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## FORMULATION OPTIMISATION OF MIXED SUGAR/PROTEIN/MALTODEXTRIN ENCAPSULANTS FOR SPRAY DRYING *L. ACIDOPHILUS* USING THE RESPONSE SURFACE METHOD

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**Abstract:** Three sugars (maltose, fructose, and lactose) have been combined in different formulations with three protein based powders (whey protein, skim milk, and soy protein) to assess the survivability of *L. acidophilus* after spray drying at 80°C followed by optional further exposure to simulated gastric intestinal juice (SGI) or bile solution. The results showed that the highest survival rate was found in a recipe consisting of 87.5% skim milk and 12.5% maltose, while the lowest rates were found in formulations containing no protein. Maltose and lactose provide higher survival rate than fructose which may reflect the higher glass transition temperature of maltose/lactose mixtures. Similar trends were found with cells rehydrated in SGI and bile solutions.

**Keywords:** maltose, fructose, lactose, heat tolerance, probiotic bacteria

### INTRODUCTION

Probiotic bacteria provide a beneficial effect to the balance of intestinal microflora by limiting colonization of pathogenic bacteria. Consequently much research has been conducted on culture identification, strain selection, properties and health benefits of probiotics. This includes cell inactivation by the manufacturing process in order to preserve and maintain live cells in the products. It has been suggested that in thermal processes cells are inactivated by one of two reasons; 1) cells are damaged by high temperature; and 2) cells are damaged by the dehydration process which involves cell membrane alteration (Santivarangkna et al., 2008a). For heat inactivation, it has been reported that critical cell components of *L. bulgaricus*, were destroyed by heating over 65°C (Teixeira et al., 1997). For the dehydration argument, a combined effect of reduction of intracellular water and an increase in solute concentration gradients can potentially lead to cell inactivation (Miles, 2006).

In activation by dehydration is most likely to be caused by damage to cell membranes. Cell membrane consists of lipid bilayers, which are normally found in the liquid-crystalline phase when cells are alive. These might be transformed into a gel phase by the drying process (Crowe et al., 1989; Santivarangkna et al., 2008a). This membrane phase transition affects the movement of embedded globular protein and increases cell membrane permeability, which are involved in cell component synthesis and transport across cell membranes. It is believed that increasing of permeability of cell

membrane occur because a) packing defects are created in the bilayer membrane by drying and b) the orientation of lipid bilayer changes to the hexagonal form as a result of the temperature change (Patist and Zoerb, 2005; Santivarangkna et al., 2008a; Crowe et al., 1989; Crowe and Crowe 1982; Crowe et al., 1983). These two mechanisms cause leaking through the cell membrane that allows undesired chemical substances to easily cross into cell. Moreover, low water content arising from dehydration reduce cell volumes causing concentration gradients between the inside and outside of cell that produces an osmotic pressure differences which can lead to cell bursting (Santivarangkna et al., 2008a). Furthermore, the membrane phase change from liquid to gel phase also influences the fluidity of the cell membrane and affects protein function.

It can be seen that there are combined effects of high temperature and dehydration on cell inactivation. Thus, to protect heat sensitive probiotic bacteria such as *L. acidophilus*, particularly for high temperature processes such as spray drying, materials used for cell entrapment play a key role in cell protection. The role of sugars and proteins are believed to be important in cell stabilization as they are believed to interact with the cell membrane. Many research groups have used sugars, such as sucrose, glucose, maltose and trehalose, to stabilise cell membranes or phospholipid bilayers, either in growth media or in drying protective agents and the results have showed that added sugar can increase cell viability after drying (Crowe et al., 1987; Lievens et al., 1994; Potts, 2001; Carvalho et al, 2003). It is accepted that

disaccharides can form glasses when drying occurs and the hydroxyl group of sugars can form hydrogen bonds with the carbonyl group of phospholipid membranes, as well as protecting proteins from the drying process by forming hydrogen bonds with protein while water is removed from cells (Ananta et al., 2005; Crow et al., 1984; Diaz et al., 1999; Lee et al., 1986; Leslie et al., 1995; Luzardo et al., 2000; Patist and Zoerb, 2005; Santivarangkna et al., 2008b; Sum et al., 2003; Villarreal et al., 2004; Winer et al., 1989).

