liquid (SFV/L). If the SFV/L methods are used, a slower freezing occurs due to atmospheric braking and conditions are similar to those of atmospheric freezing. In SFL, instead, the nozzle is directly immersed in the cryogenic liquid and freezing occurs very quickly.

1.3.1.3 Vacuum freeze-drying

After spray-freezing, lyophilization occurs. The freeze-drying process can be conducted under pressure or at atmospheric pressure. Vacuum freeze-drying is the conventional mechanism used which was discussed above, while freeze-drying occurs at atmospheric pressure, and to make it possible is necessary that the vapor pressure of the water is lower than the vapor pressure of the ice. It is a technique which does not require extreme vacuum conditions, drastically reducing the costs foreseen for the conventional process, but, on the other hand, its application requires a continuous flow of cold and dry gas in the chamber which in any case raises the operating costs. For these reasons, vacuum freeze-drying is always preferred.

1.4 Enzymes

The present work focuses on the spray freeze-drying process and on how the latter affects the particular molecules, the enzymes.

Enzymes are proteins with particular properties: they are strong biological catalysts of chemical reactions, essential in physiological processes. They are capable of increasing the reaction rate considerably. Another peculiarity of these molecules is that they are highly specific; in fact, they only react with certain substrates, where each substrate is specific to a certain enzyme. Due to these properties, enzymes are used as therapeutic targets to fight diseases: by acting on their activity thanks to the use of inhibitors or activators it is possible to fight any diseases. Moreover, they are useful because they represent alarm bells in our organism as, based on their biological activity, they give information about any diseases present.

1.4.1 Enzyme structure

Enzymes are polymers formed by the binding of α-amino acids via a peptide bond. Each amino acid consists of a carbon atom to which a hydrogen atom, an amino group, a carboxylic acid group and a R-group are attached which determines the specific properties of the different enzymes. Amino acids can be hydrophobic or hydrophilic depending on the nature of the R group. About conformation, hydrophilic amino acids are usually found

outside the structure due to their capability to form hydrogen bonds with the surrounding aqueous medium, while hydrophobic amino acids are found inside of the enzyme.

The overall structure of the enzymes can be different. Four different structures are possible: primary structure, simply the order of amino acids, secondary structure, which can be α-helix or β-sheet, tertiary structure, which describes the folding of secondary elements, and quaternary structure, which interconnects in case of multiple polypeptide chains. The structure of the enzyme is very important because the enzyme activity depends on the latter.

In particular, the structure of the enzyme changes according to the morphology of the product and this could give rise to phenomena of denaturation. The main factors that could inactivate the enzyme following changes in the structure are: temperature variations, dehydration process, surface adsorption phenomena.

As for temperature variations, secondary, tertiary and quaternary structures are dependent on hydrogen bonds: increasing the temperature reduces the strength of the hydrogen bonds and thus denaturation occurs. In the same way, even the low temperature could create problems: in particular, in the case of low temperature there is the risk of obtaining a high residual humidity: in this case, vacuum freeze-drying is a good alternative.

Furthermore, the dehydration step is harmful to the enzymes: the removal of the aqueous medium surrounding the enzyme can also lead to the breakdown of hydrogen bonds, for this reason the addition of excipients or the like is necessary to protect the protein.

Finally, the phenomena of surface adsorption occur in particular during the SFD: the enzymes tend to adsorb on the surface of the drop and this could be a limit due to possible conformational changes at the air-liquid interface.

1.4.2 Effect of excipients

In order to prevent denaturation phenomena, excipients are added to formulations containing enzymes.

Carbohydrates are the most used excipients to stabilize this type of formulation. They are responsible for a phenomenon that takes the name of the “water replacement hypothesis”: thanks to this process carbohydrates replace water in forming hydrogen bonds with proteins after the dehydration process.

Another significant phenomenon to attribute to carbohydrates is the so-called "vitrification” phenomenon. Thanks to this process the carbohydrate is kept in an amorphous state which prevents the protein from changing shape, thus avoiding denaturation due to conformational changes of the protein.

Carbohydrates are not the only solution to avoid denaturation. Due to the tendency of enzymes to adsorb at the droplet interface, surfactants are particularly used as additives in formulations. They are particularly effective in protecting enzymes following denaturation phenomena at the interface: thanks to their ability to accumulate at the interface, they can expel proteins from the air-liquid interface avoiding denaturation.

1.5 Motivation of the thesis

The main purpose of this work is to investigate the critical steps of spray-freeze drying on the biological activity of enzymes, in particular, lactate dehydrogenase (LDH) was taken as a model protein during the SFD experiments performed in this work.

In this chapter, an overview has been presented regarding the problems related to the manipulation of proteins due to their high sensitivity to stress in general. The traditional techniques used for drying are explained, focusing on why they could not be used with heat-sensitive materials. An overview of the lyophilization process is given to provide a basis for a better understanding of the spray freeze-drying process and its advantages over conventional freeze-drying. Finally, the spray freeze-drying process was described and the whole process was broken down into three steps (atomization, freezing/thawing, and spray freeze-drying) in order to identify the enzymatic activity of the protein after each step that will be described later.

In chapter 2, the formulations analysed, the experimental setup and operating procedures are described in detail. It includes enzyme activity assay of formulations after each step of the process. The particle characterization analysis techniques such as morphology and particle size distribution by scanning electron microscopy (SEM), specific surface area by Brunauer-Emmett-Teller (BET), identification of crystalline species by X-ray diffraction (XRD), and moisture content measurements (Karl Fischer titration) are also explained.

In chapter 3, the results of activity loss from the different separated process steps and particle characterization of spray freeze-dried powders are discussed. It includes the effects of process variables (liquid feed flow rate and formulation type) on the total activity loss of LDH, as well as effects on particle size and morphology.

Chapter 4 summarizes the final based on the comparison of the results. The individual steps are compared to highlight which is the most critical step for the final product, and the main findings can be used to further optimize the spray freeze-drying process for protein stabilization.

2. Materials and methods

In this section, the materials and the experimental setup used in this research are described. The description of methods used is divided into two parts regarding the enzyme activity assay after the different steps of the spray freeze-drying process and the characterization of the final dried products. The lactate dehydrogenase enzyme (LDH) was chosen for the analysis of the enzymatic activity, which was tested after each steps (atomization, freeze/thaw, and spray freeze-drying) of the process to understand which of the three steps was the most critical.

Regarding particle characterization of the final products, residual moisture content was analysed, and SEM, BET and XRD analyses were carried out.

2.1 Materials

L-Lactate dehydrogenase (LDH) that origins from rabbit muscle and all other chemicals and reagents used were purchased from Sigma-Aldrich (Milan, Italy). LDH was dialyzed against 10 mM citrate buffer at pH 6.5, using the Sigma-Aldrich Pura-A-Lyzer, 3.5 kDa MWCO kit. Dialysis was carried out at 4°C and the buffer was changed 3 times (first 2 times every 3 hours, the last, after a night). Post-dialysis LDH concentration was determined using UV/VIS spectroscopy (6850 UV/VIS Spectrophotometer; Jenway, Stone, Staffordshire, UK). The peak at 280 nm was monitored and an extinction coefficient of 1.44 mL/(mg cm) was used for calculations. The dialyzed solutions were stored in 2 mL aliquots at a concentration of 0.1 mg/mL in a -80C freezer until use.

Spray freeze-dried formulations of LDH (10 mL) were prepared by dissolving excipients (sucrose or mannitol) in a 10 mM buffer solution with or without the surfactant Tween 80. For each formulation, LDH and excipients concentrations were adjusted to 5 µg/mL and 0.5% w/w, respectively. The presence of the surfactant Tween80 at 0.01% w/v was also considered. A summary of the formulations used is reported in the following Table 2.1.

The aqueous formulations were prepared by dissolving LDH in 10mM citrate buffer to keep the pH constant during the experiments at a value of 6.5. 100 ml of citrate buffer solution of desired concentration was prepared in a beaker weighing 0.210 g of citric acid and pH was adjusted to 6.5 with the 0.5 M sodium hydroxide (NaOH) solution. The pH was adjusted to 6.5 since it was observed that the enzyme was not stable at the selected value as a result of the preliminary tests. In addition, the activity of many enzymes is strongly influenced by the pH of the reaction medium. An LDH activity assay is performed, which evaluates the reduction of NAD to NADH favoured by the pH range of 5.0 and 7.7 (Jonas, 1972).

Sucrose and mannitol, together with a buffer, were used as a cryoprotectant and lyoprotectant to control pH and provide cake resistance. Sucrose and mannitol solutions were prepared at a concentration of 5% w/w by dissolving 0.5 g of sucrose or mannitol in 10 mL of citrate buffer solution.

Tween 80 was used at a concentration of 0.01% w/v as a surfactant to minimize surface-induced denaturation.

Table 2-1. Compositions of LDH formulations

Formulation	Concentration
LDH + buffer	10 mM buffer, 5µg/ml LDH
LDH + buffer + sucrose	10 mM buffer, 5µg/ml LDH, 5% w/w sucrose
LDH + buffer + mannitol	10 mM buffer, 5µg/ml LDH, 5% w/w mannitol
LDH + buffer + tween 80	10 mM buffer, 5µg/ml LDH, 0,1% w/w tween 80
LDH + buffer + tween 80 + sucrose	10 mM buffer, 5µg/ml LDH, 0,1% w/w tween 80, 5% w/w sucrose
LDH + buffer + tween 80 + mannitol	10 mM buffer, 5µg/ml LDH, 0,1% w/w tween 80, 5% w/w mannitol

2.2 Spray freeze-drying process and separation of process steps

In this part, the operating conditions and the instrumentations used are described. To better evaluate the process of spray-freeze-drying process on the activity of LDH, the process was subdivided into three principal steps: atomization, freezing and complete spray-freeze-drying. This way of working allowed us to analyse each step of the process and to understand the most critical one.

Following the LDH activity test, the particles obtained by SFD at optimum process conditions, which ensure maximum enzyme activity, were characterized to understand the link between particle morphology and size and the enzyme activity of the end product.

2.2.1 Atomization experiments

Atomization is the step derived from the spray drying technique: by this, it is possible to achieve a controlled particle size distribution that will guarantee saving in the processing time required for both freezing and drying.

The droplet size distribution obtained by atomization represents what the final product will be as it does not change during the subsequent freezing and lyophilization steps.

For each solution, a quantity of 1 mL was atomized directly into vials through an ultrasonic atomizer via syringe pump at the flow rate of 1-10 mL/min (Figure 2.1). The selected flow rates are given in Table 2.2. In this research, the 60 kHz ultrasonic atomizer (Buchi, Switzerland) was used for the atomization of the formulations. The ultrasonic atomizer consists of two parts: the nozzle and the ultrasonic generator. This type of atomizer exploits the ultrasonic vibrations coming from the generator, which are produced after an electrical signal is sent to two piezoelectric transducers. The size of the resulting droplets mainly depends on the frequency and the flow rate. During atomization, a power of 3 W was set while changing the flow rate. Figure 2.2 shows the feed connections to the ultrasonic atomizer using a syringe pump (Model KDS 200, KD Scientific, Holliston, MA) that allows regulating feed flow rates to the desired value.

