Lyophilized yeast powder for adjuvant free thermostable vaccine delivery

Ravinder Kumar[a*], Bhushan N. Kharbikar[b], and Tejal A. Desai[b]

[a]Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Francisco, California - 94143, USA

[b]Department of Bioengineering and Therapeutic Sciences, University of California San Francisco, California, 94158, USA

[*]**Correspondence:**

Dr Ravinder Kumar, PhD

Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Francisco, California - 94143, USA

Email: raj86tau@gmail.com

Type of article:

Research Article

Keywords:

Yeast-based vaccine, *P. pastoris*, long term-stability, lyophilized, yeast powder

Running title:

Freeze-dried yeast powder for long-term storage of immunogen under the non-refrigerated condition

List of abbreviations: SEM, Scanning electron microscope; GVAP, Global Vaccine Action Plan; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19, coronavirus disease of 2019; APCs, antigen-presenting cells

Abstract

Thermolabile nature of commercially available vaccines necessitates their storage, transportation and dissemination under refrigerated condition. Maintenance of continuous cold chain at every step increases the final cost of vaccines. Any breach in the cold chain, even for a short duration results in the need to discard the vaccine. As a result, there is a pressing need for the development of thermostable vaccines. In this proof of concept study, we showed that *E. coli* curli-GFP fusion protein remains stable in freeze-dried yeast powder for more than a 13 and 6 months when stored at 30 °C and 37 °C respectively. Stability of the heterologous protein remains unaffected during the process of heat-inactivation and lyophilization. The mass of lyophilized yeast powder remains almost unchanged during the entire period of storage. Expressed protein remains intact even after two cycles of freeze and thaws. The protease deficient strain appears ideal for the development of whole recombinant yeast-based vaccines. The cellular abundance of expressed antigen in dry powder after a year was comparable to freshly lyophilized cells. SEM microscopy showed the intact nature of cells in powdered form even after a year of storage at 30 °C. Observation made in this study showed that freeze-dry yeast powder can play a vital role in the development of thermostable vaccines.

Introduction

In the last century, vaccines have proved to be one of the most important medical interventions in the fight against infectious diseases. Eradication of smallpox in 1980 (reviewed in **Fenner 1982)** and soon polio (reviewed in **Norrby *et al.* 2017)** are important success stories associated with the benefits of vaccines in public health. Widespread application of vaccines also leads to a sharp decline in newer cases of tuberculosis, hepatitis, measles, tetanus and other infectious diseases (**Versteeg *et al.* 2019)**. The importance of vaccines can also be underscored, by the fact that there is a race for vaccine development against novel coronavirus (SARS-CoV-2) responsible for present COVID-19 pandemic **(Callaway 2020)**. As per WHO estimates, every year, around 2-3 million lives are saved by vaccine application **(Cruz-Resendiz *et al.* 2020).** Still, millions of individuals (out of which majority are children below 5, years of age) die from vaccine-preventable diseases. Despite the significant efforts at every step, millions of people are left unvaccinated, thus putting them at the risk of getting the infection at some point in life. According to GVPA, 2011-2020 review report approximately 19.4 million infants did not receive lifesaving vaccines in 2018 (https://www.who.int/immunization /globalvaccine_action_plan/en/).

Among the various issues, associated with presently licensed vaccines (summarized by **Kumar and Kumar 2019),** inherent thermolabile nature of these vaccines makes their storage, transportation and dissemination a daunting task. In the field of vaccines, this is commonly known, as the "Cold Chain" problem and, at every step, maintenance of refrigerated condition (2-8 °C in general) is a must. In many instances, maintenance of continuous cold chain raises the cost of the vaccines by about 80 % (**Bandau *et al.* 2003; Das 2004**). Exposure of vaccine to sub-optimal temperature even for short duration leads to vaccine degradation and dramatic loss in efficacy or potency which forces almost 50 % of the vaccines to be discarded before their application (**Brandau *et al.* 2003; Hill *et al.* 2016**). This scenario becomes even more relevant in resource-poor settings in countries of Asia and Africa, which lack access to vaccines **(Chen and Kristensen 2009; Das 2004).** Therefore, improving the thermostability of available vaccines at ambient temperature is highly desirable.

To address, the problem of poor thermostability and short shelf life of vaccines at ambient temperature, different approaches have been taken in the past. In the case of human enterovirus type 71, biomineralization of virus particle improves thermal stability significantly **(Wang *et al.* 2013)**. In another common approach, modification in a liquid formulation was found encouraging. For example, the addition of stabilizers like deuterium oxide, proteins, $MgCl_2$, and non-reducing sugars in vaccine formulation improves their thermal stability **(Milsstein *et al.* 1997; Alcock *et al.***

2010). Similarly, the addition of anionic nanogold particles and PEG (polyethene glycol) improve the thermal stability of some of the vaccines significantly **(Pelliccia *et al.* 2016)**. Recently the coating of bacterial cells or viral particles in a thin film of sugar gave promising results **(Leung *et al*. 2019; Bajrovic *et al.* 2020).** All the above-highlighted approaches used the addition of one or more chemicals in vaccine preparation which, necessitating additional safety tests and other regulatory procedures. Moreover, the application of all the aforementioned approaches kept the vaccine stable only for a short duration depending upon vaccine and storage temperature. Use of these approaches makes the entire process lengthier and more cumbersome. Therefore, a procedure, which is simple, cost-effective, safe, while improving the thermal stability and shelf life of a vaccine at ambient temperature will be desirable.