Apart from sugar, it is suggested that proteins (particularly milk proteins) are suitable for using as coating materials in spray dried products as they have good binding properties to form wall materials (Rosenberg and Sheu, 1996). Furthermore, proteins are both partially hydrophilic (which provides water retention properties) and also partially hydrophobic (which provides fat binding properties) (Mosilhey, 2003; Kinsella, 1976). There are several works which claim that whey protein and skim milk can increase cell survival rates (Ananta et al., 2005; Picot and Lacroix, 2004). However, proteins might be denatured during high temperature processing such as during spray drying and this will alter their functionality.

This work investigates the effect of different sugars (maltose, lactose and fructose) and proteins (whey protein, soy protein and skim milk) in formulations containing maltodextrin on the survival rates of *L. acidophilus* when spray drying at an outlet temperature of 80°C, locate an optimized recipe that provides the best cell survival rate.

## MATERIALS AND METHODS

### *Microorganisms*

The freeze dried form of *L. acidophilus* NCIMB 70225 was purchased from National Collection Microbial (NICMB). Cells were activated, transferred into new Man, Rogosa and Sharpe broth (MRS broth; Oxoid, Hampshire, UK) and incubated at 37°C for 72 hours under anaerobic conditions using a Gas Pack system. Broth cultures were kept in a fridge before using as a starter culture for preparations of cell pastes or population growth experiment.

Cell pastes of *L. acidophilus* were prepared by harvesting and centrifuging cells (spin at 3000g, 4°C for 10 minutes) in the early-stationary phase, from 1L cultured broth. This was added to 5 mL of its medium broth, and mixed to obtain a homogeneous cell paste. This was then kept in the refrigerator at 4°C before using in spray drying experiments on the same day (method adapted from Chen et al., 2006).

### *Feed solution preparation for spray drying*

The following ingredients were used in experiments: maltose (Sigma, Poole, UK), fructose (myprotein.co.uk, Manchester, UK), lactose (Acros Organics, NJ), whey protein (Impact Whey Protein isolate, myprotein.co.uk, Manchester, UK), soy protein isolate (myprotein.co.uk, Manchester, UK), and skim milk (Sainsbury's supermarket, UK), and maltodextrin (myprotein.co.uk, Manchester, UK). These were prepared in 9 different ratios as shown in Table 1 with distilled water (20% w/v solids content). This is adapted from a Simplex centroid design with three components. Due to the limitations of stickiness in spray drying the maximum level of sugar used was 25%.

500 mL of solution was stirred until become homogeneous and then pasteurised at 60°C for 30 minutes. A cell paste of *L. acidophilus* (1 mL) was individual inoculated into the mixed solution and the solution then stirred until it become homogeneous. The cell concentration in the feed was typically 10<sup>8</sup> CFU/mL.

Table 1. Ratio of mixed drying protective agents consisting of sugars, proteins and maltodextrin (followed Simplex centroid design with three components).

TRT	Ratio		
	*Sugar	**Protein	Maltodextrin
1	0.25	0.00	0.75
2	0.125	0.00	0.875
3	0.00	0.00	1.00
4	0.25	0.375	0.375
5	0.125	0.4375	0.4375
6	0.00	0.50	0.50
7	0.25	0.75	0.00
8	0.125	0.875	0.00
9	0.00	1.00	0.00

\*Sugar = maltose/fructose/lactose; (lactose was only use in the mixed with whey protein and maltodextrin).

\*\* Protein=impact whey protein isolate/soy protein isolate/skim milk.

### *Spray drying study*

Spray drying was performed in a tall-form co-current spray drier of 12 ft height x 4 ft diameter (Spray Processes, Bedford UK). A peristaltic pump (Watson-Marlow 510U) was used to deliver the feed solution to the atomiser. The atomisation was performed by a twin-fluid nozzle, using compressed air as the atomising gas. Ambient air was directly heated in a burner using natural gas, allowing control

of the inlet air temperature. A HEPA filter was used to filter bacteria from the outlet air stream. The operation was started by warming the chamber for 10-15 minutes before feeding distilled water and the inlet and outlet temperatures were set by adjusting the liquid feed and air flow rate. The outlet temperature was effectively regulated by variation of the liquid feed flow rate. A drying outlet air temperature of 80°C was used, as this was found to be a good operating temperature according to a previous study. Once the required outlet temperature was reached, the solution was fed into the drying chamber. The dried powders were collected in a cyclone separator and transferred to a sealed tight sterilized amber bottle, and were taken for analysis. All experiments were carried out in duplicate.

