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Fabrication of oral acid resistant Fermosorb (ferment + sorbent)-type preparations can be simplified using industrial *Bacillus subtilis* culture filtrates as an enzyme source

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Abstract

In our recent article [Biziulevičius, G.A. and Žukaitė, V., 1999. Int. J. Pharm. 189, 43–55] we described a novel approach in design of oral preparations intented for intestinal delivery of enzymes, based on reversible immobilization of the latter onto the polymer matrix. Fermosorb (ferment + sorbent)-type preparations, produced in such a way, are characterized as two-component delayed-release enzyme formulations being stable at acidic pH and thus ensuring the protection of an active substance in the environment of the gastric region and liberating the active substance through dissociation of the enzyme–polymer complex at neutral pH values characteristic for the intestines. In the present paper we report our updated findings showing that the technology of fabrication of two Fermosorb-type preparations manufactured (namely Fermosorb and Polyferm) can be simplified (as well as their production cost reduced) substituting the acetone precipitated enzyme preparation solutions, currently used as an enzyme source, by their precursors — industrial *Bacillus subtilis* culture filtrates. Moreover, we give a description of how one more Fermosorb-type preparation aimed at intestinal delivery of amylolytic enzymes can be produced in a similar manner. Determination of immobilization conditions has revealed, that irrespectively of the enzyme origin (lytic, proteolytic or amylolytic) and its source (1% acetone precipitated preparation solution or culture filtrate), optimal for immobilization v/w ratio of the liquid phase and the polymer matrix (Biocarb L) remains the same and is equal to 10:1 (approximate). The main differences have been found to be in optimal for immobilization pH values as well as process duration in regard to the two enzyme sources applied. In case of proteolytic and amylolytic enzymes only one of the variables was different (process duration for the first ones, optimal pH for the second ones), while in case of lytic enzymes both variables were different. The percentage of the enzymes activity uptaken from the reaction mixture formed by either of the enzyme sources and the polymer matrix ($\approx 60\%$) as well as activity losses at drying the enzyme–polymer complexes ($\approx 20\%$), tolling in the final activity yield of near 40%, were found to be very similar.

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Oral acid resistant enzyme preparations are conventionally prepared in the form of tablets or pellets coated with enteric polymers (Mayer and Viernstein, 1994; Schulz and Schmidt, 1995; Kleine, 1997). Recently we proposed a novel approach in design of oral preparations intended for intestinal delivery of enzymes by describing (Biziulevičius and Žukaitė, 1999) a simple technology for the pilot-scale preparation of pH-dependent reversibly dissociating acid stable enzyme–polymer complex Fermosorb (ferment + sorbent). The technology described was based on immobilization of lytic (degrading microbial cell walls) enzymes, using 1% lysosubtilin, a commercial acetone precipitated preparation derived from *Bacillus subtilis* SK-52 submerged culture filtrate, solution in 10 mM acetate buffer of pH 5.0, onto commercially available highly porous carboxylic cation exchanger Biocarb L. In general, Fermosorb-type oral preparations are characterized as two-component delayed-release enzyme formulations (enzymes reversibly immobilized onto polymer matrix) being stable at acidic pH and thus ensuring the protection of an active substance in the environment of the gastric region and liberating the active substance through dissociation of the enzyme–polymer complex at neutral pH values characteristic for the intestines. Two representatives, Fermosorb and Polyferm (polymer + ferment) namely, have already been shown to be highly effective for prophylaxis and treatment of gastrointestinal disorders in newborn calves (Zotkin et al., 1987a,b; Sisyagin et al., 1988a,b; Biziulevičius and Žukaitė, 1999). Both are authorized for use throughout the former Soviet Union. Up till now these veterinary medicinal products were manufactured using acetone precipitated enzyme preparation solutions and substitution of the latter by industrial culture filtrates hopeful from the technological and economical points of view still remained a future perspective.

In this communication we describe our updated findings showing that the technology of fabrication of known Fermosorb-type preparations can be simplified (as well as their production cost reduced) substituting the currently used acetone precipitated enzyme preparation solutions by their precursors-industrial culture filtrates. We also give a description of how one more Fermosorb-type preparation aimed at intestinal delivery of amylolytic enzymes can be produced in a similar manner.

Lysosubtilin, protosubtilin and amylosubtilin (the latter two being acetone precipitated preparations of proteolytic and amylolytic enzymes derived from *B. subtilis* 103 and *B. subtilis* 65 culture filtrates, respectively) as well as the corresponding culture filtrates were acquired from the State Joint-Stock Enterprise 'Biosintezė' formerly called the Vilnius Experimental-Industrial Plant of Enzyme Preparations (Vilnius, Lithuania). Biocarb L (a beaded cross-linked copolymer of methacrylic acid and triethylene glycol dimethacrylate) was purchased from the All-Union Research Institute of Chemical Technology (Moscow, Russia). Optimal immobilization conditions were determined according to (Biziulevičius and Žukaitė, 1999) with the exception that, instead of the acetate buffer applied for dissolution of enzyme preparations, a diluted acetic acid was used for the adjustment of pH in culture filtrates. Culture filtrates for pilot-scale procedures were processed analogously. Pilot-scale fabrication of Fermosorb-type preparations was performed as described in detail previously (Biziulevičius and Žukaitė, 1999). Briefly, enzymes were immobilized, using 250-l amounts of 1% enzyme preparation solution or culture filtrate onto Biocarb L, in a 0.6-m^3 closed metal vessel having an inert plastic-coated inner surface under constant mechanical agitation with a propeller stirrer (not less than 600 rpm) at room temperature and at optimal conditions for immobilization (for the latter see below). Then the procedures of vacuum-filtration and oven-drying followed. Enzyme activities both at determination of optimal immobilization conditions and in the course of the downstream processing were controlled by the methods used in industrial production of the appropriate enzymes