It is well known and established, that the freeze-drying or lyophilization of biomolecules and biopharmaceuticals (proteins, lipids, nucleic acids) improves their stability as well as shelf life significantly **(Emami *et al.* 2018; Liao *et al.* 2004; Song *et al.* 2017).** Lyophilization has been shown to improve the stability of whole cells (example bacterial cell) and viral particles that are regularly used in conventional vaccines preparation **(Wang *et al.* 2012; Maa *et al.* 2004; Garmise *et al.* 2007)**. Although purified protein antigen can also be lyophilized and can be used as vaccine but poor immunogenicity and fast body clearance of pure protein remains an important concern**.** Apart from that application of purified antigen (subunit vaccines) will require use of adjuvant and will involve protein purification which will further increase cost, time and will affect the number of doses that can be available. On top of that lyophilized protein antigen will again require cold chain (**reviewed by Vartak *et al.* 2016; Kumru *et al.* 2014**). The natural adjuvant nature of yeast cell walls makes it possible to use lyophilized recombinant cells without the addition of an external adjuvant **(Stubbs *et al.* 2001)**. Unlike subunit vaccine, the yeast-based vaccines do not require protein purification. Yeast cells are efficiently taken up, by APCs **(Xiang *et al.* 2006)**. Handling of recombinant yeast is much easy and safer compared to infectious biological entities. Most importantly, the application of inactivated yeast cells is found safe and well-tolerated in human subjects **(Gaggar *et al.* 2014)**.

This forced us to investigate, whether whole recombinant yeast lyophilized powder can keep the heterologous protein (acting as an immunogen) intact when stored, at ambient temperature. In this proof of concept study, we showed that protein antigen remains stable in lyophilized yeast powder for more than a year when stored at 30 °C. Stability of heterologous fusion protein remains unaffected during the process of heat-inactivation and lyophilization. Apart from this, the mass of lyophilized yeast powder remains essentially unchanged. The observations from this study will

136 help in developing a thermostable vaccine with long shelf life even under non-refrigerator
137 condition (2-8 °C) using a yeast-based platform.

138 **Materials and methods**

139 **Yeast strains**

140 Haploid auxotrophic PPY12h (*arg4 his4*) **(Gould *et al.* 1992)** and protease deficient SMD1163
141 (*pep4 prb1 his4*) **(Gleeson *et al.* 1998)** *Pichia pastoris* strains, were used in the entire study**.**

142 **Media**

143 YPAD media (1 % yeast extract, 2 % peptone, 0.05% adenine and 2 % dextrose), SD+CSM-His
144 (0.17 % YNB without amino acid and ammonium sulfate, 0.5 % ammonium sulfate, 0.08 % CSM-
145 His, 2 % agar, 2 % dextrose) **(Kumar 2019)**.

146 **Cloning of *E. coli* curli protein**

147 Synthetic construct coding for *E. coli* curli was synthesized by a commercial vendor (Genewiz,
148 New Jersey, USA) into a pUC57-Amp vector. Fragment coding of curli ORF was excised from
149 the vector and subcloned into *Pichia pastoris* integrating vector pIB2 **(Sears *et al.* 1998)** into which
150 cycle 3 GFP, was cloned previously **(Crameri *et al.* 1996)**. The sequence of ORF was the same
151 as that of the original construct for which sequence, was deposited in Genebank with the following
152 accession number MH264502 **(Kumar 2018)**. Expression of the CSGA-GFP fusion protein was
153 under the constitutive GAP promoter (Glyceraldehydes-3 phosphate dehydrogenase). The
154 combined mass of the fusion protein was 42.9 kDa. It is, important to note that no codon
155 optimization for the construct was used in this study.

156 **Yeast transformation**

157 *Pichia pastoris* (now officially *Komagataella phaffii* or *K. phaffii*) **(Kurtzman 2009; Kumar *et*
158 ***al.* 2020)** transformation was performed as described previously **(Kumar 2019)** and briefly
159 mentioned here. The final plasmid (pRK10) was linearized by digestion with *EcoNI* which cleaves
160 within the *HIS4* marker gene. Linearized plasmid was, transformed into PPY12h, and SMD1163
161 strain using the electroporation method (GenePulser Xcell from Biorad). Transformants were
162 selected, on *His*⁻ plates and positive transformants were confirmed, both by the fluorescent
163 microscopy and western blot.

164 **Protein extraction from regular cycling cells**

165 Presence of *E. coli* CSGA-GFP protein in *P. pastoris* was confirmed by detecting CSGA-GFP
166 fusion protein using polyclonal rabbit anti-GFP antibodies (from Life Technologies USA; cat # A-
167 11122). Amount of protein was normalized, based on either the number of cells or by dry mass of
168 cells. Cells were treated, with 12.5 % TCA and samples were incubated at -80 °C for one hour or

169 overnight. On completion of incubation, samples were thawed, at room temperature and, the
170 samples were centrifuged, at 12000 g for 8 min. The supernatant was, discarded and, the pellet
171 was, washed twice with 80 % chilled acetone. Finally, protein pellet was air-dried and was
172 resuspended, in 150 μL 1 % SDS and 0.2 N NaOH. Then 150 μL 2X dye (100 mM Tris HCl pH6.8,
173 200 mM DTT, 4 % SDS, 0.2 % bromophenol blue, 20 % glycerol) was added to the sample and
174 samples were heated for 5 min at 95 °C using dry heating block. Samples were cooled down, to
175 room temperature, vortexed, spanned and, an equal amount or volume of samples was loaded, in
176 each well of 10 % SDS-PAGE along with pre-stained protein marker (Biorad, cat # 161-0376).
177 Samples were run at constant 100V till the dye front reaches the bottom of the gel **(Kumar 2019)**.