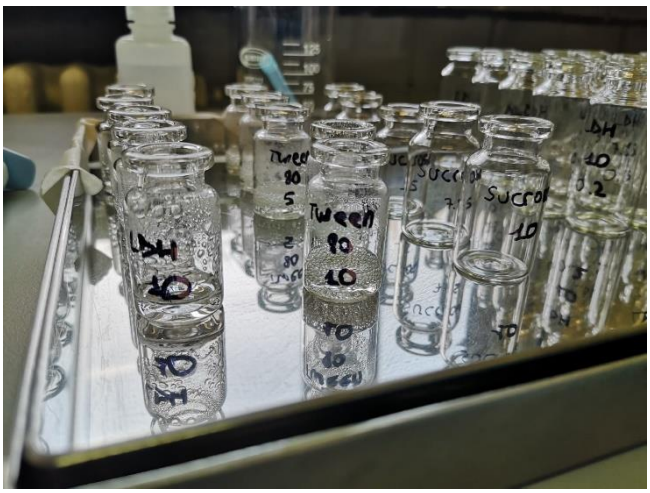

Figure 2.1: Atomization of the solutions directly into the vials

Table 2-2. Flow rate analysed

Flow rate	1 ml/min
	2,5 ml/min
	5 ml/min
	7,5 ml/min
	10 ml/min

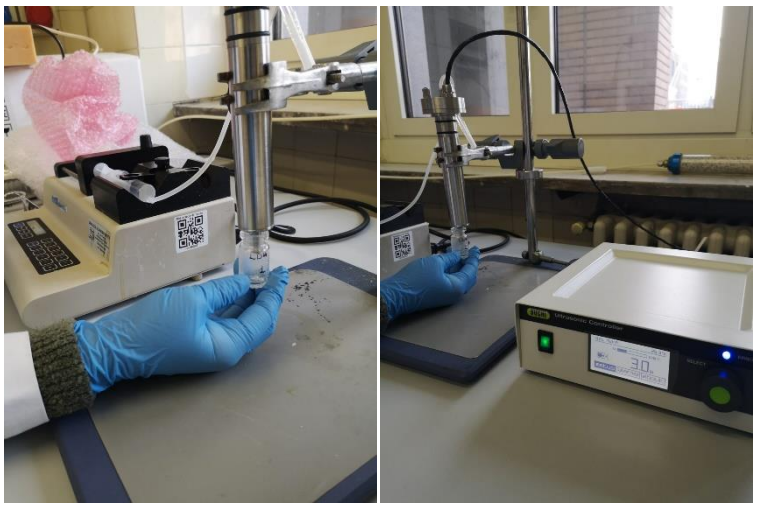

Figure 2.2: The configuration of atomization step. On the left the syringe pump and the 60 kHz ultrasonic atomizer, on the right the 60 kHz ultrasonic atomizer and the ultrasonic generator.

2.2.2 Freeze/thaw experiments

After analysing the atomization process, one more step was added which is freeze/thaw.

In the case of freezing, the operational choice made was SFV/L. This technique involves atomizing the solution into a vapor over a cryogenic liquid, which is liquid nitrogen in this study, and having fully frozen particles when they come into contact with the cryogenic liquid.

The operating conditions for the freeze/thaw step are the same as for atomization. The purpose of this step is to evaluate the effect of freeze/thaw on the enzymatic activity of LDH, knowing the effect of atomization alone. Therefore, a certain level of liquid nitrogen was filled into the vials used for atomization to form a vapor phase, and then the solutions were atomized. The solution was subjected to thawing in a water bath immediately after freezing (Figure 2.3).

Figure 2.3: Thawing of frozen particles in the water bath after freezing.

2.2.3 Spray freeze-drying experiments

The drying part was carried out in the REVO freeze dryer by Millrock Technology (Figure 2.4).

Figure 2.4: REVO Millrock Technology freeze dryer.

Looking at the main parts of the equipment, this consists of: (i) the lyophilization chamber, (ii) the vacuum pump, (iii) the condenser and (iv) the compressor for the refrigeration cycle.

Samples are loaded into the main chamber. Thermocouples are used to control the temperature of the product.

The vacuum pump is activated before starting the primary drying to reduce the pressure to the desired value. The condenser recovers the water eliminated by sublimation.

The first step in traditional freeze-drying is freezing, where the samples are loaded into the chamber and the temperature is lowered. In the case of SFD, however, freezing occurs externally together with atomization and liquid nitrogen. What is done, therefore, equipment is prepared in such a way that the temperature of the shelf is -50 °C before the vials are placed on the shelf, so that the frozen product does not thaw until the primary drying begins.

The frozen particles were then loaded into the freeze dryer. For the production of spray freeze-dried sucrose and mannitol formulations, the primary drying was conducted at a temperature of -20°C and 10°C, respectively. The chamber was kept under a vacuum of 10 Pa for sucrose and 20 Pa for mannitol formulations during freeze-drying.

The end of the primary drying was determined by using a Pirani vacuum gauge that is a thermo conductive type probe to detect the pressure in the chamber. This type of probe is calibrated on a composition in the chamber at 100% nitrogen, thus providing an incorrect measurement of the pressure in the chamber during the entire primary drying phase. When the primary drying is at its end point, the composition in the chamber changes from 100% aqueous vapor to 100% nitrogen, favouring a correct pressure measurement by the Pirani. When that happens, the ratio between the pressure measured by a Pirani probe and the pressure measured by a capacitance manometer tends towards unity and the primary drying end point is thus determined.

Secondary drying for both formulations was carried out at 20°C for 5 hours, keeping the pressure at the set value. At this stage, the bound water was removed.

To analyse the enzyme activity of each formulation at the same time after SFD, all formulations were freeze-dried in one cycle. In this case, the primary drying was set at -20°C for 10 Pa, taking into account the sucrose formulations. At the end of the entire process, the samples obtained were characterized by the physical state seen in the following Figures 2.5, 2.6, 2.7.

Figure 2.5: LDH formulation (left) and LDH + sucrose formulation (right) after SFD

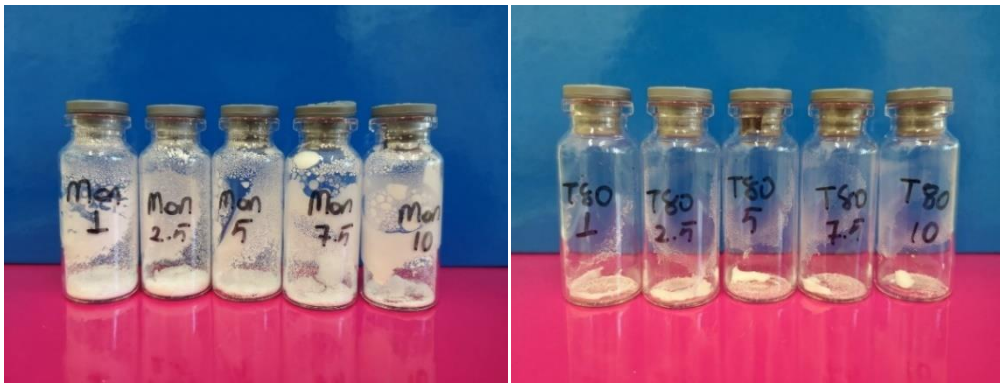

Figure 2.6: LDH + mannitol (left) and LDH + Tween 80 (right) formulations after SFD

Figure 2.7: LDH + Tween 80 + mannitol (left) and LDH + Tween 80 + sucrose (right) formulations after SFD

2.3 Enzyme Activity Assay and Characterization of the spray freeze-dried product

In this section, all the analyses that have been done on the samples will be illustrated. The main purpose of this work is to evaluate the enzymatic activity of LDH, after the SFD process. In addition to this, however, it was decided to proceed with the powder characterization obtained from solutions containing the excipients and the surfactant used for the activity assay: this is to understand how sucrose, mannitol, and tween 80 influenced the structure obtained and the link detected.

2.3.1 Enzyme Activity

LDH activity assay is carried out by exploiting the redox capacity of LDH, which allows the conversion of NAD into NADH, which is specifically detected by colorimetry analysis using a spectrophotometer at 450 nm.

For this analysis, it was used the L-Lactate Dehydrogenase Activity Assay Kit by Sigma-Aldrich which consists of LDH Assay Buffer, LDH Substrate Mix, NADH Standard 0,5 µmole, and LDH Positive Control.

To conduct this analysis, the concentration of LDH in solution must be decreased to 40 ng/ml. Before testing, the solutions were diluted from the initial concentration of 5 µg/ml to the desired value with a dilution factor equal to 127,5. This step was accomplished through two different dilution steps in buffer solution:

1. 0,5 ml of samples were diluted in 0,75 ml of buffer solution (A)
2. 0,2 ml of the previous dilution (A) were mixed in 10 ml of buffer solution (B)

When performing the dilution process, care must be taken during the mixing phase because LDH is extremely sensitive Mixing is done using a pipette, gently taking and releasing the sample into the buffer solution.

After the solutions were prepared and the desired concentration was reached, the next step was to prepare the 96-pots plate, which is then inserted into the spectrophotometer. The Plate used is reported in Figure 2.8.

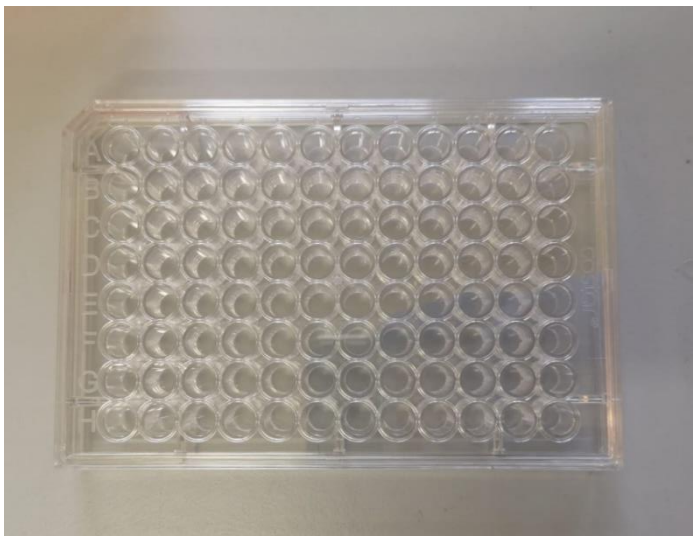

Figure 2.8: 96-pots plate used for enzymatic activity assay of LDH.