#### *Determination of powder moisture content*

The moisture content of the spray dried powders was determined according to AOAC method 960.18 (OMA) by oven drying at 105±1°C for 24 hr. The percentage moisture content values were then used to calculate cell survival rates on a dry basis.

#### *Determination of cell numbers and cell survival rate*

Approximately 2 g of dried cells were rehydrated in PBS and incubated for an hour at 37°C under anaerobic conditions. Then samples were serially diluted with PBS and cells counted by the pour plate method on MRS agar. The number of viable cells was expressed in colony forming units per gramme of dried sample (CFU/g).

The percentage of bacteria surviving spray drying was calculated as follows (Rodríguez-Huezo et al., 2007):

$$\% \text{ Survival} = N / N_0 \times 100 \quad (1)$$

where

N is Log cell number of bacteria after spray drying/passing simulated gastric juice/bile solution (Log CFU/g. dried sample)

N<sub>0</sub> is Log cell number of bacteria before spray drying (Log CFU/g. dried sample)

#### *Simulated gastric intestinal juice and bile solution tests*

Simulated gastric intestinal juice (SGI) was prepared by dissolving 0.3% of pepsin (Sigma, Poole, UK) in 0.5% v/v NaCl solution and then adjusting to pH 2.0 with 1M HCl or 1 M NaOH. The SGI was sterile-filtered through a 0.2 micron membrane and kept in the fridge before using.

To prepare bile solution, 10% w/v of bovine bile (Fluka, Poole, UK) was diluted to 2% v/v and sterilised at 121°C for 15 minutes. The bile solution was kept in the fridge before using.

Approximate 1 g of dried cell were dissolved in 9 mL of simulated gastric juice or 2 % bile solution and then incubated at 37°C for 1 hour under anaerobic condition (in screw cap bottle). The sample was then taken for plate count analysis on MRS agar as described earlier.

#### *Response surface methodology*

Response surface models were evaluated by fitting experimental data to second order polynomial expressions using STATISTICA 9. 3D Surface plotting was performed using the same software.

## RESULTS AND DISCUSSION

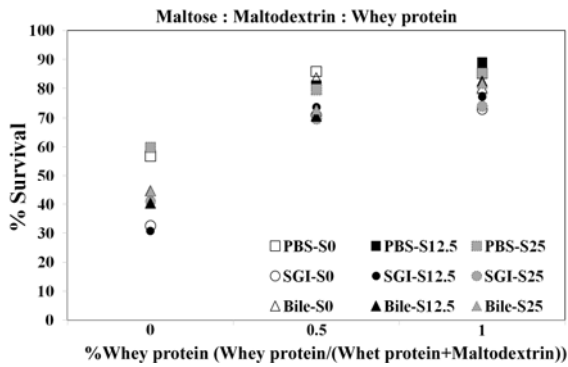
This work studied the basic survival, and additional acid and bile resistance of *L. acidophilus* in the early stationary phase (10<sup>th</sup> hour of incubation time) after spray drying at an air outlet temperature of 80°C. Ratios of mixed ingredients consisting of sugar (maltose, fructose and lactose), protein (whey protein, soy protein and skim milk), and maltodextrin were set up along the coordinate point following Simplex centroid design (Table 1). Cell survival rates were investigated and compared for the different ingredient formulations. In addition, experimental data of cell survival after rehydration in PBS were plotted by the surface technique to find the optimal recipe for cell survival after spray drying.

#### *Cell survival rates after spray drying*

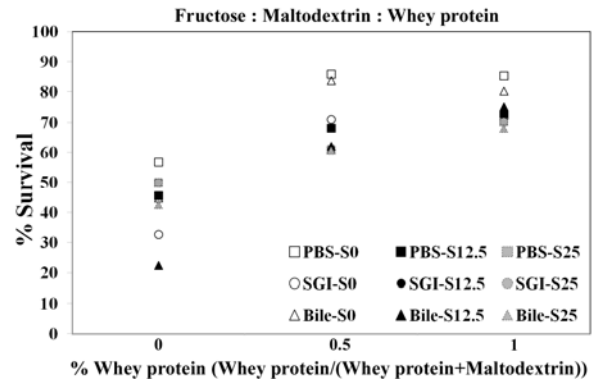
Comparisons of cell survival rates of encapsulated *L. acidophilus* with mixed ingredients after rehydration with PBS, exposure to SGI and 2% bile solution for an hour are shown in Figs. 1, 2 and 3 for formulations containing maltose, fructose and lactose respectively.