178 **Western blot**

179 On completion of SDS-PAGE run, proteins were transferred onto nitrocellulose membrane by wet
180 transfer (at constant 100 V for 1 hour at 4 °C) as described elsewhere **(Towbin *et al.* 1979)**. The
181 blotting membrane was incubated in blocking buffer (5 % nonfat skimmed milk powder in TBST)
182 (TBST, 137 mM NaCl, 2.7 mM KCl, 19 mM Tris base, 0.1 % Tween 20) for 1 hour and then
183 incubated with anti-GFP antibodies overnight at 4 °C under gentle shaking condition. Primary
184 antibodies were removed and, the membrane was washed thrice with TBST. The membrane was
185 again incubated with IRDye®800CW goat anti-rabbit secondary antibodies (from LI-COR, USA;
186 cat # 926-32211) for one hour at room temperature. The membrane was again washed thrice with
187 TBST and blot was scanned using LI-COR Odyssey software (from LI-COR, Nebraska, USA) as
188 per manufacturer instructions.

189 **Heat inactivation of recombinant *P. pastoris***

190 A single colony of recombinant *P. pastoris,* expressing *E. coli* CSGA-GFP under GAP promoter
191 was inoculated into 5 mL of YPAD tube. The tube was incubated, at 30 °C, 230 rpm for overnight
192 growth (source of inoculum). The overnight grew culture was used for the inoculation of 500 mL
193 YPAD in 2.8 L flasks. The flasks were incubated, at 30 °C, 230 rpm for 48 hours. Cells were
194 pelleted by centrifugation at 3000 *g* for 5 minutes. Cells were washed twice with sterile water.
195 Finally, the cell pellet was, resuspended in 25 mL sterile water and tube was incubated in a water
196 bath maintained at 56 °C for 90 min for heat-inactivation of yeast cells **(Haller *et al.* 2007).** After
197 90 min, tubes were taken out of a water bath, cooled to room temperature. A small volume (10 μL)
198 of cell suspension was taken out and plated on YPAD plate to check for the presence of viable
199 cells. The remaining cell suspension was, centrifuged at 3000 *g f*or 5 min, the supernatant was,
200 discarded and, pellet in tubes was put for lyophilization as described below.

201 **Lyophilization (freeze-drying) of whole recombinant *P. pastoris***

202 Recombinant *Pichia pastoris* was lyophilized as described elsewhere **(Patterson *et al.* 2015)** and
203 briefly mentioned here. Cells were harvested in 50 mL falcon tube by centrifugation at 3000 g for
204 5 minutes and removal of supernatant. Lyophilization was performed on the cell pellet using the
205 AdVantage 2.0 Bench Top Freeze Dryer/Lyophilizer (from SP Scientific). Samples in tubes were
206 regularly checked, for the formation of lyophilized powder. On completion of lyophilization, tubes
207 were, taken off and the combined mass of tube and lyophilized yeast powder was noted.

208 **Storage of freeze-dried yeast powder**

209 On completion of the process of freeze-drying, tubes were removed from the lyophilizer and caps
210 were tightly closed. Tubes were kept in cardboard boxes and stored in a separate incubator (in the
211 dark away from direct sunlight, no humidity) operating at 30 °C and 37 °C. Then the weight of
212 tubes was noted, at a regular interval. All steps were performed under normal lab environment (i.e.
213 not in an inert and sterile atmosphere).

214 **Protein extraction from lyophilized powder**

215 A known amount of lyophilized yeast powder was, taken in a sterile Eppendorf tube. The powder
216 was resuspended in 200 uL of 12.5 % TCA and stored at -80 °C for 1 hour or overnight. The
217 subsequent procedure is the same as described above for protein extraction.

218 **Freeze and thawing of lyophilized yeast powder**

219 As above, a known amount of lyophilized yeast powder was, taken into fresh sterile Eppendorf
220 tubes. Tubes were, stored at -20 °C and -80 °C for, two hours followed by incubating the tubes at
221 30 °C for two hours. This process was repeated, depending on the number of freeze-thaw cycles.
222 A known amount of yeast powder was also taken and incubated at 30 °C as a control. Protein
223 extraction was performed as described above.

224 **Field Emission Scanning Electron Microscopy**

225 The shape and surface morphology of lyophilized yeast cells stored at 30 °C for a year was
226 analyzed, by Field Emission Scanning Electron Microscopy (ZEISS Gemini Sigma 500 VP, Carl
227 Zeiss Microscopy LLC, NY, USA). Samples were prepared, by mounting a small amount of
228 lyophilized yeast powder on a double-sided carbon tape on a metal SEM stub. Gently spread the
229 yeast powder across the surface of the tape. Compressed air was, used to remove loose lyophilized
230 yeast cells. Yeast samples were sputter-coated with a 3 nm thin layer of Gold-Palladium for
231 ensuring the conductivity for electrons beam. A beam strength of 5.0 kV and a working distance
232 in the range of 8–9 mm was, used to visualize yeast samples.

