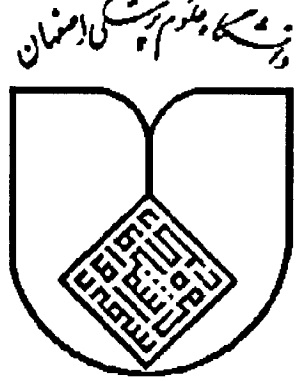

Isfahan University of Medical Sciences and Health Services

Faculty of Pharmacy and Pharmaceutical Sciences Research Centre

"Department of Pharmaceutical Biotechnology"

A Thesis for obtaining the Degree of Doctorate in Pharmacy (Pharm.D.)

Project Number: 384171

Title:

Immobilization of CTAB treated whole cells of *E.coli* 11105 using Chitosan

Supervisors:

Dr. Dariush Abedi (Ph.D)

Dr. Naser Tavakoli (Ph.D)

Dr. Hasan Korbekandi (Ph.D)

Written By:

Mohammad Reza Bagherinejad

September 2009

List of Contents

Dedication.....II

Acknowledgment.....III

Contents.....IV

List of Tables.....X

List of Diagrams.....XI

List of Figures.....XII

List of Abbreviation.....XIV

Abstract.....1

1. Introduction.....3

1.1.Biocatalysts and Industry.....4

1.2.Penicillin acylase.....5

1.3.Penicillin G acylase.....6

1.4.Catalytic mechanism.....8

1.5.Permeabilized cell.....10

1.6.Enzyme immobilization.....11

1.7.Immobilization methods.....14

1.7.1. Cross-linking method.....16

1.7.2. Entrapment method.....18

1.8.Chitosan.....18

1.9.Chitosan gel preparation.....23

1.9.1. Solvent evaporation method.....23

1.9.2. Neutralization method.....23

1.9.3. Cross-linking method.....24

1.9.4. Ionic gelatin method.....24

1.10. Previous studies.....25

1.11. Objectives.....26

2. Materials and Methods.....27

2.1.Materials and instrument.....28

2.1.1. Materials.....28

2.1.2. Instruments.....30

2.2.Reagents and Solutions.....31

2.2.1. 6-APA standard solutions.....31

2.2.2. Biomass standard solutions.....31

2.2.3. *ρ*-Dimethyl amino benzaldehyde (*ρ*-DBA) solution.....32

2.2.4. Penicillin G potassium solution.....32

2.2.5. Phosphate buffer solutions.....32

2.3.Microorganism.....33

2.4.Medium.....33

2.5.Fermentation.....34

2.6.Cells growth and Enzyme production.....35

2.7.Treatment with N-cetyl-N, N, N-trimethyl ammonium bromide.....36

2.8.Preparation of chitosan beads.....36

2.9.Effect of medium pH on loading of the biocatalyst.....38

2.10. Hydrolytic activity assay.....38

2.11. Conversion calculating.....39

2.12. Morphological characterization.....39

2.13. Operational stability.....40

2.14. Statistical data analysis.....40

3. Results.....41

3.1.Standard Graph of 6-APA.....42

3.2.Biomass Standard Graph.....43

3.3.Growth and Enzyme production curve during shaking flask fermentation.....44

3.4.Effect of CTAB concentration on cell permeabilization.....46

3.5.Effect of Glutaraldehyde concentration on cell immobilization47

3.6.Beads size and shape.....48

3.7.Characterization of chitosan-immobilized biocatalysts.....50

3.8.Effect of pH on loading of the biocatalyst.....52

3.9.Statistical analysis.....53

3.10. Hydrolysis activity of different forms of the biocatalyst...55

3.10.1. Hydrolytic activity of intact cells.....56

3.10.2. Hydrolytic activity of treated cell with CTAB.....57

3.10.3. Hydrolytic activity of the cells immobilized using NaOH-Methanol method 58

3.10.4. Hydrolytic activity of the cells immobilized using glutaraldehyde...59

3.11. Operational stability.....60

4. Discussion.....61

4.1.Cell culture.....62

4.2.Effect of CTAB concentrations on cells activity.....63

4.3.Effect of glutaraldehyde concentration on treated *E.coli* cells immobilization.....65