In the first row (A1, A2, A3, …, A12 pot) prepare the tare line:

1. Pots A1 e A2: 50 µl buffer (from reaction kit)
2. Pots A3 e A4: 2 µl NADH standard + 48 µl buffer
3. Pots A5 e A6: 4 µl NADH standard + 46 µl buffer
4. Pots A7 e A8: 6 µl NADH standard + 44 µl buffer
5. Pots A9 e A10: 8 µl NADH standard + 42 µl buffer
6. Pots A11 e A12: 10 µl NADH standard + 40 µl buffer

From B row onwards, the samples have been inserted. In particular, in B row is put the zero solution, that is a solution that has not undergone any process to have a reference.

The various samples were inserted starting from row C, filling 3 pots for each sample in order to have greater accuracy in the results. For each sample, zero solution included, are inserted in the pots 0,05 ml.

Tests on enzymatic activity were conducted using the Multiskan Sky spectrophotometer produced by Thermo Fisher Scientific illustrated in Figure 2.9

Figure 2.9: Multiskan Sky spectrophotometer by Thermo Fisher Scientific.

Once the plate is ready, it is necessary to prepare Multiskan Sky starting the incubation in such a way as to bring the internal temperature to 37°C, the temperature at which the analysis takes place.

In the meantime, the Master Kit Reaction is prepared in an Eppendorf: with a pipette take 60 µl of substrate mix and inject in the Eppendorf; also with a pipette, take 1,44 ml of buffer (the one from the reaction kit) and inject in the Eppendorf and mix gently. This is the standard amount that could be changed according to the amount required for the analysis, considering 50 µl of Kit for each pot.

It is important to pay attention when the Master Kit Reaction is put inside pots because the reaction from NAD to NADH is extremely fast: for this reason, the Kit is added to the samples using a multichannel pipette.

When the samples are ready, the plate is covered and put inside the Multiskan Sky spectrophotometer and the analysis starts. Generally, this test takes 30 minutes and the absorbance is measured every 5 minutes.

In the following table 2.3, all the data reported until now are summarized.

Table 2-3. Operative conditions of the enzymatic assay.

Operative conditions	
Sample volume	50 µl
Sample dilution factor	127,5
Reaction time	30 min
Temperature	37°C
Wavelength	450 nm
Master Kit Reaction volume (for each pot)	50 µl

The enzymatic activity percentage was calculated based on that obtained by analysing the zero solution, that is the solution that has not undergone any process.

The absolute value of NAD obtained from the conversion process from NADH by LDH was detected by the spectrophotometer. It was done the difference between the value obtained from the last measurement and that of the first measurement of the instrument. For each sample, three measurements were made, then the average between the three differences was made. Once this value was obtained, the ratio between it and the value obtained from the mean of the differences for the zero solution was made. From these results, the percentage was calculated in the way shown in the following.

$$\Delta A_{450} = A_{450\,(final)} - A_{450\,(initial)}$$

$$Residual\ enzymatic\ activity\ \% = \frac{\Delta A_{450,sample}}{\Delta A_{450,zero\ solution}}\ x\ 100$$

2.3.2 SEM

Scanning Electron Microscopy (SEM) is a test process that scans a sample with an electron beam to produce a magnified image to evaluate the morphology of the product; in this case, it is used to determine the dimension of the final particles after the process.

The particle size is mainly influenced by the atomization and the pore size by the freezing process at which ice crystals are formed, which are removed by means of sublimation, leaving pores behind. The SEM analysis is used to determine the morphology and the size of the final particles.

SEM analysis uses electron beams to investigate a sample at the nanoscale. The main types of signals detected are backscattered electrons (BSE), secondary electrons (SE) and X-ray, which are the most used.

The beam is generated by an electronic source that emits a flow of primary electrons concentrated by a series of electromagnetic lenses and deflected by an objective lens. The latter, in addition to further refocusing the beam, imposes a controlled deflection on it, so as to allow the scanning of areas of the sample. These electrons are captured by a special detector and converted into electrical impulses which are sent in real-time to a screen where a black and white image with high resolution and large depth of field is shown.

In the end, the particle size is measured directly on the images obtained.

In this work, SEM was performed through a desktop SEM Phenom XL at an accelerating voltage of 15 kV and different magnifications. The particles were coated with 8 nm platinum using a spray coating. To determine the geometric diameter of the particles, the SEM images were analysed using the ImageJ software.

In Figures 2.10, 2.11, and 2.12, examples of SEM images are reported. In particular, SEM images of formulations atomized at 5 mL/min are shown, those that contain, 5%w/w mannitol and 0,01%w/w Tween 80 and 5%w/w sucrose, 5%w/w mannitol, respectively.

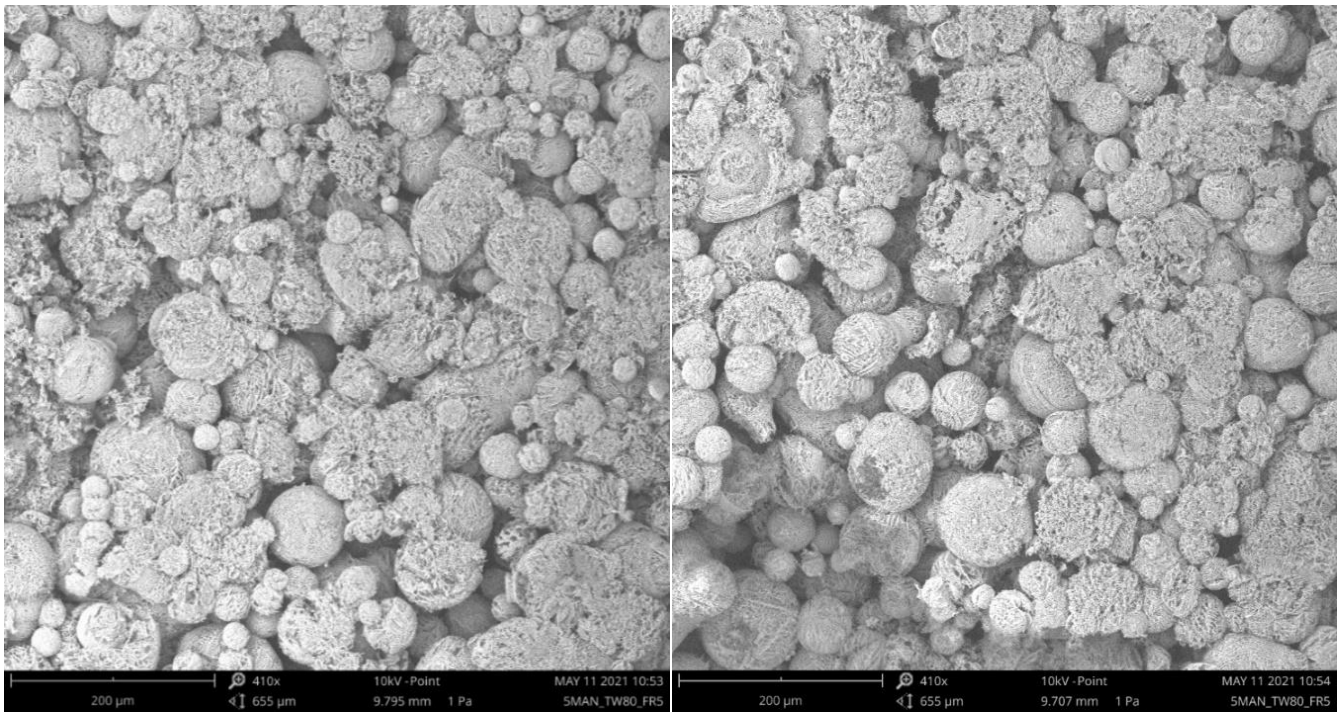

Figure 2.10: SEM image of SFD powder of 5% w/w mannitol with 0,01 %w/w Tween 80 formulation , atomized at 5 ml/min.

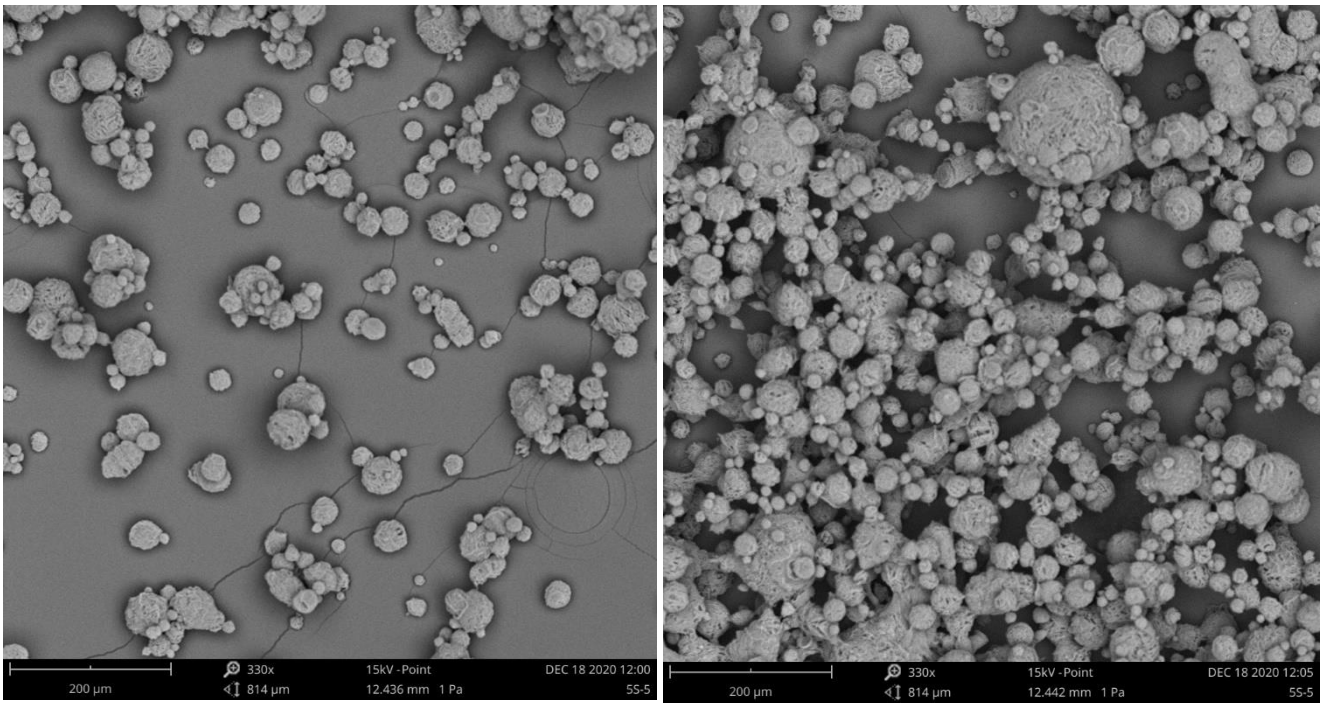

Figure 2.11: SEM image of SFD powder of 5% w/w sucrose formulation atomized at 5 ml/min.