233 **Fluorescence Microscopy**

234 Expression of *E. coli CSGA-GFP* fusion protein in both SMD116 and PPY12h *Pichia pastoris*
235 was confirmed, by fluorescence microscopy. For each strain, 100 uL cycling cells, were taken into
236 sterile Eppendorf tube, cells were pellet down by centrifugation at 3000 g for 3 min. The
237 supernatant was discarded, and the cell pellet was resuspended, in 1 mL sterile Milli Q water. 5
238 uL of cell suspension was transferred, onto a glass slide. Cells were fixed by adding an equivalent
239 volume of 1 % agarose. Images were captured, using plan apochromat 100× 1.40-NA oil
240 immersion objective on a motorized fluorescence microscope (Axioskop 2 MOT plus; Carl Zeiss)
241 coupled to a monochrome digital camera (AxioCam MRm; Carl Zeiss). Images analysis was
242 performed, using Axio vision software **(Kumar 2019)**.

243 **Results**

244 **The basic assumption behind the study**

245 In our previous study, we showed that stationary phase yeast cells were able to keep the
246 heterologous protein intact when stored at room temperature (23-25°C) for a year **(Kumar,2018)**.
247 Unfortunately, the gradual degradation of cells over time suggests that long-term storage of protein
248 antigen even within cells in a liquid environment is not feasible **(Kumar 2018)** Therefore, we
249 looked at other ways for long-term storage of cells at ambient temperature. It is a common
250 observation that active dry yeast powder sold commercially is stable (a significant proportion of
251 cells retain viability) for almost two years when stored under non-refrigerated conditions (at 23-
252 25 °C). Even under recommended conditions of storage and handling most of the available
253 vaccines remain potent for up to 1.5 to 3 years. Therefore it raised the possibility that dry yeast
254 powder can act as a simple yet an important, means for long-term storage of immunogen (vaccine)
255 under room temperature (ideally around two years) **(Clenet 2018; Chen *et al.* 2010; Galazka *et*
256 *al.* 1998; Chen and Zehrung 2013)**. Through this proof of concept study, we tested whether,
257 lyophilized yeast cells can be used, to store protein immunogen at ambient for year or more. For
258 this, we expressed *E. coli* curli-GFP fusion protein (*CSGA-GFP*) in *P. pastoris*. Curli is a bacterial
259 surface protein (fimbri) involved in cell adhesion and biofilm formation **(Barnhart *et al.* 2006;
260 Nguyen *et al.* 2014)**. Curli is capable of raising immune response, thereby acting as a good
261 immunogen (**Barnhart *et al.* 2006**) and a suitable, candidate for the proof of concept study.

262 **Expression of *E. coli CSGA-GFP* in *Pichia pastoris***

263 *E. coli* curli was subcloned into *P. pastoris* integrating vector pIB2 as described in the materials
264 and methods section. Cartoon presentation of the final plasmid used for the expression of *E. coli*
265 *CSGA-GFP* fusion protein is shown, in Figure 1A. For checking the expression of the CSGA-GFP

266 fusion protein, we randomly selected eight colonies from the transformant plate and patched them
267 on a fresh selection plate (CSM-HIS plate). Next day, the expression of the fusion protein was first
268 confirmed, by fluorescent microscopy (Figure 1B for PPY12h background). Later in a separate
269 experiment, the same plasmid was transformed, into SMD1163 strain. Integration of plasmid and
270 expression of CSGA-GFP was confirmed by fluorescent microscopy (Figure 1C).

271 Expression of the fusion protein, CSGA-GFP, was further confirmed by western blot. 2 OD_{600nm} of
272 cells were used for protein extraction for western blot. Blot image in Figure 1D shows that all the
273 selected colonies were positive. Ponceau-S stain image of the same blot is shown, as a loading
274 control (Figure 1E) which confirm the proper loading of protein in each well. Specific anticipated
275 bands are highlighted, by an arrow pointing towards them. We do not get any band in empty vector
276 control strain used as a negative control. The absence of any signal from negative control shows
277 the specificity of GFP antibodies used in the experiment. The expression of the heterologous
278 protein was endogenous. Overall we can say that we were able to express the fusion protein in *P.*
279 *pastoris*.

280 **Heat inactivation and lyophilization do not affect the stability of expressed antigen**

281 Yeast species especially *S. cerevisiae* and *P. pastoris* are non-pathogenic and are in the list of
282 GARS and routinely used for the production of diverse biomolecules for human consumption
283 (**Ramchuran *et al.* 2005; Basanta *et al.* 2010**). Even then. direct administration of live
284 recombinant yeast cells into human subjects is not advisable from the point of safety. However,
285 the level of immune response mounted on the application of whole recombinant yeast is
286 independent of live or dead nature of yeast (**Lu *et al.* 2004; Franzusoff *et al.* 2005**) raising the
287 possibility for application of inactivated recombinant yeast. Heat-inactivation of yeast is rapid,
288 simple and more convenient compared to chemical based-inactivation of bacterial or viral particles
289 for vaccine preparation. The combined effect of heat-inactivation and lyophilization on the
290 stability of heterologous protein was missing and through this study, we tried to fill that gap.

291 Heat inactivation of yeast cells was performed as described in the materials and methods. Heat
292 inactivation was confirmed by plating small volume of cell suspension on YPAD plates (Figure
293 2B) along with untreated control cycling cells (Figure 1A). Our present western blot data showed
294 that the stability of heterologous proteins remains essentially unaffected during heat-inactivation
295 (Figure 2C) and heat-inactivation followed by lyophilization (Figure 2E). Proper loading and
296 transfer of protein are shown by Ponceau-S stained blots (Figure 2D and 2E respectively). Protein
297 amount was normalized, by the weight of cells used for protein extraction. Results of Figure 2C

298 are in accordance with our previous study **(Kumar 2018)**. Based on the present data, it can be said
299 that together heat-inactivation and lyophilization have no affect protein stability.