4.4.Effect of chitosan solution concentrations on beads preparation.....68

4.5.Beads preparation.....69

4.6.Morphological study.....69

4.7.Effect of pH on immobilization of *E.coli* cells in chitosan.....71

4.8.Operational stability.....72

4.9.Hydrolytic activity.....73

4.10. Conclusion.....78

4.11. Recommendation for future work.....79

5. REFERENCES.....80

6. Appendix.....86

6.1.Appendix 1.....87

6.2.Appendix 2.....91

6.3.Appendix 3.....95

6.4.Appendix 4.....97

7. Farsi Abstract.....100

List of Tables

Table 1.1. Some of the more important medicinal applications of immobilized enzyme systems.....13

Table 1.2. A summary of studies on immobilization of different biocatalysts.....25

Table 2.1. Materials used to produce treated *E.coli* cells immobilized in chitosan.27

Table 2.2. Instruments used to produce treated *E.coli* cells in chitosan.....29

Table 2.3. The optimized medium for maximum production of PGA in *E.coli* cells.....32

Table 3.1. Homogeneous subsets of effect of CTAB concentration data.....53

Table 3.2. Homogeneous subsets of effect of glutaraldehyde concentration data...54

Table 3.3. Homogeneous subsets of effect of pH on loading biocatalyst data.....54

List of Diagrams

Figure 3.1. Standard values of 6-APA.....40

Figure 3.2. Biomass standard values of *E .coli* 11105.....41

Figure 3.3. Time course of the *E.coli* cells growth.....42

Figure 3.4. Enzyme production time course during *E.coli* cells growth.....43

Figure 3.5. Effect of CTAB concentrations on activity of biocatalyst.....44

Fig 3.6. Effect of glutaraldehyde concentrations on biocatalyst loading.....45

Figure 3.11. Effect of pH on loaded biocatalyst.....50

Figure 3.12. Conversions of intact cells.....52

Figure 3.13. Conversions of treated cells.....53

Figure 3.14. Conversions of beads prepared using NaOH:Methanol (80/20).....54

Figure 3.15. Conversions of beads prepared using glutaraldehyde.....55

Figure 3.16. Operational stability of beads immobilized using glutaraldeyde and CTAB-treated *E.coli* cells.....56

List of Figures

Figure 1.1 differences between the action of penicillin acylase and *β*-lactamase....6

Figure 1.2 post-translational processing of penicillin acylase.....8

Figure1.3. Proposed mechanism of penicillin acylase hydrolytic activity.....9

Figure1.4. Schematic diagrams of different forms of immobilized enzymes.....16

Figure 1.5. Structure of chitin, chitosan and cellulose.....20

Figure 2.1. The bioreactior which was used for *E.coli* 11105 cells growth.....33

Figure 3.2. The procedures used for producing beads.....35

Figure 3.7. Beads prepared using glutaraldehyde without magnification.....47

Figure 3.8. Treated cells beads without using glutaraldehyde.....47

Figure 3.9. SEM from white beads prepared by NaOH-Methanol without glutaraldehyde.....49

Figure 3.10. SEM from brown beads prepared by using glutaraldehyde.....49

Figure 5.1. SEM from brown beads prepared using glutaraldehyde.....84

Figure 5.2. SEM from white beads prepared without glutaraldehyde.....84

Figure 5.3. SEM from brown beads prepared using glutaraldehyde.....85

Figure 5.4. SEM from white beads prepared without glutaraldehyde.....85

Figure 5.5. SEM from brown beads prepared using glutaraldehyde.....86

Figure 5.6. SEM from white beads prepared without glutaraldehyde.....86

Figure 5.7. Chitosan beads prepared at pH 7.8 and 45 rpm.....88

Figure 5.8. Brown beads prepared glutaraldehyde.....89

Figure 5.9. White beads prepared without using glutaraldehyde.....90

Figure 5.10. Fermentor used for *E.coli* cells cultivation for production of biomas.92