Fig. 1 clearly shows an upward trend of survival rate when increasing both protein and maltose contents. Considering role of protein in mixed encapsulants, it seemed that skim milk provided the highest cell protection compared with whey protein and soy protein. The results showed that 91% (in PBS), 90% (in SGI), and 96% (in bile solution) survival rates were found in 12.5% maltose mixed with 87.5% skim milk, while the lowest survival rates were found in recipe containing no protein.

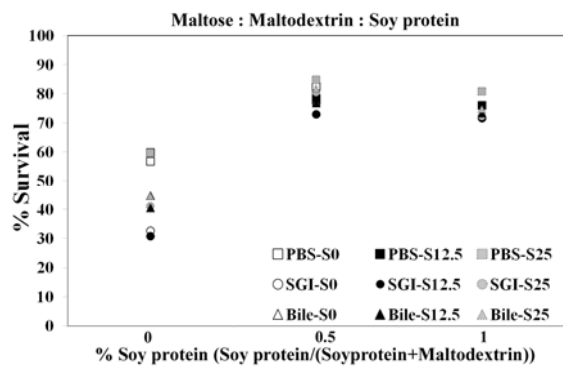
Broadly similar results are shown in Fig. 2, i.e. increasing the protein ratio gives a higher survival rate with 100% skim milk providing the highest survival after rehydration in PBS (87%), exposure to SGI (82%), and bile solution (84%). However, it seems that fructose content does not affect the cell survival rate.



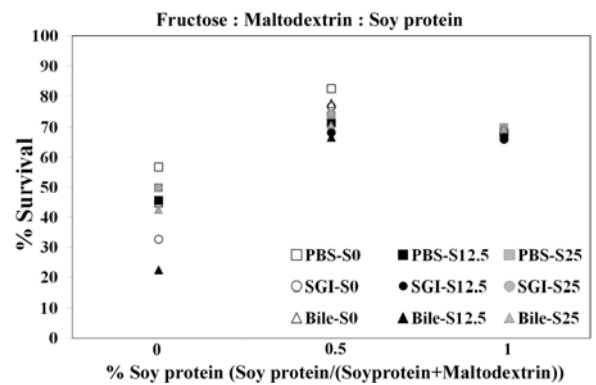
(a)



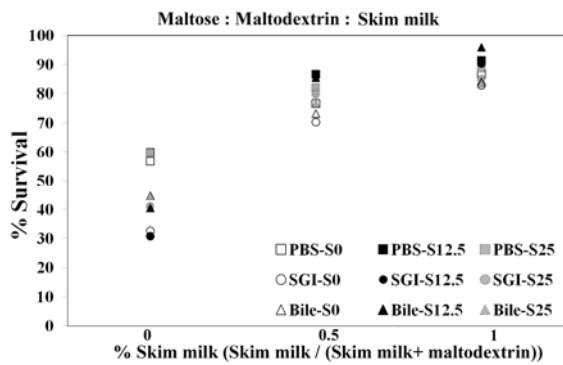
(a)



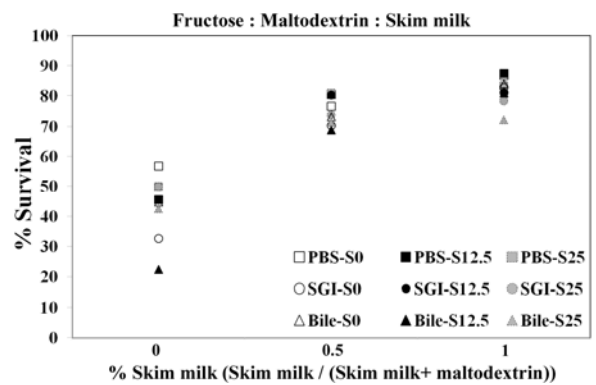
(b)



(b)



(c)



(c)

Fig. 1. % Survival of encapsulated *L. acidophilus* with maltose, maltodextrin and (a) whey protein, (b) soy protein and (c) skim milk after rehydration in PBS, exposure to SGI and bile solution. Where S0=0% maltose, S12.5=12.5% maltose and S25=25% maltose.