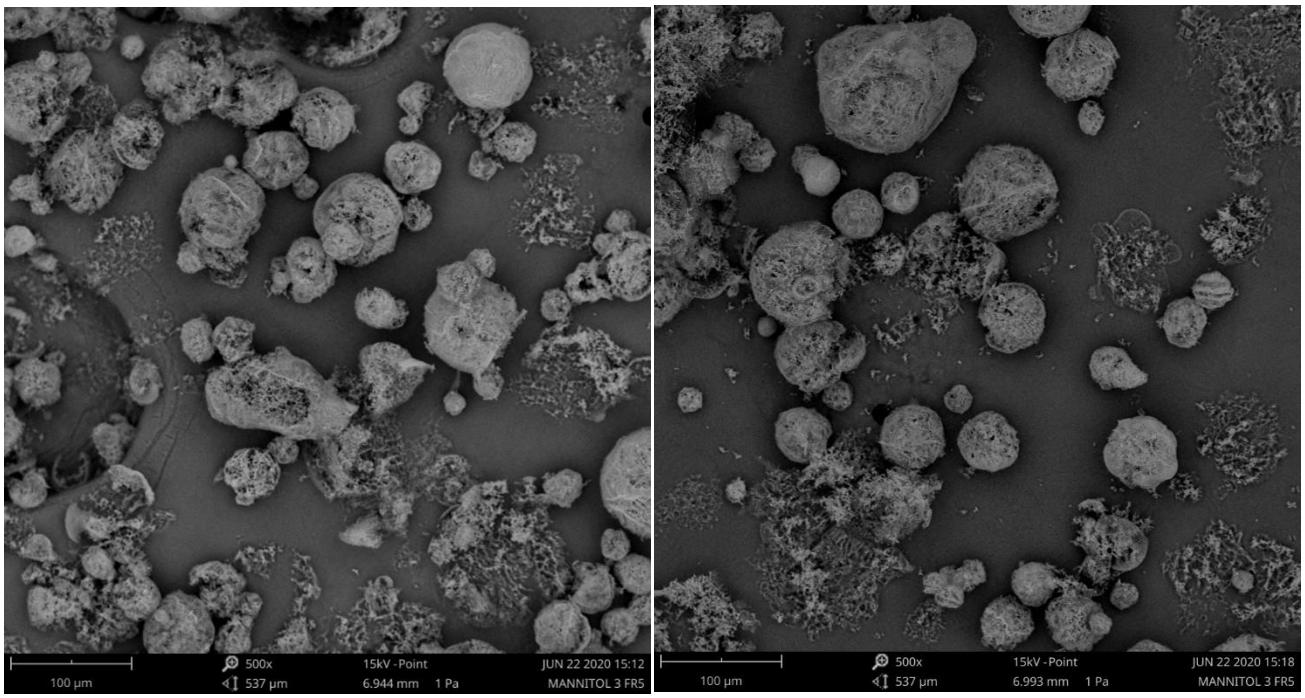

Figure 2.12: SEM image of SFD powder of 5% w/w mannitol formulation atomized at 5 ml/min.

The particles diameter of analysed formulations was determined from 200 particle counts of the SEM images using ImageJ software. An example of measurement is shown with the red arrow in Figure 2.13 The measurement was done six times for each particle.

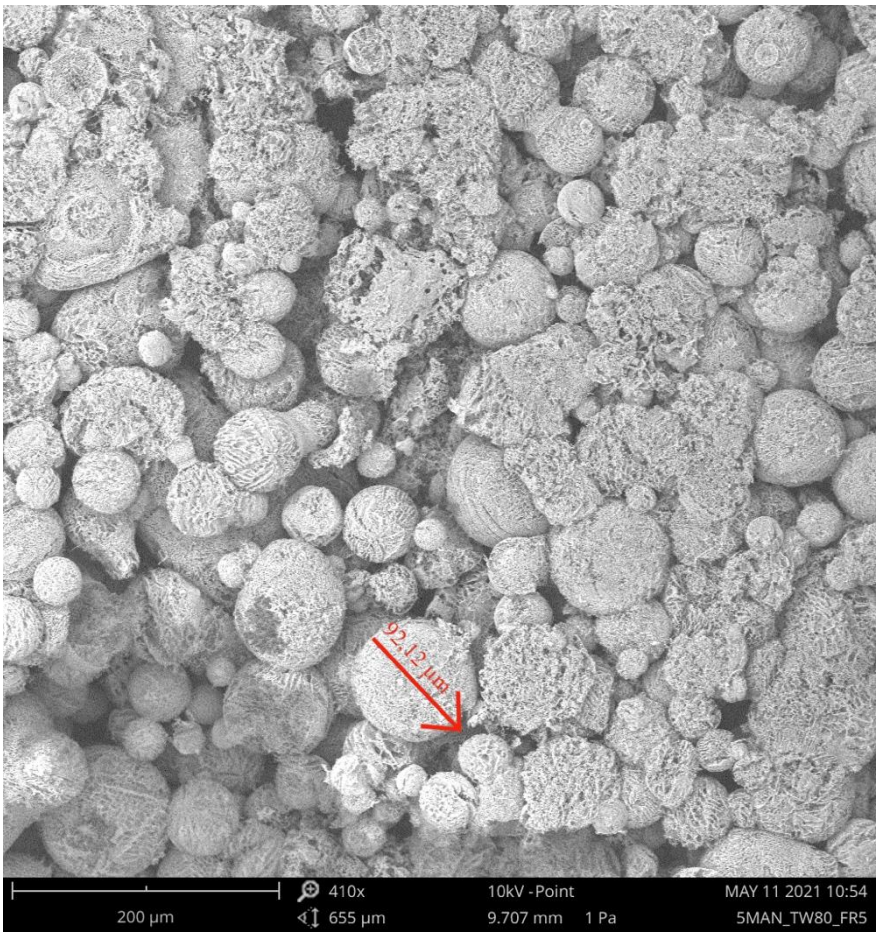

Figure 2.13: Example of measurement of the particles diameter on a SEM image of 5% w/w mannitol with Tween 80 at 0,1 %w/w, atomized at 5 ml/min

2.3.3 Specific surface area (BET)

BET theory is an abbreviation of the Brunner-Emmett-Teller theory used to measure the surface area of solid or porous materials

The BET analysis exploits the adsorption of a non-reactive gas on the surface of the powder in order to determine its surface area. In order to conduct the analysis, the solid material must be cooled using a cryogenic liquid. The temperature of the solid sample is kept constant, or under isothermal conditions, while the pressure or concentration of the adsorbent gas is increased.

As the relative pressure increases, the molecules adsorb on the surface forming a thin layer that will eventually cover the entire surface in a monolayer layer. Knowing the cross-sectional area of the adsorbate, it is possible to calculate the accessible surface area.

A Micromeritics ASAP 2020 apparatus was used, considering a sample of 200 mg of powder. The sample was loaded into the glass BET sample cell and then, the degassing

process was performed at 40 ° C for 3 hours. The nitrogen adsorption-desorption isotherms were measured at a temperature of 77 K over a relative pressure range of 0.05-0.30.

2.3.4 X-ray powder diffraction (XRD)

Mannitol has a high propensity to crystallize during lyophilization and the crystalline form provides structural support and elegance to the resulting cake. Also, recent studies have demonstrated that crystalline mannitol decreases the reconstitution time of high concentration lyophilized protein formulations. Instead, when held amorphously, mannitol can act as a cryoprotectant. Usually, mannitol crystallizes as a mixture of anhydrous polymorphous, depending on the formulation and the processing parameters. However, mannitol can also crystallize in such a way that two mannitol molecules associate with a water molecule form a hemihydrate. In this case, it could have implications on the performance of the product, so it is very important to analyse the final structure.

X-ray powder diffraction (XRD) is a technique used for the characterization of crystalline materials, identifying the atomic and molecular structure of a crystal. It is used only on crystalline materials, for this reason, this analysis was not carried out on formulations containing sucrose due to its amorphous nature.

During XRD analysis, an X-ray beam is directed towards a sample and the scattered intensity is measured as a function of the outgoing direction. The principle of operation of an X-ray diffractometer is based on Bragg reflection or diffraction.

X-rays are generated in a cathode ray tube by heating a filament to produce electrons, accelerating electrons to a target by applying a voltage, and bombarding the target material with electrons. When the electrons have enough energy to remove the electrons from the inner shell of the target material, characteristic X-ray spectra are produced. These X-rays are collimated and directed on the sample. When the geometry of the incident X-rays hitting the sample satisfies the Bragg equation, constructive interference occurs, and an intensity peak occurs. A detector records and processes this X-ray signal and converts the signal into a count rate which is then sent to a monitor.

The information that is obtained from a diffractometric measurement are essentially two: the angle for which the peak occurs diffraction of an X-ray beam incident on the surface and the width of the diffraction peak.

X-ray diffraction (XRD) patterns were characterized using Cu-Kα radiation with a wavelength of 1.54054 at 40 kV and 40 mA from an X-ray diffractometer. The sample powder was placed in a sample holder and scanned from 5 to 65 ° (2θ) at every 0.026 °.

2.3.5 Residual moisture content

The residual moisture (RM) content in the final product is important to ensure adequate shelf life. In general, there is a specific threshold for the RM value: if it is too high it causes chemical and physical degradation of the product, but at too low residual moisture content, degradation increases again and aggregation of the protein could start. During secondary drying, unfrozen water remaining in the glassy matrix is removed by isothermal desorption. The end point of the secondary drying is adjusted to achieve the desired RM.

RM was determined by automated Karl Fischer titration. Approximately 50-100 mg of powder was dissolved in 2-3 mL of Karl Fischer reagent and then injected into the Karl Fischer titrator cell to react with the titration reagent under magnetic stirring. Thanks to this

process, the final amount of water content was determined on the basis of the dried weight of the sample.

2.4 Statistical analysis

A statistical analysis was performed in order to be able to select the best and most significant results and evaluate the effective correlations between the analysed parameters

Minitab 17 software was used to find correlations between:

- The different atomization flow rates,
- The different formulations,
- The different steps of the entire SFD process.

The discriminating parameter to assess whether there is a significant difference between the compared results is the p value: if this value is greater than 0,05, it means that there is no significant difference between the compared values.

3. Results and conclusions

In this chapter, the activity losses of LDH at each different step and in the whole SFD process and morphology formation of particles during SFD were discussed. The purpose is to map the influence of the main stresses imposed on the LDH at each three steps.

After enzyme activity analysis, the results were analysed by one-way analysis of variance (ANOVA) to determine the optimum condition for performing particle characterization.

3.1 Enzyme activity assay

3.1.1 Stability of LDH after atomization

The atomization method is shown in section 2.2.1. To summarize briefly, an ultrasonic atomizer operating at a frequency of 60 kHz and a power of 3W was used by varying the feed flow rate (Table 2.2).

The atomization step is the one that determines the size of the final particles obtained. Evaluation of different flow rates during atomization showed how enzyme activity and particle size were affected in the final product. However, before performing a characterization on the finished product, it was investigated how the atomization step affects the enzymatic activity and therefore the amount of enzyme remaining after the single atomization treatment at the air-water interface.