Immobilization of CTAB treated whole cells of *E. coli* 11105 on chitosan

D. Abedi[a], N. Tavakoli[b], H. Korbekandi[a] and M. R. Bagherinejad

a)Department of Pharmaceutical Biotechnology b) Department of Pharmaceutics

Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran, 81745-359

Introduction: Penicillin G Acylase (PGA) catalyses the transfer of acyl group from one nucleophile (PG) to another (Water) and produces 6-APA and the corresponding side chain (PAA). There are so many problems, which cause short operational life time for the enzyme. Entrapment of permibilized whole cells in a matrix is one of the methods of choices for immobilization. Chitosan possesses distinct chemical and biological properties, which makes it a suitable matrix for entrapment and immobilization of PGA. In this study, a new combinatory method for immobilization of PGA was proposed and optimized using N-cetyl-N, N, N-trimethylammoniumbromide (CTAB) as the permeabilizing agent, chitosan as the matrix, and gluteraldehyde as the cross-linking agent for industrial use. The main aim of our study was 6-APA production using permeabilized *E.coli* 11105 cells, immobilized on chitosan.

Method: In the first step, *E.coli* (ATCC 11105) cells were permeabilized using a solution of CTAB (0.1% w/v) for 45 min under gentle stirring (45 rpm) and then immobilized using gluteraldehyde (5% w/v) and chitosan (3% w/v) as matrices. These conditions were established after preliminary trials with CTAB and glutaraldehyde concentrations in the range of 0.05-0.25% (w/v) and 1-9% (v/v). After beads preparation, hydrolytic activity of the new biocatalyst was assayed using Balasingham-Ehrlich method.

Results: Permeabilization of the cells caused 9% increase in PGA conversion compared to the intact cells after 15 min. Although, immobilization on chitosan decreased the conversion compared to un-immobilized treated cells (13%), the new biocatalyst showed good operational stability, and maintained more than 90% of the initial activity after 20 cycles. Optimum conditions for immobilization of *E.coli* cells were: CTAB 0.1 % w/v, Glutaraldehyde 5% v/v and pH 7.8.

Conclusion: A new combinatory method was successfully developed and optimized for immobilization of PGA on chitosan. The produced biocatalyst showed a good hydrolytic activity and operational stability, and it seemed that this method can be developed for industrial scales.

Key words: Chitosan, Penicillin G Acylase, *E.coli* (ATCC 11105), Immobilization, Glutaraldehyde, N-cetyl-N,N,N-trimethyl Bromide (CTAB),

5. REFERENCES:

1. Verweij J., De Vroom. E. Status and prospects in the use of biocatalysis in the synthesis of β-lactam antibiotics. Recl. Trav. Chim. Pays. 1993; 112: 66-81.

2. Van Langen, L. M. Penicillin acylase: Properties, Application and Prospects in the biocatalyst production of fine chemicals. Thesis. Delft University of Technology. Netherlands. 2001: 1-129.

3. Wegman, M. A. Alternative routes to activate side-chain donors of β-lactam antibiotics. Thesis. Delft University of Technology. Netherland. 2001.

4. Bruggink A., Roos E.C., Vroom E. Penicillin Acylase in the Industrial Production of β-Lactam Antibiotics. Org. Proc. Res. Dev. 1998; 2: 128-133.

5. Savidge T.A., Enzymatic conversion used in the production of penicillin and cephalosporins. NewYork; Dekker, 1984; 171-224.

6. Nam D.H., Kim C., Ryu D.D.Y. Reaction kinetics of cephalexin synthesizing enzyme from *Xanthomonas citri*. Biotechnol. Bioeng. 2004; 27: 953-960.

7. Verheart R.M.D., Reimens A.M., Van der lann J.M. Antibacterial and toxicological evaluation of β-lactam antibiotics. Appl. Environ. Microbial. 1997; 63: 3412-3418.