300 **The protease deficient yeast strain improves the stability of the expressed fusion protein**

301 In our previous section, we showed that process of heat-inactivation and heat-inactivation followed
302 by lyophilization does not affect the stability of CSGA-GFP in yeast cells. But we do see a very
303 prominent band around 25 kDa region of the blot. Whether the observed bands were due to degra-
304 dation of the fusion protein on heat-inactivation and lyophilization, or whether they were a result
305 of protease activities in cytosol and vacuoles was not clear. To sort out this, we transform the same
306 construct into protease deficient strain (SMD1163), which lacks Prb1 (cytosolic) and Pep4 (vacu-
307 olar). Our present western blot data pointed towards the idea that observed lower bands are the
308 result of vacuolar protease action (Figure 2G). Like previous blots, we again observed lower bands
309 in PPY12h strain used as a control (Figure 2G, well1), but failed to detect the same bands in
310 SMD1163 (Figure 2G, well 2,3). The presence or absence of lower bands in PPY12h and
311 SMD1163 respectively is not due to the difference in the amount of protein loaded into each well
312 was confirmed by Ponceau S stained blot image (Figure 2H). Therefore, it can be concluded that
313 lower observed bands are not due to heat-inactivation or lyophilization but may be due to the action
314 of cellular proteases. Our observation is supported by the fact that, during stress, autophagic path-
315 ways get activated, which forces the degradation of cellular components in vacuoles or lysosomes
316 **(Klionsky *et al.* 2007; Takeshige *et al.* 1992; Kumar *et al.* 2020)**. Therefore lower bands detected
317 in PPY12h and their absence in SMD1163 may be due to autophagic degradation of fusion proteins
318 in vacuoles and GFP (26.7 kD), which is quite stable in vacuoles (whose free release in the vacuole
319 is used in autophagic assays) was detected by antibodies **(Klionsky *et al.* 2007; Takeshige *et al.*
320 **1992)**.

321 **Mass of freeze-dried yeast powder remains unchanged during storage**

322 After confirming that heat-inactivation, as well as freeze-drying, do not affect the stability of
323 expressed protein, we increase the volume of culture to 1 L. PPY12h strain expressing CSGA-GFP
324 was grown in bulk volume (1 L). Cells were, harvested heat-inactivated and freeze-dried as
325 described in Materials and Methods. After lyophilization, tubes were taken off from lyophilizer,
326 screwed the cap and weighed. Initial mass was taken and stored at 30 °C for one year. The weight
327 of the tube was, then checked, regularly and sometimes the tubes were opened, for a short time.
328 Table 1 is showing the data for the mass of powder stored at 30 °C. Similarly, we checked, the
329 weight of freeze-dried yeast powder stored at 37 °C for six months (Table 2). Present data showed
330 that mass of freeze-dried powder remains essentially unchanged under both conditions of storage.

331 The slight variation observed in weight may be due to moisture that might be entered into tubes
332 when tubes were open for some time. Note in Table 1 data is shown for two conditions. In one
333 condition cells were lyophilized after-heat inactivation (Table 1 second and third column) and in
334 another, cells were lyophilized without heat-inactivation (table 1 fourth and fifth column). Based
335 on the data shown in tables, it can be said, that the mass of stored lyophilized cells does not change
336 significantly.

337 **Cells in lyophilized powder retain their intactness**

338 Next we asked whether cells in lyophilized powder also remain intact by checking the morphology
339 and surface appearance of cells using SEM microscopy. A small amount of lyophilized powder
340 (figure 3A cells were lyophilized without heat-inactivation and, figure 3B cells were lyophilized
341 after heat-inactivation) was taken and SEM microscopy was performed. SEM images showed that
342 the cells remain intact in lyophilized powdered form when stored at 30 °C for more than a year
343 (figure 3C and 3D). Although cells were intact under both the condition, we observed a difference
344 in the texture of the powder. Freeze dry powder of cells without heat-inactivation appears less
345 compact and less dense (figure 3A) whereas heat-inactivated powder appear more compact and
346 denser (figure 3B). Thus, it can be said, that cells in lyophilized powdered form remain intact for
347 a year even when stored under ambient or room temperature.

348 **Expressed antigen remains stable in lyophilized yeast powder stored at 30 °C temperature**

349 Although SEM data in the previous section confirmed intact nature of lyophilized yeast cells after
350 a year of storage at 30 °C, whether expressed protein also remained intact was unknown. To check
351 the stability of the expressed protein, we took equal amounts of lyophilized powder from both the
352 condition and extracted the protein. An equal amount of whole cell lysate was loaded on 10 %
353 SDS-PAGE and, the fusion protein was detected using anti-GFP antibodies. Our present western
354 blot data showed that expressed protein remains stable in powdered yeast stored at 30 °C for a year
355 (figure 4). The expressed protein remains stable under both the conditions in which cells were
356 lyophilized without heat-inactivation (figure 4A) and in which cells were lyophilized after heat-
357 inactivation (figure 4B). Before incubation in blocking buffer, blots were stained with Ponceau S
358 for showing the loading control (figure 5C and 5D for figure 4A and 4B respectively).