The enzyme activity obtained as a result of different flow rates is given in Table 3-1.

Table 3-1 Residual enzymatic activity of LDH formulations after atomization step

	Residual enzymatic activity (%) of LDH formulations					
Flow rate [ml/min]	**LDH + buffer**	**LDH + buffer + sucrose**	**LDH + buffer + mannitol**	**LDH + buffer + tween 80**	**LDH + buffer + tween 80 + sucrose**	**LDH + buffer + tween 80 + mannitol**
1	24,56 ± 8,06 ^{c}	47,67 ± 6,36^{b}	23,89 ± 2,45^{c}	72,91 ± 2,52^{a}	68,44 ± 5,51^{a}	69,29 ± 3,44^{b}
2,5	35,30 ± 1,90^{bc}	74,22 ± 4,21^{a}	27,47 ± 3,48^{bc}	103,71 ± 7,46^{a}	61,38 ± 6,05^{a}	77,72 ± 6,39^{ab}
5	41,42 ± 2,75^{ab}	64,93 ± 3,57^{a}	24,93 ± 1,73^{c}	90,82 ± 4,67^{a}	61,97 ± 5,99^{a}	79,28 ± 3,09^{ab}
7,5	36,80 ± 2,54^{abc}	69,36 ± 2,84^{a}	33,33 ± 3,58^{ab}	98,48 ± 25,09^{a}	59,92 ± 6,54^{a}	86,83 ± 0,50^{a}
10	49,26 ± 6,39^{a}	64,49 ± 5,74^{a}	36,20 ± 2,69^{a}	86,52 ± 6,72^{a}	66,02 ± 8,04^{a}	89,56 ± 7,69^{a}

Each of the formulation excipients sucrose and mannitol, together with the surfactant Tween 80 had a clear and significant effect on the enzymatic activity of LDH (Figure 3.1)

At first glance, as expected, the loss of activity of LDH was found to be much lower when there was only LDH-containing formulation in the buffer solution, because the effect of the excipients or surfactant on stabilizing protein was not included.

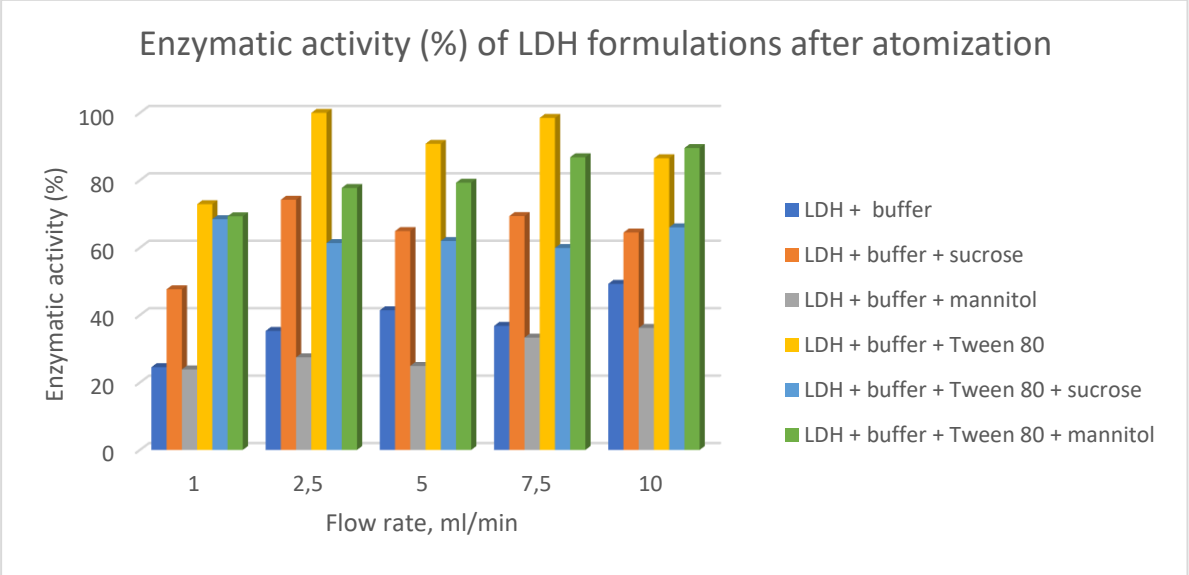

Figure 3.1: Residual enzymatic activity of each LDH formulation after the atomization step of the SFD process

In the case of a formulation containing mannitol, the results are similar to those with only LDH without excipients and/or surfactant, in fact this excipient is more beneficial in the freezing phase by acting as a cryoprotectant. There is an improvement when looking at formulations with sucrose instead, as it is a stabilizer for the solution.

Considering the formulations containing Tween 80, it was observed that the enzyme activity was preserved to a great extent. During atomization, the splitting of the liquid into many small particles increases the air-liquid interface. Tween 80 preserves the enzyme thanks to its capacity to accumulate at the air-liquid interface, which is typical of a surfactant, guaranteeing a much higher recovery of enzymatic activity than formulations that do not contain it.

Looking at the effect of flow rate, it is seen that enzyme activity is higher at higher flow rates, especially for 10 ml/min. This could be explained by the lower ultrasonic vibration energy to which the feed solution is exposed in case of higher flow rates. Because when the flow rate/frequency value is low, the ultrasonic vibration energy increases and causes more mechanical stress, which has a greater negative impact on enzyme activity.

A statistical analysis was carried out in order to clarify if there were significant differences among flow rates and formulations. In table 3-1, letters are reported from a to c indicate within each column whether there are significant differences between results for each flow rate.

As a result of the statistical analysis, it was seen that there was no significant difference ($p>0.05$) among the flow rates for only the two formulations, which are Tween 80 and Tween 80 plus sucrose. This result is due to the fact that the formulations in question are those that present the best results in terms of enzymatic activity, therefore it is possible that having obtained a high protection of LDH, the latter is not sensitive to variations in flow rate. Also, for formulation containing mannitol and Tween 80, even if there is a difference, this is not so huge.

In terms of the effect of atomization on different formulations, statistically, LDH formulation containing tween 80 without excipients was found to be the best formulation to preserve enzyme activity ($p<0.05$).

3.1.2 Stability of LDH after freeze/thaw

The freezing step was accomplished by spraying the liquid into the steam over the liquid nitrogen in the vials. This stage can be considered the most important stage because it is the stage where ice crystals form and phase separation occur. It should also be noted that after freezing, the frozen product was thawed in a water bath.

Table 3-2 reports the enzyme activity of LDH for different formulations at different flow rates after freeze/thaw.

Table 3-2 Residual enzymatic activity after atomization and freeze/thaw steps

	Residual enzymatic activity (%) of LDH formulations					
Flow rate [ml/min]	**LDH + buffer**	**LDH + buffer + sucrose**	**LDH + buffer + mannitol**	**LDH + buffer + tween 80**	**LDH + buffer + tween 80 + sucrose**	**LDH + buffer + tween 80 + mannitol**
1	$0,34 \pm 0,17^{c}$	$29,49 \pm 0,58^{c}$	$53,78 \pm 5,59^{bc}$	$45,76 \pm 1,07^{d}$	$62,78 \pm 2,28^{c}$	$99,77 \pm 3,39^{a}$
2,5	$3,36 \pm 0,12^{a}$	$57,52 \pm 2,02^{a}$	$56,05 \pm 2,52^{a}$	$64,92 \pm 2,44^{b}$	$70,95 \pm 1,26^{bc}$	$98,49 \pm 7,98^{a}$
5	$1,64 \pm 1,22^{bc}$	$24,49 \pm 1,12^{c}$	$64,44 \pm 3,06^{ab}$	$58,06 \pm 1,46^{c}$	$90,25 \pm 5,25^{a}$	$104,92 \pm 2,78^{a}$
7,5	$2,21 \pm 0,17^{ab}$	$23,82 \pm 1,54^{c}$	$64,78 \pm 8,97^{bc}$	$63,89 \pm 0,49^{b}$	$73,35 \pm 2,19^{bc}$	$90,04 \pm 24,79^{a}$
10	$0,83 \pm 0,17^{bc}$	$51,07 \pm 4,38^{b}$	$59,45 \pm 1,28^{c}$	$72,84 \pm 3,12^{a}$	$82,06 \pm 7,13^{ab}$	$98,37 \pm 4,21^{a}$

Freeze/thaw resulted in a significant reduction in LDH activity in the LDH formulation without excipients and/or Tween 80, values near to zero.

During the freezing step, the presence of Tween 80 is not so relevant: it always preserves the enzyme activity during atomization but has no effect during freezing, in fact formulations containing tween 80 showed minor differences compared to the atomization step.

As can be seen in Figure 3.2, sucrose and mannitol play key roles in the freeze/thaw step. They are added to the formulations in order to act as cryoprotectants.

In particular, sucrose protects the enzyme during freezing by a mechanism called water replacement: During the formation of hydrogen bonds with protein, sucrose molecules replace water; in this way, when water separation occurs due to the formation of ice crystals, the protein does not tend to rearrange its structure as it binds with sucrose and not water.

The action of mannitol, on the other hand, is a little different and is linked to its ability to crystallize during freezing. The mannitol crystals provide a "kinetic" barrier to phase separation, thus inhibiting the phase separation phenomena that could occur during freezing, which would lead to denaturation. In other words, mannitol reduces the molecular mobility of proteins, stopping their tendency to modify their structure.

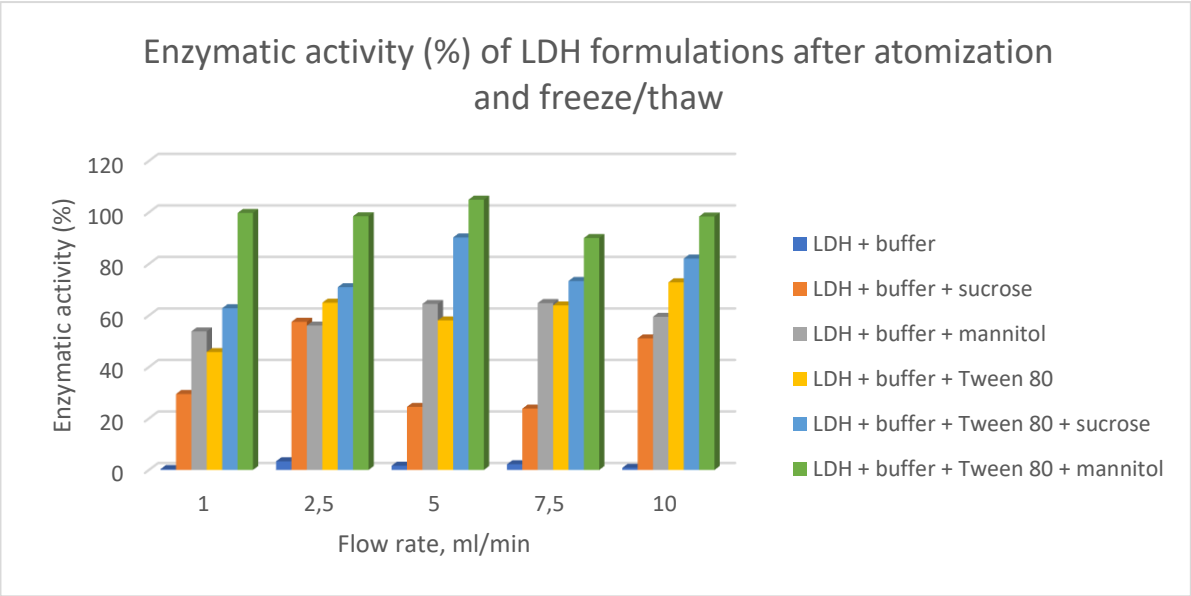

Figure 3.2: Residual enzymatic activity of each LDH formulation after the atomization and freeze/thaw steps of the SFD process

As is shown in Table 3-2, a statistical analysis was carried out in the freeze/thaw step as well. As in the atomization step, the letters from a to d indicate the differences among the results of each column.