8. Shamolina T.A., Svedas V.K. Reactivation of heterodimer and individual subunits of penicillin acylase from *E.coli* after inactivation on urea. Biochem. J. 2000; 65: 672-676.

9. Abedi D., Korbekandi H., Haghighi B., Kalantari S. A preliminary study on production of Penicillin G Acylase from *E.coli* 11105. Thesis. Isfahan University of Medical Sciences. 2006.

10. McVey C. E., Walsh M. A., Dodson G. G., Wilson K. S., Brannigan J. A. Crystal Structures of Penicillin Acylase Enzyme-Substrate Complexes: Structural Insights into the Catalytic Mechanism. J. Mol. Biol. 2001; 313(1): 139-150.

11. Rojendhran J., Gunasekaran P., Recent biotechnological interventions for developing improved Penicillin G Acylase. J. Biosci. Bioeng. 2004; 97: 1-13.

12. Youshko M. I., van Langen L. M., de Vroom E., van Rantwijk F., Sheldon R. A., Svedas V. K. Highly Efficient Synthesis of Ampicillin in an "Aqueous Solution-Precipitate" System: Repetitive Addition of Substrates in a Semicontinuous Process. Biotechnol. Bioeng. 2001; 73(5): 426-430.

13. Youshko M. I., van Langen L. M., de Vroom E., van Rantwijk F., Sheldon R. A., Svedas V. K. Penicillin Acylase-Catalyzed Ampicillin Synthesis Using a pH Gradient: a New Approach to Optimization. Biotechnol. Bioeng. 2002; 78(5): 589-593.

14. Fonseca L. P., Cabral J. M. Penicillin Acylase Release From *Escerichia coli* Cells by Mechanical Cell Disruption and Permeabilization. J. Chem. Technol. Biotechnol. 2002; 77: 159-167.

15. Felix H. Permeabilization cell. Anal. Biochem. 1982; 120: 211-234.

16. Tabatabaie M., Fazli B. M., Abedi D. Immobilization of penicillin G acylase using Cross-Linked Enzyme Aggregates (CLEA). Thesis. Isfahan University of Medical Science. 2004.

17. Krajewsk B. Application of chitin and chitosan-based materials for enzyme immobilization: a review. En. Micro. Technol. 2004; 35: 126-139.

18. Baker S.A., Petch G.S. Enzyme and Immobilized Cell in Biotechnology. Benjamin Cummings Publishing. London 1985.

19. Tische W., Wedekind F. Immobilized enzyme: Methods and Applications. Top. Cur. Chem. 1999; 200: 95-126.

20. Flickinger M.C., Drew S.W. Encyclopedia of bioprocess technology. 1999; 1063-1068.

21. Nelson J.M., Griffin E.G. Adsorption of invertase. J. Am. Chem. Soc. 2004; 38: 1109-1115.

22. Chitaba I., Wingard L.B. Immobilized microbial cells. Appl. Biochem. Bioeng. 1983; 4: 66-69.

23. De Main A.L., Davis J. Manual of Industrial Microbiology and Biotechnology. 9th Edition. NewYork; ASM Press. 1999; 67-93.

24. Chetan B.P., Vilas G.G. Adsorption and immobilization of Penicillin G Acylase on chitosan beads. Sep. Sci. Technol. 2004; 39: 2655-2675.

25. D'Souza S.F. Immobilized enzymes in bioprocess. Nuclear Agriculture and Biotechnology Division. 2006; 1-18.

26. Zaks A. Industrial biocatalysis (Review). Cur. Op. Chem. Bio. 2001; 5: 130-136.

27. Scejter A., Bar-Eli A. Preparation and properties of cross-linked water-insoluble catalase. Arch. Biochem. Biophys. 1970; 136: 325-339.

28. Keneddy J.F., Cabral J.M.S. Immobilized enzymes. Solid phase biochemistry. Analytical and synthetic aspects. NewYork. John Wiley and Sons. 1983; 253-391.