359 Encouraged from the stability of the protein in lyophilized powder stored at 30 °C for a year, we
360 checked whether expressed protein also remains stable in lyophilized yeast cells stored, at 37 °C
361 for six months. Our present western blot data showed that expressed protein remains stable in
362 powdered yeast even when stored at 37 °C at least for six months (figure 4E). Loading control is
363 shown in figure 4F. Therefore, it can be, concluded that expressed protein remains stable in

364 lyophilized yeast cells when stored at a temperature ranging from 30 °C -37 °C for quite a long
365 time.

366 **Freeze and thaw do not affect protein stability in powdered yeast**

367 Most of the commonly used vaccines are stored at 2-8 °C, while some of them are also stored at -
368 15 °C to -50 °C (e.g. measles, mumps and rubella or MMR, is stored at +8 °C to -50 °C,
369 https://www.merckvaccines.com/mmr/storage-handling/). However for most vaccines, exposure
370 to sub-optimal temperature (i.e. more than 2-8 C or below freezing temperature) even for a short
371 duration is known to reduce vaccine potency dramatically) **(Brandau *et al.* 2003; Hill *et al.* 2016)**.
372 An ideal vaccine should remain stable and retain potency even when stored under the non-
373 refrigerated condition as well as under accidental freeze condition. For investigating the effect of
374 freeze and thaw on the stability of the heterologous protein in lyophilized yeast cells, known
375 amount of yeast powder was taken into separate tubes. Tubes were stored at -20 °C and -80 °C for
376 two hours. Freeze and thaw were performed in one cycle and two-cycle. For each condition,
377 samples were taken, in duplex. After a freeze, thawing was performed by incubating samples at 30
378 °C for two hours. Our present western blot data showed that expressed protein in lyophilized yeast
379 powder remain stable following one as well as two cycles of freeze and thaw at both -20 °C (figure
380 5A) and -80 °C (figure 5B). Ponceau S stained image of the membrane (as a loading control), is
381 shown next to the blot. A known amount of yeast powder, stored at 30 °C separately was taken, as
382 a control. Conditions of freeze and thaw are mentioned, in the materials and methods section.
383 Based on present data, it can be said that expressed protein remains stable both under the non-
384 refrigerated condition for a year as well as under freeze and thaw condition.

385 **The abundance of expressed protein after a year was similar to fresh cells**

386 In the previous section, we showed that the expressed CSGA-GFP fusion protein was stable in
387 lyophilized yeast powder when stored at 30 °C for a year and also survived two cycles of freeze
388 and thaw. But whether the expressed protein deteriorated over time and to what extent still needed
389 to be determined. To answer this question, we compared the level of expressed protein in yeast
390 powder stored for a year to the freshly lyophilized yeast cells. Before comparing, the level of fusion
391 protein we checked the overall protein content in the two samples (Figure 6A). Although an equal
392 amount of whole-cell lysate, was loaded for each sample, we still see a slightly low level of
393 proteins in a sample from year-old powder (well 6,7) compared to freshly lyophilized cells (well
394 4,5). Apart from this we also observed relatively more background in a year old sample. Whether
395 the observed difference in the level of proteins and background is due to the difference in protein
396 extraction efficiencies or protein degradation is unclear. But we do find a problem in re-suspending

397 the powder entirely even on vigorous vortex. We also loaded an equal amount of cell lysate from
398 normal cycling cells as control (well 2,3).
399 After looking at the overall proteome of lyophilized cells stored for a year and freshly lyophilized
400 cells, we proceed to compare the level of the expressed fusion protein in two samples. Our present
401 data (Figure 6B) shows that level of the CSGA-GFP fusion protein is almost similar in freshly
402 lyophilized cells (figure 6B, well 4) to that of one-year-old powder (figure 6B, well 2,3). Note we
403 used the same sample for western blot which, were used in running SDS-PAGE shown in figure
404 6A. And the slight difference which appears in two samples may be due to the issues mentioned
405 above. Loading control is shown, by Ponceau-S stained image of the same blot (figure 6C). To
406 capture any possible difference in samples blot images were captured at low intensity of exposure
407 (data for high exposure, is not shown).

408 **Discussions**

409 Storage, transportation and distribution of vaccines before their final application are still presents
410 difficulties. On one hand, exposure of vaccine to temperatures more than the recommended
411 temperatures (generally 2-8 °C) leads to vaccine degradation, denaturation and finally loss of
412 vaccine potency or efficacy **(Brandau *et al.* 2003; Hill *et al.* 2016)**. On the other hand, exposure
413 of vaccines to below freezing conditions also affect vaccine potency **(Lloyd *et al.* 2015; Kumru**
414 ***et al.* 2014)**. Therefore, maintenance of optimum conditions from point of manufacturing till the
415 final application is required which is more difficult in less well-developed countries. A solution to
416 this is the development of thermostable vaccines. Availability of thermostable vaccines will be an
417 important, step in global immunization step **(Lee *et al.* 2017**). The thermostable nature of future
418 vaccines will not only make vaccine transport, storage and distribution more convenient and
419 economical but, will also help in saving a huge, amount of life-saving vaccines. Currently, the
420 development of thermostable vaccines are yet unmet, with little success in which thermal stability
421 was observed (only for a few months at best and that too in few cases) (**Leung *et al.* 2019; Chu *et***
422 ***al.* 2016; Mistilis *et al.* 2017; Hassett *et al.* 2013, 2015; Chen *et al.* 2010; Ohtake *et al.* 2010,**
423 **2011; Lovalenti *et al.* 2016**). By using thin-film coating, one study was able to keep the adenovirus
424 stable at room ambient temperature for three years (**Bajrovic *et al.* 2020).** It will be interesting to
425 see whether this approach can be applied to other vaccines. But this study showed the possibility
426 that a vaccine may be stored and transported at ambient temperature. So far, most of the approaches
427 used for enhancing the thermal stability and shelf life of vaccines at ambient temperature require
428 the addition of different chemicals. Assessing the safety of each chemical added in formulation
429 requires more regulatory clearance, time and money. Moreover, whether the developed