Fig. 2. % Survival of encapsulated *L. acidophilus* with maltose, maltodextrin and (a) whey protein, (b) soy protein and (c) skim milk after rehydration in PBS, exposure to SGI and bile solution. Where S0=0%fructose, S12.5=12.5% fructose, S25=25% fructose.

Fig. 3 shows survival rates for recipes consisting of lactose, whey protein and maltodextrin. It was also found here that increasing the whey protein content provided higher survival rate. However, similarly to fructose the lactose content had a minimal effect on survival rates. Indeed the highest survival rates, after rehydration in PBS (86%), exposure to SGI(73%), and bile solution (84%), were found in recipes containing no lactose.

Overall, it is found that rates of cell survival after rehydration with PBS are in the range 42-91%. The formulation with 12.5% maltose mixed with 87.5% skim milk gives the highest rate at 91%, while the lowest rate was found in a recipe consisting of 25% lactose mixed with 75% maltodextrin (42%). After passing SGI and bile solution, the results showed % of survival of spray dried cell are in range of 27-90% (in SGI) and 22-95% (in bile solution). Similar to rehydration process, the highest survival was found

in a recipe consisting of 12.5% maltose: 87.5% skim milk, either in SGI (90%) or bile solution (96%), whilst the lowest survival rates after passing SGI (27%) and bile solution (22%) were from the mixture of 12.5% lactose: 87.5% maltodextrin and 12.5% fructose:87.5% maltodextrin, respectively.

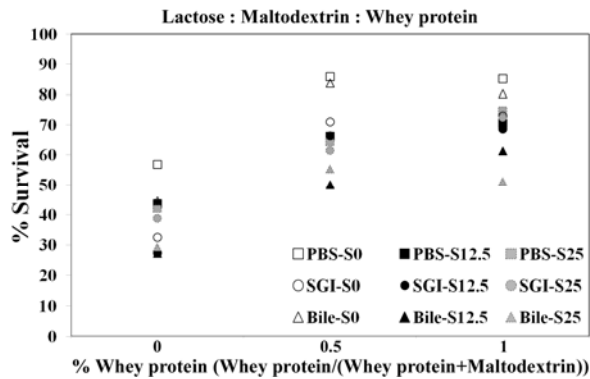


Fig. 3. % Survival of encapsulated *L. acidophilus* with lactose and whey protein after rehydration in PBS, exposure to SGI and bile solution. Where S0=0% lactose, S12.5=12.5% lactose, S25=25% lactose

As the results show that high protein recipes gave high survival rates, this might be explained by the protein possibly interacting with the globular protein in the cell membrane and aid water retention which maintains the conformation of that protein. This finding was supported by other work groups claimed that proteins are hydrophilic substance which has water retention properties and also are hydrophobic substance which has fat binding properties (Rosenberg and Sheu, 1996; Mosilhey, 2003; Kinsella, 1976). It is clear that skim milk (also containing lactose and casein) gives better survival rates than soy protein and whey protein. However, soy protein and whey protein also provide high survival rate, but might have slight difference due to their different structures which affect cell protection ability (as well as possible influences of casein and lactose). However, due to the problem that it was not possible to spray dry formulations based upon 100% soy protein (because of its high viscosity), it is not possible to absolutely claim that skim milk is better than soy protein.

Turning to the influence of the type of sugar used; it was found that maltose provided better cell protection than the other sugars, and particularly, better than fructose. This might because maltose and lactose are disaccharides which have higher glass transition temperatures ( $T_g$ ) (maltose = 87°C, lactose= 97°C (Roos and Karel, 1991), compared to fructose. It was supported by work which claimed that disaccharides, especially with higher glass transition temperatures, can form the glassy state during drying process and can stabilise cell

membrane. They also perform a water replacement property by forming hydrogen bonds with polar groups at cell membrane (Lerbret et al., 2007; Carpenter and Crowe, 1989). However, maltose has a greater protecting effect than lactose despite lactose having a higher  $T_g$  than maltose.

These results also imply that using sugars only might not protect cell from high temperature, acid and bile environments. This is supported by some workers who claimed that maltose, lactose and monosaccharide did not protect solid supported lipid bilayers (Albertorio et al., 2007). It was also reported that maltose does not interact with the polar head group of lipid membranes (Linders et al., 1997). However, the results indicate that it is possible that maltose affects cell membrane protection when combined with protein, as the mixed material consisting of 12.5% maltose showed the highest value of survival rate of any experiment.