29. Felt O., Bori P., Gurny R. Chitosan: a unique polysaccharide for drug delivery. Drug Dev. Ind. Pharm. 1998; 24: 979-993.

30. Single A.K., Chawla M. Chitosan: some pharmaceutical and biological aspect. Pharm. Pharmacol. 2001; 53: 1047-1067.

31. Tharanathan R.V., Kittur F.S. Chitin: the undisputed biomolecule of great potential. Crit. Rev. Food Sci. Nut. 2003; 43: 61-87.

32. Kawamura Y., Yoshida H., Asai S., Kurahashi I., Tanibe H. Effect of chitosan concentration and precipitation bath on the material properties of porous crosslinked chitosan beads. Sep. Sci. Technol. 1997; 32: 1959-1974.

33. Peter M. Application and environmental aspect of chitin and chitosan. J. Macro. Sci. Pure Appl. Chem. 1995; 32: 629-640.

34. Shahidi H., Arachi J.K.V., Jeon Y.J. Food application of chitin and chitosan. Food Sci. Technol. 1999; 10: 37-51.

35. Varshosaz J., Jafarian D. A., Golafshan S. Colon delivery of Mesalasin using bio-adhesive microsphere of chitosan. Thesis. Isfahan University of medical sciences. 2004.

36. Paul W., Sharma C.P. Chitosan, a drug carrier for the 21th century: a review. STP Pharm. Sci. 2001; 10: 5-22.

37. Balasingham K., Warburton D., Dunnil P., Lilly M.D. The isolation and kinetics of penicillin amydase from *E.coli*. Biochem. Biophys. Acta. 1972; 276: 250-256.

38. Tabatabaie M., Fazeli M., Setaiesh N. Biosynthesis of 6-APA using immobilized cell. Thesis. Tehran University. 2000.

39. Kheirolomoom A., Atjmand M., Fazelinia H., Zakeri A. Isolation of penicillin G Acylase from *E.coli* ATCC 11105 by physical and chemical treatments. Biochem. Engin. J. 2001; 8: 223-227.

40. Prabhune A.A., Rao B.S., Pandle A.V., SivaRaman H. Immobilization of permeabilized *Escherchia coli* cell with penicillin acylase activity. En. Micro. Technol. 1992; 14: 161-163.

41. Norouzian D., Javadpour S., Moazami N., Akbarzadeh A. Immobilization of whole cell Penicillin G Acylase in open pore gelatin matrix. En. Micro. Technol. 2002; 30: 26-29.

42. Ruey-Shin Juang, Feng-Chin Wo. Ru-Ling Tseng. Solute adsorption and enzyme immobilization on chitosan beads prepared from shrimp shell wastes. Bio. Technol. 2001; 80: 187-193.

43. Monsan P. Optimization of glutaraldehyde activation of a support. J. Mol. Cat. 1978; 3: 371-380.

44. Oyrton A. C., Monteiro J., Airoldi C. Some studies of crosslinking chitosan-glutaraldehyde interaction in homogeneous system. Int. J. Bio. Macro. 1999; 26: 119-128.

45. Zubriene A., Budriene S., Gorochovceva N., Romaskevic T., Matulionis E., Dienys G. Immobilization of hydrolases onto chitosan microparticles. Chemija. 2003; 14: 226-230

46. Braun J., Le Chanu P., Le Goffin F. The immobilization of Penicillin G Acylase on chitosan. Biotech. Bioeng. 1998; 33: 242-246.

47. Priera E.B., Zanin G.M., Castro H.F. Immobilization and catalytic properties of lipase on chitosan for hydrolysis and estrification reactions. Braz. J. Chem. Eng. 2003; 20: 1-20.

48. Altun G.D., Cetinus S.A. Immobilization of pepsin on chitosan beads. Food Chem. 2007; 100: 964-971.

7. Farsi Abstract

تثبیت سلولهای اصلاح شده باکتری *E.coli* 11105 با CTAB در کیتوزان

دکتر داریوش عابدی، دکتر ناصر توکلی، دکتر حسن کربکندی، محمدرضا باقری نژاد.