The only formulation did not differ in the results is depending on the flow rate was the formulation containing LDH with mannitol and Tween 80. It is also possible to assume that a high degree of protection has been achieved during the whole process, as the solution is insensitive to the effect of the atomization flow rate.

In addition, the formulation containing mannitol and Tween 80 was found to be better than other formulations statistically as the formulation that best preserved the enzyme activity.

3.1.3 Stability of LDH after spray freeze-drying

The final part of determining the critical steps applied to LDH was the effect of full spray freeze-drying on enzyme activity.

The following results show that the SFD process completely destroys the enzyme if there are no excipients and or surfactants. In Table 3-3, the results are summarized. A statistical analysis was carried out and the results are shown with a letter from a to c and indicate whether there are relevant differences between the results of each column.

Table 3-3 Residual enzymatic activity of LDH formulations after SFD process

Residual enzymatic activity (%) of LDH formulations						
Flow rate [ml/min]	**LDH + buffer**	**LDH + buffer + sucrose**	**LDH + buffer + mannitol**	**LDH + buffer + tween 80**	**LDH + buffer + tween 80 + sucrose**	**LDH + buffer + tween 80 + mannitol**
1	1,82 ± 0,10^{b}	4,34 ± 1,23^{b}	0,33 ± 0,06^{b}	12,61 ± 0,53^{b}	60,82 ± 8,53^{a}	70,53 ± 3,13^{b}
2,5	2,23 ± 0,12^{a}	5,90 ± 6,50^{ab}	0,37 ± 0,13^{b}	16,47 ± 0,57^{ab}	91,75 ± 6,82^{a}	89,66 ± 3,73^{ab}
5	0,87 ± 0,06^{c}	2,10 ± 1,36^{b}	0,90 ± 0,21^{a}	15,79 ± 1,35^{ab}	67,30 ± 1,98^{a}	104,12 ± 11,18^{a}
7,5	0,71 ± 0,19^{cd}	5,97 ± 0,53^{ab}	0,21 ± 0,04^{b}	15,20 ± 2,55^{ab}	90,00 ± 2,80^{a}	64,24 ± 10,63^{b}
10	0,46 ± 0,10^{d}	14,00 ± 2,31^{a}	0,22 ± 0,06^{b}	16,82 ± 1,20^{a}	97,40 ± 40,61^{a}	91,79 ± 17,67^{ab}

Formulations containing only LDH and containing either mannitol or sucrose showed significantly the lowest enzyme activity compared to the other formulations ($p<0.05$).

The results for formulation containing only Tween 80 are slightly better than those previously described, but the real improvement comes with the combination of Tween 80 with excipients, both sucrose and mannitol.

This can be explained if we consider the nature of Tween 80 and what its mechanism of action is. During the dehydration process, the protein undergoes thermal denaturation, which does not change its secondary structure, but exposes functional groups on the surface, which are otherwise confined within the protein structure, which can promote the aggregation of more protein molecules. The presence of a hydrophobic segment in the surfactant allows the proteins to bind to the hydrophobic sites to which they are exposed on the surface during denaturation, thus protecting it from self-assembly. Tween 80 absorbs abnormally exposed hydrophobic regions of unfolded proteins and alters local surface tension to facilitate protein degradation (Ji, Sun, Yu, Wang, Zheng, Wang, Niu; 2009).

Therefore, in the light of the above, the formulations containing either sucrose or mannitol with Tween 80 give the best results, thanks to the action of the surfactant in the atomization and drying steps and excipients in the freezing step.

In figure 3.3, the significant differences ($p<0.05$) among the formulations are better highlighted.

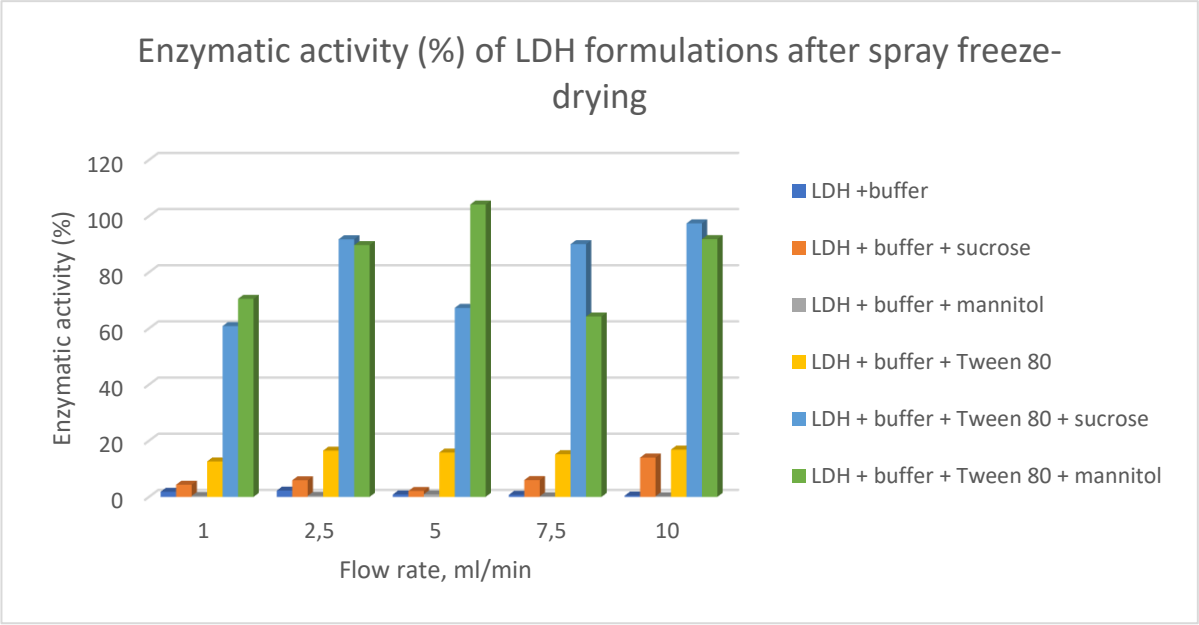

Figure 3.3: Residual enzymatic activity of each LDH formulation after the SFD process

From the statistical analysis, the formulation of sucrose with Tween 80 was not affected by the different flow rates ($p>0.05$), among the formulations, the best was the formulation containing mannitol and Tween 80 ($p<0.05$).

3.2 Particles characterization

Considering the characterization of the particles, the analyses performed are: SEM, BET, XRD and residual moisture content. These analyses were performed only for formulations atomized at 5 ml/min: this selection was made in light of the fact that it was the flow rate that gave the best results for all formulations from the statistical analysis of the enzyme activity result. Particles size is important because this parameter impacts the amount of encapsulated drug possible and the availability of release immediately upon hydration: it is, therefore, desirable to minimize particle size.

The results obtained from the SEM analysis are summarized in Figure 3.4, 3.5, and 3.6.

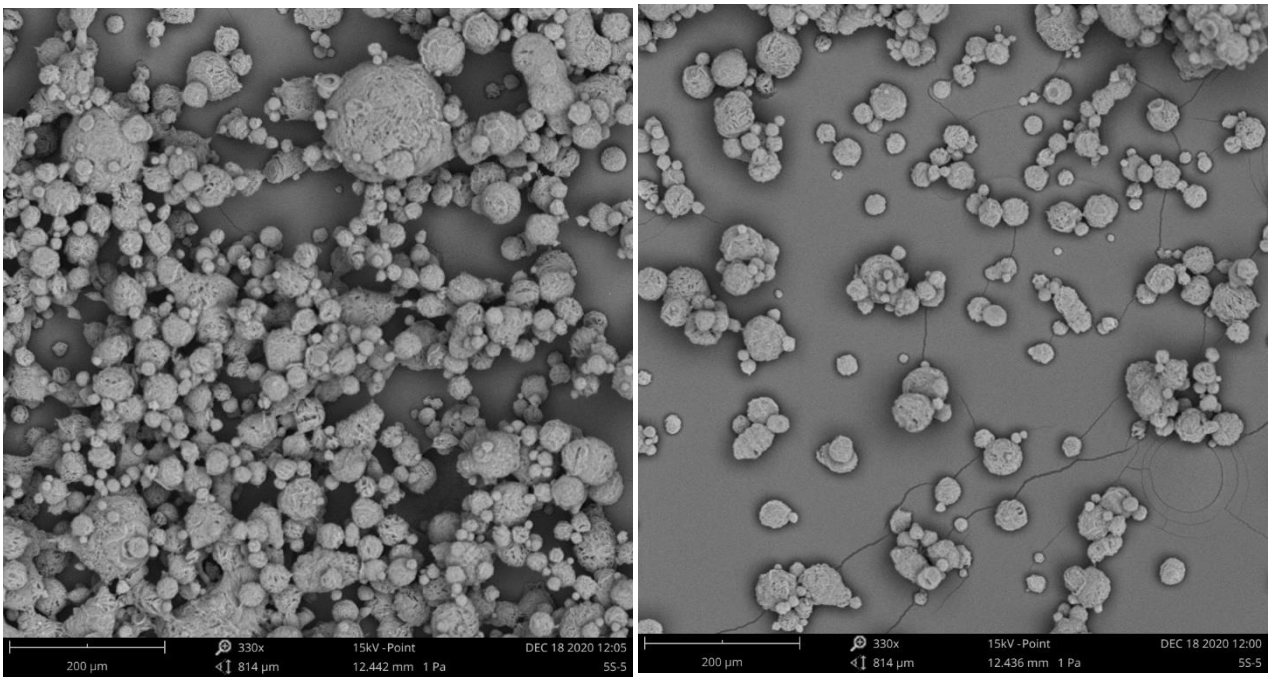

Figure 3.4: SEM images of a solution containing 5%w/w sucrose, atomized at 5 ml/min.