430 formulation applies to a wide range of vaccines also remain a matter of future research. Unlike
431 conventional vaccine preparation which requires growth of bacterial culture or viral particles
432 followed by formalin-based inactivation which itself is quite lengthy, growth and heat-inactivation
433 of yeast are rapid and more economical.

434 The degradation of expressed immunogen by vacuolar proteases may pose a serious issue in the
435 development of a whole recombinant yeast-based vaccine. The degradation of immunogen will
436 not only reduce the level of the immunogen in cells but may also affect the level of the immune
437 response. It is, therefore, important to use protease deficient strain (like SMD1163) **(Gleeson *et*
438 ***al.* 1998**). Compared to budding yeast, the post-translation modification *P. pastoris* is closer to
439 those of higher eukaryotes **(Cregg *et al.* 1993)**. The fact that P. pastoris is better fermenter is other
440 added advantages **(Cereghino and Cregg, 2000)** when a large amount of vaccine is needed such
441 as during a pandemic.

442 Surprisingly, we do not observe any bacterial contamination in yeast powdered stored for the year
443 even though we opened the tubes several times in a year. This can be the result of heat-inactivation
444 followed by lyophilization. This observation showed, that lyophilized yeast powder can be, stored
445 for a long time even in the absence of antibiotics. Several conventional vaccine preparations
446 involve the use of antibiotics such as MMR which not only increase the cost of vaccines production
447 but also cause an allergic response in some people **(Chung, 2014)**.
448 Although we have shown the data for the stability of heterologous protein for a year (at 30 °C) and
449 six months (at 37 °C), we expect that expressed protein may remain stable for much longer (as a
450 result we are still keeping our samples at the aforementioned condition). Further, it will be
451 interesting to see whether recombinant yeast lyophilized powder stored at ambient for a year or
452 more can mount a specific immune response *in vivo* models like a mouse. Previous observation
453 that APCs can process freeze-dried yeast cells and can mount an immune response relates to our
454 present and future study in this direction **(Patterson *et al.* 2015).** It is almost impossible to mimic
455 the real scenario, but our present data showed that lyophilized yeast powder, can prevent
456 immunogen degradation due accidental freeze and thaw that was lacking in other approaches used
457 for the development of thermostable vaccines. Yeast-based vaccines, which are in different phases
458 of clinical trials may present opportunities where the present method for developing thermostable
459 vaccines can be adopted directly **(Kumar and Kumar, 2019; Ardiani** *et al.* **2010).** Moreover,
460 there is no obvious barrier why our approach cannot be directly adopted for subunit vaccines where

protein immunogen have been already characterized (**Agwale *et al.* 2002; Zhang *et al.* 2015; Schiller and Lowy, 2015**).

Overall, we have shown that a dry yeast powder is a simple yet effective way for long-term storage of vaccines (immunogen) under non-refrigerated condition. Data shown in this report showed that yeast-based approach for thermostable vaccine development prevents vaccine deterioration both at high temperature (above 2-8 °C) as well as below freezing condition (below 0 °C) which is common in countries of Europe, North America and parts of Asia. We believe that this cost effective freeze-dried yeast powder approach may be one solution to the cold chain problem and will go towards attaining the universal immunization program.

Acknowledgements

We are thankful to the University of California San Francisco (UCSF) San Francisco, California-USA for providing space and other necessary facilities towards completing this manuscript. We are also thankful to Prof. Geoff Lin-Cereghino from Pacific University, Stockton, California-USA for providing SMD1163 strain, Prof. Robert Stroud from UCSF for electroporation of yeast cells. We are also thankful to the staff at the University of California Berkeley Electron Microscope Laboratory for advice and assistance in electron microscopy, sample preparation and data collection. We are specially thankful to Prof. Jennifer C. Fung for allowing us to carry out this work in her lab.

Funding

The author declares that no funding agency to be reported.

Authors Contribution

RK conceived, designed, performed the experiments, analyzed the data and wrote the manuscript. BNK perform SEM and fluorescence microscopy. TAD contributed resources for this work.

Conflict of interest

The authors declare that no conflict of any kind exists.

Declaration

Authors declare that the present study does not involve any human subjects or animal handling by any author mentioned in this study.