مقدمه:

پنی سیلین جی آسیلاز آنزیمی است که گروه آسیل یک نوکلئوفیل را به یک نوکلئوفیل دیگر منتقل میکند و محصول واکنش 6-پنی سیلانیک اسید و فنیل استیک اسید میباشد. استفاده صنعتی از آنزیم ها به صورت مستقیم به علت ناپایداری آنها بسیار محدود است. تثبیت سلول کامل نفوذ پذیر شده در یک شبکه پلیمری یکی از روشهای حل این مشکل میباشد. در این میان کیتوزان دارای ویژگیهای منحصر بفردی است که آن را به عنوان یک گزینه مناسب مطرح میسازد.

در این مطالعه، یک روش جدید جهت تثبیت آنزیم بکار گرفته میشود که در حقیقت ترکیبی از چند روش میباشد. در این روش سلول باکتری *E.coli* (ATCC 11105) به کمک ماده ستیل تری آمونیوم بروماید (CTAB) نفوذ پذیر میگردد و سپس در حضور ماده گلوتارآلدهید در پلیمر کیتوزان تثبیت میشود. هدف از این طرح معرفی یک بیوکاتالیست جدید برای مصارف صنعتی است. هدف اصلی در این مطالعه تولید 6-پنیسیلانیک اسید به کمک سلولهای نفوذپذیر شده و تثبیت شده در کیتوزان بود.

روش کار:

در این مطالعه، جهت نفوذ پذیر کردن سلولهای باکتری *E.coli* آنها در دمای اتاق و چهل و پنج دور در دقیقه در مجاورت ماده CTAB به مدت چهل و پنج دقیقه قرار میگیرند و سپس به

کمک محلول سه درصد اسیدی کیتوزان و گلوتارآلدهید پنج درصد تثبیت میگردند. جهت بهینه سازی عملکرد بیوکاتالیست حاصل اثر غلظتهای مختلف ماده نفوذپذیر کننده (پنج صدم تا بیست و پنج صدم درصد وزن در حجم) و همچنین غلظتهای متفاوت گلوتارآلدهید (یک تا نه درصد حجم در حجم) مورد بررسی قرار گرفت. بعد از تهیه گویچه های تثبیت شده سلولی، جهت اندازه گیری فعالیت آنزیمی از روش بالاسینگهام استفاده گردید.

نتایج:

بر اساس نتایج حاصل، نفوذپذیر کردن سلول باعث میشود در بازه زمانی پانزده دقیقه، درصد تبدیل نه درصد نسبت به سلول دست نخورده افزایش یابد. اگرچه تثبیت سلول نفوذپذیر شده در کیتوزان با استفاده از گلوتارآلدهید نسبت به سلول غیر تثبیت شده ولی نفوذپذیر شده، سیزده درصد کاهش درصد تبدیل دارد ولی بیو کاتالیست حاصل دارای پایداری بالایی بوده به گونه ای که بعد از بیست بار استفاده مجدد تنها ده درصد از فعالیت اولیه خود را از دست میدهد که این موضوع جهت مصارف صنعتی از اهمیت بالایی برخوردار است. غلظت بهینه ماده نفوذپذیر کننده پنج صدم درصد و گلوتارآلدهید پنج درصد بود و pH مطلوب جهت تثبیت هفت و هشت دهم گزارش گردید.

بحث و نتیجه گیری:

در این مطالعه یک روش جدید که خود در بر گیرنده چند روش مختلف قبلی است جهت تثبیت سلولهای نفوذپذیر شده باکتری *E.coli* در کیتوزان با موفقیت بکار گرفته شد. بیوکاتالیست حاصل دارای فعالیت و پایداری مناسبی بود که این مشخصات، آن را جهت کاربری صنعتی مطلوب میسازد.

کلید واژه ها:

کیتوزان، گلوتارآدهید، ستیل تری آمونیوم بروماید (CTAB)، پنی سیلین جی آسیلاز(PGA)، تثبیت، *E.coli* 11105