(lytic activity according to Kislukhina (1976), while proteolytic and amylolytic ones according to Rukhlyadeva et al. (1967)). Activity of enzyme–polymer complexes was measured after 1 or 2 h desorption of enzymes with an approximately tenfold amount of 50 mM phosphate buffer, pH 7.2, for wet and dry samples, respectively.

Table 1
Optimal pH values and process duration for immobilization of different enzymes onto Biocarb L (v/w ratio 10: 1)

Enzymes	Enzyme source	pH	Process duration (h)
Lytic	1% lysosubtilin solution	5.0[a]	1[a]
	Culture filtrate	5.2	3
Proteolytic	1% protosubtilin solution	4.6[b]	3[b]
	Culture filtrate	4.6	8
Amylolytic	1% amylosubtilin solution	4.6	2
	Culture filtrate	4.4	2

[a] The results are the same as the defined values controllable in industrial fabrication of Fermosorb (Biziulevičius and Žukaitė, 1999).

[b] The results are the same as the defined values controllable in industrial fabrication of Polyferm (Biziulevičius and Žukaitė, unpublished data).

Table 2
Enzyme activity yields as determined after fulfilment of the immobilization process applying different enzymes (stage 1) and after drying the enzyme–polymer complexes formed (stage 2)[a]

Enzymes	Enzyme source	Enzyme activity yield (%)	
		Stage 1	Stage 2
Lytic	1% lysosubtilin solution	60.5[b]	40.3[b]
	Culture filtrate	60.7	40.4
Proteolytic	1% protosubtilin solution	59.7	39.6
	Culture filtrate	58.8	38.5
Amylolytic	1% amylosubtilin solution	60.3	40.0
	Culture filtrate	60.9	40.8

[a] The results are the average values obtained from the analysis of at least five batches running, with the difference between the corresponding values being no more than 2%.

[b] The results are the same as described in our previous study (Biziulevičius and Žukaitė, 1999).

Determination of immobilization conditions revealed, that irrespectively of the enzyme origin (lytic, proteolytic or amylolytic) and its source (1% acetone precipitated preparation solution or culture filtrate), optimal for immobilization v/w ratio of the liquid phase and Biocarb L is 10:1 (approximate), i.e. being the same as observed in our previous study (Biziulevičius and Žukaitė, 1999). This is not surprising as, firstly, the enzyme preparations applied are being manufactured using related producers and in a similar manner (Kislukhina, 1972; Yamane, 1989) and, secondly, the activities of all 1% acetone precipitated enzyme preparation solutions tested and corresponding culture filtrates are of the same magnitude. As submerged *B. subtilis* culture filtrates alongside with purposive enzymes contain several other secondary metabolites (Yamane, 1989) that are absent (or their quantities diminished) in acetone precipitated preparation solutions, it was quite probable that optimal pH values for immobilization as well as process duration might be quite different in regard to those two enzyme sources. Results presented in Table 1 confirm the rightness of this presumption. In case of proteolytic and amylolytic enzymes only one of the variables was different (process duration for the first ones, optimal pH for the second ones), while in case of lytic enzymes both variables were different. Following analogous presumption we were also interested in the percentage of enzymes activity taken up from reaction mixture formed by either of the enzyme sources and Biocarb L as well as activity losses at drying the enzyme–polymer complexes. The comparative results are presented in Table 2 and these disclose at least two facts. First, application of any of the two enzyme sources gave similar results. Second, the main losses were observed at immobilization stage ($\approx$ 40%) and at drying ($\approx$ 20%), totalling in the final activity yield of near 40%.

Analysis of the enzyme–polymer complexes formed revealed, that *B. subtilis* secondary

metabolites other than the purposive enzymes (as well as proteins liberated from the biomass due to lysis) present in industrial culture filtrates were not adsorbed (or insignificantly adsorbed) onto the polymer matrix at the optimal immobilization conditions described above. Thus, potential for adsorption of contaminating proteins as well as potential toxicity due to such contamination were avoided and this was confirmed by the preliminary in vivo safety assessment studies of the formulations produced (Biziulevičius and Žukaitė, unpublished data).

In conclusion, results of this study show that introduction of a technological approach directed to substitution of acetone precipitated enzyme preparation solutions by industrial culture filtrates into manufacture is quite realistic. An answer to an unsolved question regards fabrication of Fermosorb-type preparations possessing amylolytic activity (these are of great interest for veterinarians fighting with gastrointestinal disorders caused by starch-related nutrient indigestibility) may also be given. Moreover, research in the field of other Fermosorb-type preparations (e.g. catalyzing degradation of non-starch-like polysaccharides) is already under way.

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