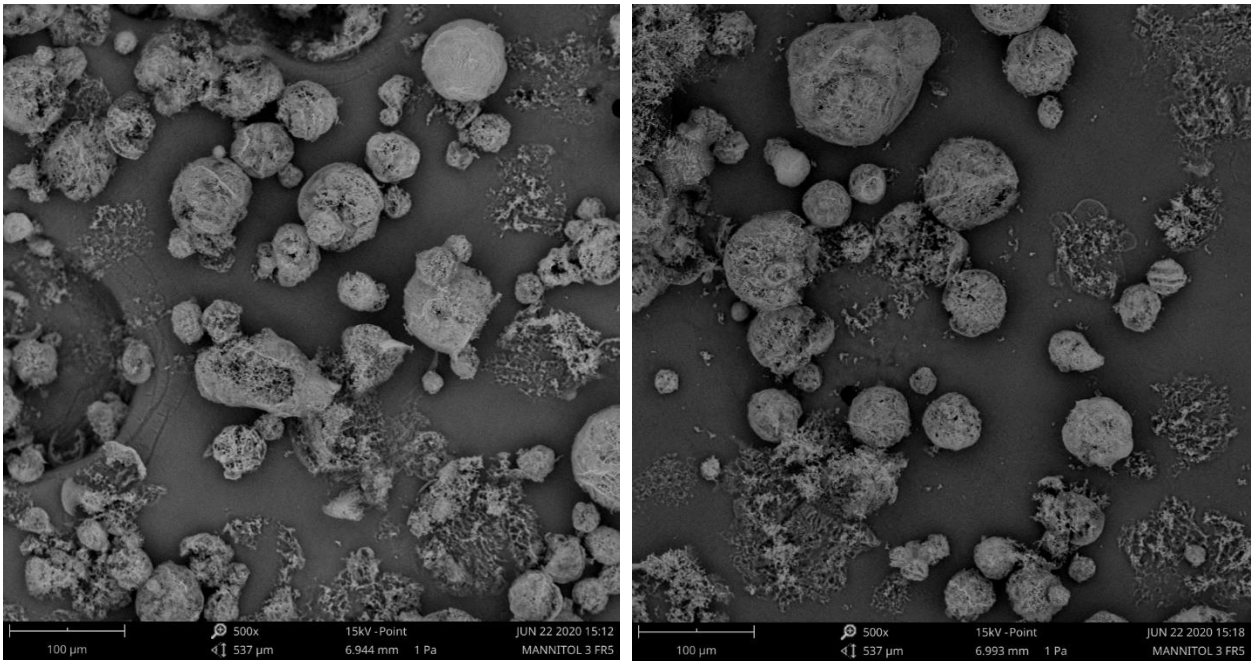

Figure 3.5: SEM images of a solution containing 5%w/w mannitol, atomized at 5 ml/min.

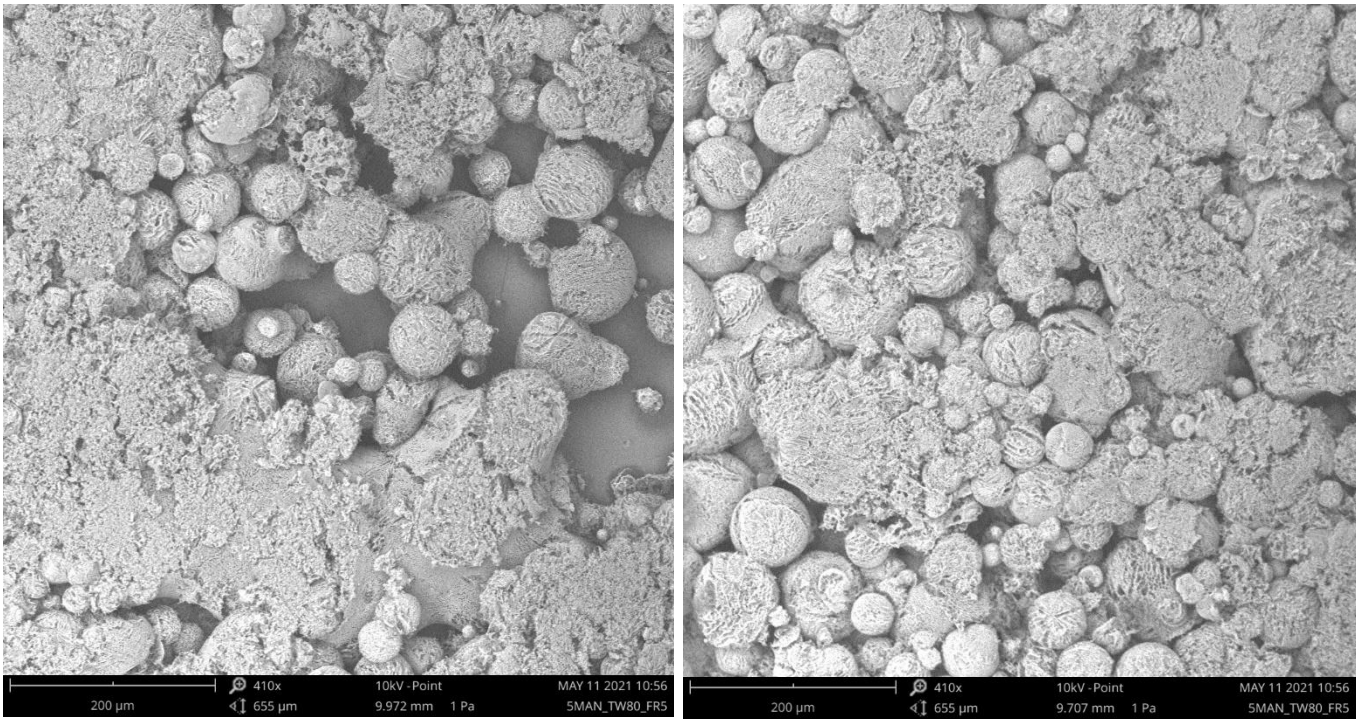

Figure 3.6: SEM images of a solution containing 5%w/w mannitol and 0,1%w/w Tween 80, atomized at 5 ml/min.

The particles were mainly spherical. When they seem destroyed, this can be attributed to the particle deformation that took place during sample preparation to conduct the analysis. In the case of formulations with Tween 80 there are more particles, with a more spherical shape. Also, the particles obtained with Tween 80 are larger than that with sucrose and mannitol.

An average diameter was calculated for each formulation and the results are shown in Table 3-4, while the results in terms of particles size distribution are reported in Figure 3.7, 3.8, and 3.9.

The formulation containing sucrose gives the smallest value in terms of particle size, followed by mannitol. Thanks to its amorphous structure, sucrose prevents the formation of larger particles during atomization by giving a water exchange mechanism.

Table 3-4: Mean diameter results from SEM analysis

Formulation	Mean diameter (µm)
5%w/w sucrose	21,88 ± 13,25
5%w/w mannitol	32,48 ± 19,25
5%w/w sucrose + 0,1%w/w Tween 80	-
5%w/w mannitol + 0,1%w/w Tween 80	61,90 ± 22,36

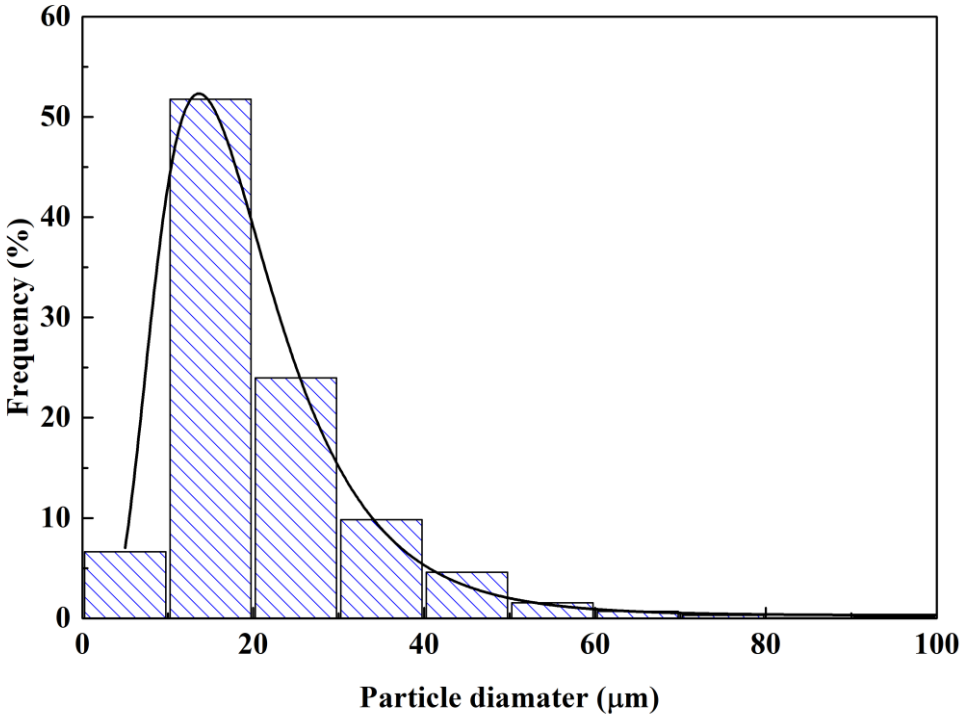

Figure 3.7: Particle size distribution of 5%w/w sucrose at 5ml/min.

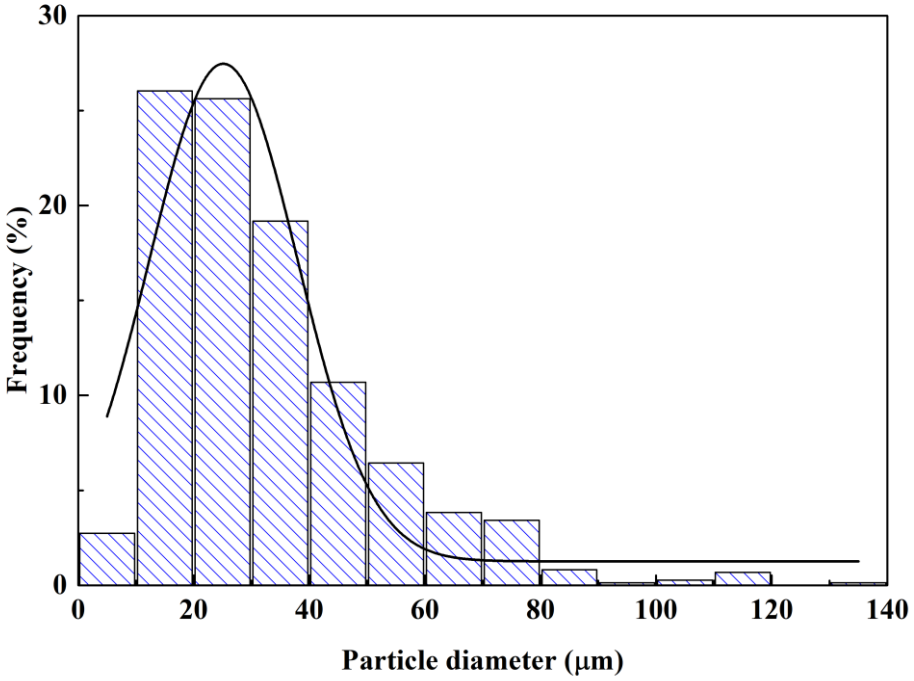

Figure 3.8: Particle size distribution of 5%w/w mannitol at 5 ml/min.