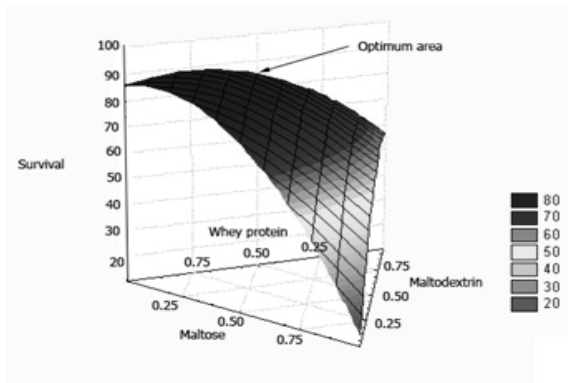
#### Response surface methodology

Survival rates of spray dried *L. acidophilus* after rehydration in PBS were fitted to cubic models, as shown in Table 2, and are replotted as 3D surface plots Figs. 4-6. These allow a different way to view how the rate of survival varies with changing component ratios.

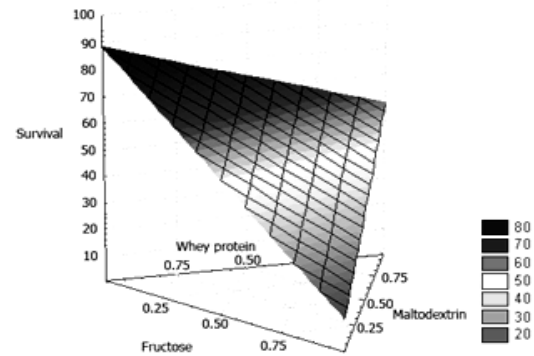
Table 2. Fitted model of % Survival rate of spray dried *L. acidophilus* encapsulated in mixed ingredients by using RSM.

*INGREDIENTS	FITTED MODEL
MT:MD:WP	14.39*MT+56.83*MD+85.82*WP +73.34*MT*MD+97.10*MT*WP +55.54*MD*WP-65.05*MT*MD*WP
MT:MD:SP	73.02*MT+61.18*MD+87.06*SP
MT:MD:SM	-100.92*MT+56.25*MD+86.67*SM +223.82*MT*MD+259.61*MT*SM +22.94*MD*SM+176.95*MT*MD*SM
FT:MD:WP	11.49*FT+59.45*MD+88.33*WP
FT:MD:SP	30.14*FT+58.65*MD+86.14*SP
FT:MD:SM	65.06*FT+33.24*MD+92.65*SM
LT:MD:WP	11.03*LT+56.89*MD+89.65*WP

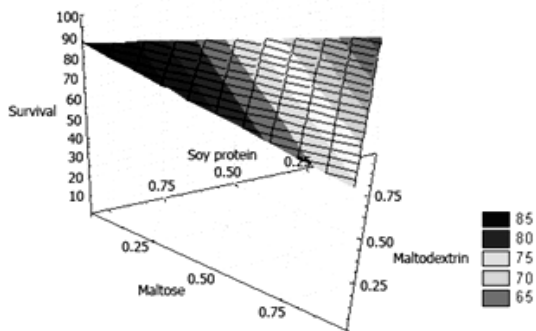
\*MT=maltose; MD=maltodextrin; LT=lactose; WP=whey protein; SP=soy protein; SM=Skim milk



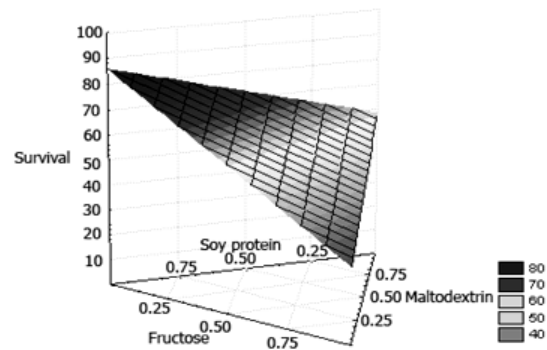
(a)



