There is a variety of non-digestible oligosaccharides produced worldwide, intended for use as functional food ingredients. Amongst these, galactooligosaccharides (GOS), which are generally recognized as safe, have now been definitely established as prebiotic ingredients. Synthesis of GOS from lactose fermentation by *Pseudozyma tsukubaensis* and *Pichia kluyveri* was investigated. In this study, a sequential strategy of two experimental designs was used to optimize galactooligosaccharides production. First, Plackett-Burman screening design was applied to evaluate the effects of lactose, yeast extract, urea and inoculum concentrations, pH and agitation rate on the response of GOS yield synthesized by both yeasts. The preliminary Plackett-Burman design allowed to identify urea concentration and pH as statistically significant variables in the range studied. In order to find the optimal conditions for GOS synthesis, a central composite rotational design was performed. The independent variables were lactose, yeast extract and urea concentrations and the response take into account was GOS yield produced by *P. tsukubaensis*. The optimized condition for this prebiotic synthesis was: lactose, yeast extract and urea at concentrations of 26.0, 0.8 and 1.8% respectively. Under the optimized and validated conditions the transgalactosylation activity of *P. tsukubaensis* resulted in 28.35% of GOS yield at 24h with a GOS production of 73.71 g/L and approximately 50% of lactose hydrolysis. GOS producing by fermentation process can help to overcome the difficulties and expenses associated with enzyme extraction /purification from microorganism cells. It also helps to improve the development of a low-cost process for lactose hydrolysis.