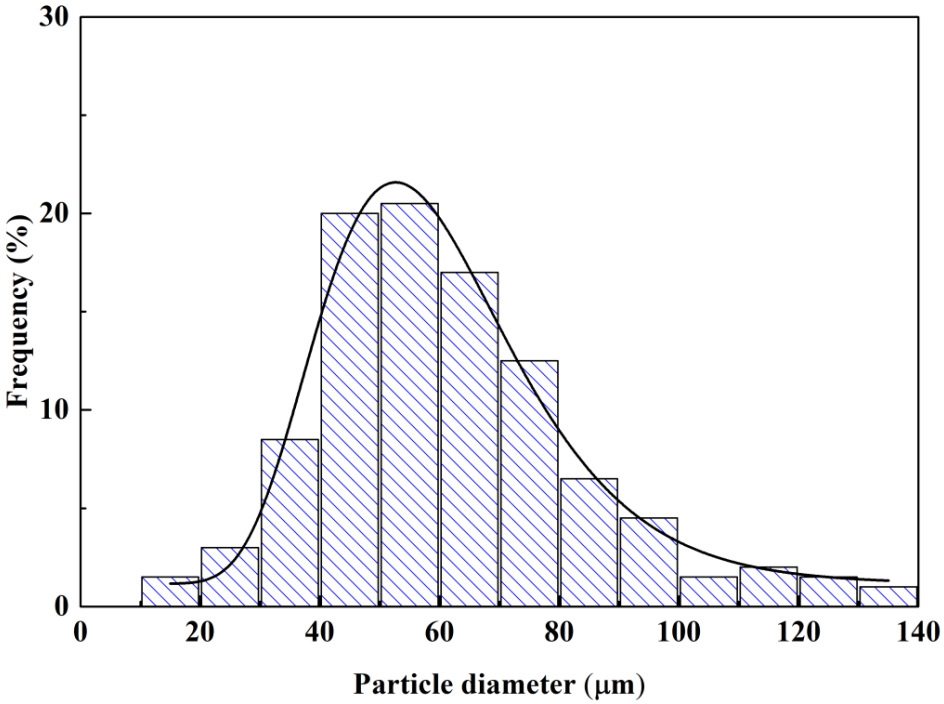

Figure 3.9: Particle size distribution of 5%w/w mannitol and 0,1%w/w Tween 80 at 5 ml/min.

The formulation containing sucrose and Tween 80 atomized at 5 mL/min was not analysed by SEM because the powder melted immediately as in contact with the air.

In general, sucrose tends to cause problems during drying due to its natural stickiness, but, this effect is enhanced in presence of Tween 80. It can be thought that the reason for this behaviour lies in the effect of the surfactants that modify the surface tension of the solution. In fact, a study has shown that the difference in surface tension of a solution containing sucrose is not so relevant with and without Tween 80. In this case, the kinetics of the motion rather than the equilibrium surface tension play a dominant role. Since Tween-80 has a much higher diffusion than protein, it can move to the surface much faster (Adhikari, Howes, Wood, Bhandari, 2009).

Another important parameter for particle characterization is the specific surface area. As can be seen in Table 3-5, formulations containing mannitol achieve the best results, while sucrose tends to restore non-porous structures, resulting in a low specific surface area.

Mannitol, plays a crucial role in the rearrangement of the structure during its crystallization process: in particular, it increases the porosity of product and therefore the specific surface area.

Table 3-5: Specific surface area from BET analysis

Formulation	Specific surface area (m^2/g)
5%w/w sucrose	1,37 ± 0,02
5%w/w mannitol	7,53 ± 0,05
5%w/w sucrose + 0,1%w/w Tween 80	1.05± 0.02
5%w/w mannitol + 0,1%w/w Tween 80	9.84 ± 0.03

The residual moisture content is representative of how effective freeze-drying has been. Usually, a good value for spray freeze-dried product is around 3%w/w (Mutukuri, Wilson, Taylor, Topp, Zhou, 2020); about this work we were able to obtain the smallest value for mannitol formulations, around 2%w/w, like is summarized in Table 3-6.

Table 3-6: Residual moisture content

Formulation	Moisture content (%)
5%w/w sucrose	6,08 ± 0,24
5%w/w mannitol	1,81 ± 0,24
5%w/w sucrose + 0,1%w/w Tween 80	5,83 ± 0,14
5%w/w mannitol + 0,1%w/w Tween 80	2,52 ± 1,79

From the results obtained in terms of residual moisture content it is clear that formulations containing mannitol give the best results, this may be due to the fact that the excipients have a greater capacity to retain water if in an amorphous state, less so in the case of crystalline structure.

The decrease in moisture content with Tween 80 found in the case of formulations with sucrose, on the other hand, can be attributed to the hydrophobic interactions of the surfactant. If mannitol is present, the predominant effect derives from the behaviour of the latter.

The spectra obtained from XRD are shown in Figure 3.10. This type of analysis was conducted only on formulations containing mannitol, atomized at 5 ml/min.

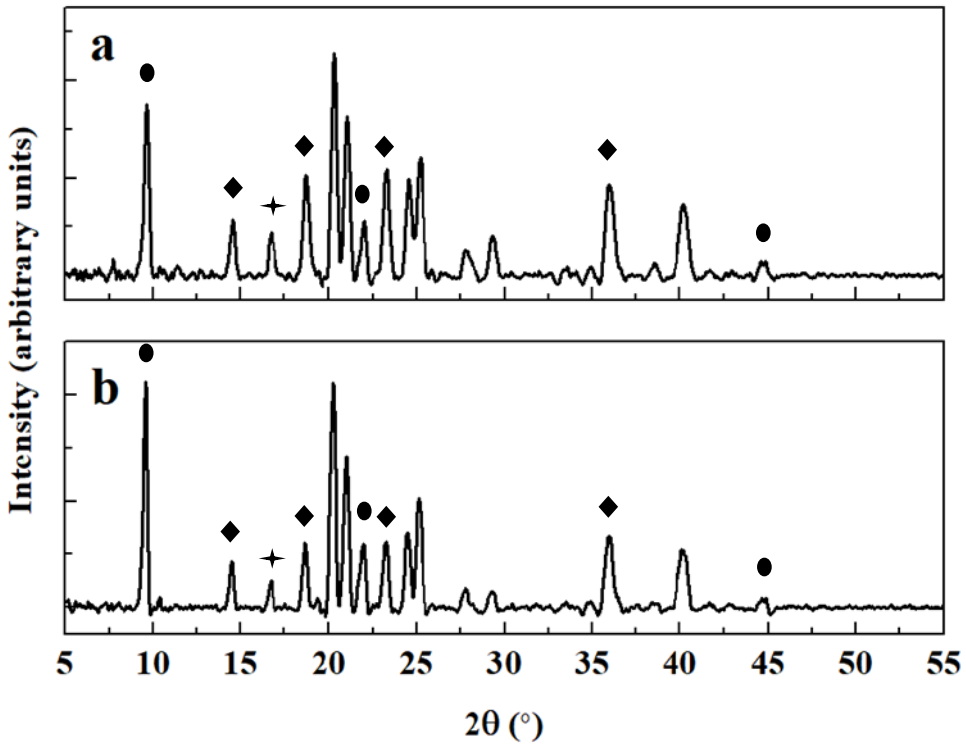

Figure 3.10: XRD results of formulations: a) 5%w/w mannitol and b) 5%w/w mannitol and 0,1%w/w Tween 80.

The symbols ● and ◆ represent, respectively, the polymorphous δ and β forms, while the symbol ✦ represents the hemihydrate form.

From Figure 3.10, it is possible to note that Tween 80 did not affect the crystallization of mannitol, since the same peaks were detected in the mannitol formulation without Tween 80. In particular, it can be seen that mannitol crystallizes in the δ form for values approximately equal to 10, 22, and 45 °, while it has peaks at approximately 15, 19, 27, and 35 ° representing a crystallization in the β form. For values around 14 °, the hemihydrate form of mannitol was observed.

3.3 Process performances

If we want to have an overview of the results obtained from all the analyses conducted, we could certainly say that LDH must be placed in formulations containing excipients and/or surfactants to maintain the highest possible enzyme activity during spray freeze-drying process.

The analyses on the enzymatic activity are the ones that gave us the most important results, the basis on which the characterization of the final product was then conducted.

Generally, the denaturation encountered when processing proteins are of two types: interface denaturation or bulk denaturation. This study demonstrates how the combination of a surfactant with an excipient, can overcome these types of problems.

Thanks to the Tween 80 as a surfactant, most of the denaturation at the interface is blocked, guaranteeing a high level of protection of the enzymatic activity in the atomization and freeze-drying steps. On the other hand, sucrose and mannitol, allow avoiding denaturation in the bulk, resulting for example, from cryo-concentration during freezing.

In the light of what has been said, it is evident that the combination of Tween 80 and sucrose/mannitol is the best formulation to use in spray freeze-drying.

Following the characterization of the final product, it was found that the presence of Tween 80 did not influence the structure of the final structure. This, therefore, leads us to decisions that will fall into the choosing the sucrose or mannitol-based formulations while keeping the presence of surfactant in solution constant. while maintaining constant, however, the presence of surfactant in solution.

Considering the results obtained, the smallest particle size and the lowest specific surface area were obtained in the sucrose-based formulation compared to the mannitol-based formulations.

3.4 Conclusions

Spray freeze-drying is a revolutionary process that combines the characteristic advantages of spray drying and freeze-drying techniques. It is the most suitable process for producing powdered foods or pharmaceuticals, ensuring a high level of control over the size and porosity of the final product.

Thanks to the versatility of the operating conditions and the equipment used, it allows to obtain a wide range of products. This work showed that each different processing step in spray freeze-drying can result in a loss in protein activity. In this study, an ultrasonic atomizer and spray freeze-drying into vapor over a liquid nitrogen technique were used, but other combinations could be used to study new operating conditions and obtain different product properties. Different formulations and flow rates were studied to see their effects on enzyme activity. It is possible to assume that the best formulation was that with mannitol and Tween 80: this formulation gives the best results of enzymatic activity mainly at 5ml/min. In terms of particles characterization, the combination of mannitol and a surfactant, results in an average mean diameter of 61,90 μm, a specific surface area equal to 9,84 m^2/g and a residual moisture content of 2,52 %.

In conclusion, spray freeze drying is a complete and extremely versatile process that could revolutionize the “modus operandi” of the pharmaceutical industry known so far.

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