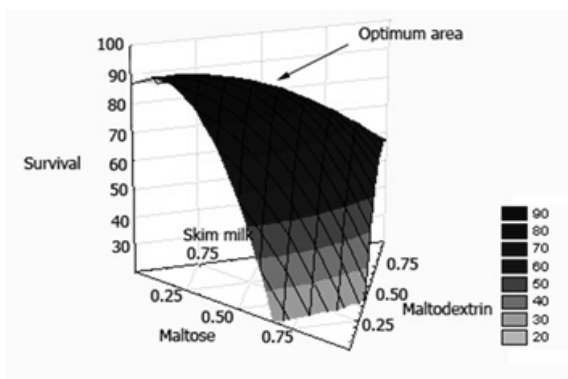
(a)



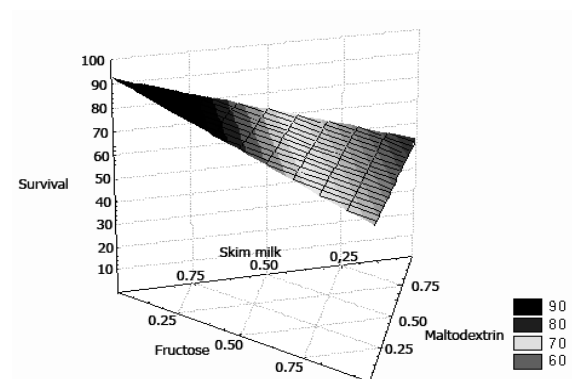
(b)



(b)



(c)



(c)

Fig.4. Surface plot of mixed ingredients consisting of maltose, maltodextrin and (a) whey protein, (b) soy protein, and (c) skim milk.

Fig. 5. Surface plot of mixed ingredients consisting of fructose, maltodextrin and (a) whey protein, (b) soy protein, and (c) skim milk.

Although one should not extrapolate outside the data area, a number of observations can be made:

For recipes containing fructose (Fig. 5) and soy protein a similar “saddle-point” is seen. However, for whey and skim milk a formulation with 100% protein looks best. The data for lactose and whey (Fig. 6) shows very similar trends to that of fructose).

For recipes containing of maltose (Fig. 4), the optimum formulation (for whey and skim milk) is generally 12.5% maltose and the remainder protein. However, for soy protein the optimum position includes maltodextrin (at about 30%).

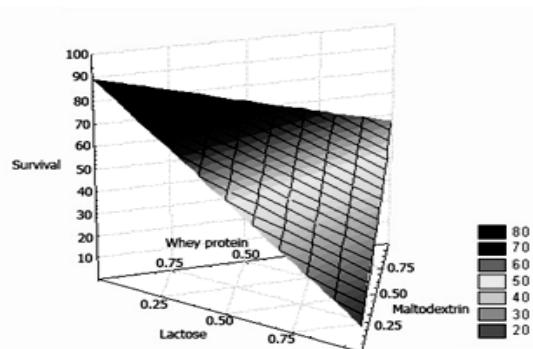


Fig. 6. Surface plot of mixed ingredients consisting of lactose, maltodextrin and whey protein.

### CONCLUSIONS

This work examined the cell survival rate of spray dried *L. acidophilus*, encapsulated with different ratios of protective agents. Maltose, fructose and lactose were used to study the role of sugars on cell protection, while whey protein, soy protein and skim milk were used to represent different proteins.

The results showed that in general, higher levels of protein gave a higher survival rate. For powders rehydrated in PBS the highest survival rate (91%), was found in a recipe consisting of 87.5% skim milk and 12.5% maltose, while the lowest rates (45-60%), were found in formulations containing no protein. Results for soy protein and whey protein were very similar whilst skim milk gave slightly better survival rates – this may be due to the presence of lactose and casein in skim milk. Formulations containing maltose generally performed better than those containing fructose and lactose (especially when lactose was not present), which may reflect the higher glass transition temperature of maltose mixtures. Similar trends were found with cells rehydrated in SGI and bile solutions, although in formulations containing no protein the survival rates were significantly lower than when cells were rehydrated in PBS. Although sugars alone used in the recipe could not show their capabilities to protect cell from heat, acid and bile, high cell survival was found when combined with protein. BY using RSM, it is showed that the optimum protein in excipient should be in range of 50-100% (whey protein), 75-100% (skim milk) and 30-75% (soy protein). This strongly suggests that the presence of protein does protect cells in these harsher environments. Maltose, fructose and lactose can be used in the mixture up to 25%, however at higher contents than this will cause excessive stickiness problems during spray drying.

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