ORCID

Dr Ravinder Kumar, PhD; https://orcid.org/0000-0003-1922-0030

Dr Bhushan N. Kharbikar, PhD; https://orcid.org/0000-0003-0394-0308

References

495 1. Agwale SM, Shata MT, Reitz MS Jr et al. A Tat subunit vaccine confers protective
496 immunity against the immune-modulating activity of the human immunodeficiency virus
497 type-1 Tat protein in mice. *PNAS USA* 2002;**99**:10037-41.
498 2. Alcock R, Cottingham MG, Rollier CS *et al*. Long-Term Thermostabilization of Live
499 Poxviral and Adenoviral Vaccine Vectors at Supraphysiological Temperatures in
500 Carbohydrate Glass. *Science Translational Medicine* 2010;**2**:19ra12
501 3. Ardiani A, Higgins JP, Hodge JW. Vaccines based on whole recombinant Saccharomyces
502 cerevisiae cells. *FEMS Yeast Res*. 2010;**10**:1060-9.
503 4. Bajrovic I, Schafer SC, Romanovicz DK et al. Novel technology for storage and
504 distribution of live vaccines and other biological medicines at ambient temperature. *Sci*
505 *Adv* 2020;**6**:eaau4819.
506 5. Barnhart MM, Chapman MR. Curli biogenesis and function. *Annu Rev Microbiol*.
507 2006;**60**:131-147.
508 6. Basanta A, Gómez-Sala B, Sánchez J et al. Use of the yeast Pichia pastoris as an expression
509 host for secretion of enterocin L50, a leaderless two-peptide (L50A and L50B) bacteriocin
510 from Enterococcus faecium L50. *Appl Environ Microbiol*. 2010;**76**:3314-24.
511 7. Brandau D, Jones L, Wiethoff C et al.. Thermal stability of vaccines. *Journal of*
512 *Pharmaceutical Sciences* 2003;**92**:218-231.
513 8. Callaway E. The race for coronavirus vaccines: a graphical guide. *Nature*. 2020;**580**:576-
514 577.
515 9. Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast
516 Pichia pastoris. *FEMS Microbiol Rev*. 2000;**24**:45-66.
517 10. Chen D, Kapre S, Goel A et al. Thermostable formulations of a hepatitis B vaccine and a
518 meningitis A polysaccharide conjugate vaccine produced by a spray drying method.
519 *Vaccine* 2010;**28**:5093-9.
520 11. Chen D, Kristensen D. Opportunities and challenges of developing thermostable vaccines.
521 *Expert Rev Vaccines* 2009;**8**:547-557.
522 12. Chen D, Zehrung D. Desirable attributes of vaccines for deployment in low-resource
523 settings. *J Pharm Sci* 2013;**102**:29-33.
524 13. Chu LY, et al. Enhanced Stability of Inactivated Influenza Vaccine Encapsulated in
525 Dissolving Microneedle Patches. *Pharm. Res.* 2016;**33**:868–878.
526 14. Chung EH. Vaccine allergies. *Clin Exp Vaccine Res* 2014;**3**:50-57.

527 15. Clénet D. Accurate prediction of vaccine stability under real storage conditions and during
528 temperature excursions. *Eur J Pharm Biopharm* 2018;**125**:76-84.
529 16. Crameri A, Whitehorn EA, Tate E et al. Improved green fluorescent protein by molecular
530 evolution using DNA shuffling. *Nat Biotechnol* 1996;**14**:315-9.
531 17. Cregg JM, Vedvick TS, Raschke WC. Recent advances in the expression of foreign genes
532 in Pichia pastoris. *Biotechnology (N Y)* 1993;**11**:905-10.
533 18. Cruz-Reséndiz A, Zepeda-Cervantes J, Sampieri A et al. A self-aggregating peptide:
534 implications for the development of thermostable vaccine candidates. *BMC Biotechnol*.
535 2020;**20**:1.
536 19. Das P. Revolutionary vaccine technology breaks the cold chain. *Lancet Infect Dis*
537 2004;**4**:719.
538 20. Emami F, Vatanara A, Park EJ et al. Drying Technologies for the Stability and
539 Bioavailability of Biopharmaceuticals. *Pharmaceutics* 2018;**10**:131.
540 21. Fenner F. A successful eradication campaign. Global eradication of smallpox. *Rev Infect*
541 *Dis* 1982;**4**:916-930.
542 22. Franzusoff A, Duke RC, King TH et al. Yeasts encoding tumour antigens in cancer
543 immunotherapy. *Expert Opin Biol Ther* 2005;**5**:565-75.
544 23. Gaggar A, Coeshott C, Apelian D et al. Safety, tolerability and immunogenicity of GS-
545 4774, a hepatitis B virus-specific therapeutic vaccine, in healthy subjects: a randomized
546 study. Vaccine 2014;**32**:4925-31.
547 24. Galazka A, Milstien J, Zaffran M. Thermostability of Vaccines.Global Programmefor
548 Vaccines and Immunization (World Health Organization, Geneva) (1998).
549 25. Garmise RJ, Staats HF, Hickey AJ. Novel dry powder preparations of whole inactivated
550 influenza virus for nasal vaccination. *AAPS Pharm Sci Tech* 2007;**8**:2-10.
551 26. Gleeson MA, White CE, Meininger DP et al. Generation of protease-deficient strains and
552 their use in heterologous protein expression. *Methods Mol Biol* 1998;**103**:81–94.
553 27. Gould SJ, McCollum D, Spong AP et al. Development of the yeast *Pichia pastoris* as a
554 model organism for a genetic and molecular analysis of peroxisome assembly. *Yeast*
555 1992;**8**:613-628.
556 28. Haller AA, Lauer GM, King TH et al. Whole recombinant yeast-based immunotherapy
557 induces potent T cell responses targeting HCV NS3 and Core proteins. *Vaccine*
558 2007;**25**:1452–63.

559 29. Hassett KJ, et al. Glassy-State Stabilization of a Dominant Negative Inhibitor Anthrax
560 Vaccine Containing Aluminum Hydroxide and Glycopyranoside Lipid A Adjuvants. *J.*
561 *Pharm. Sci.* 2015;**104**:627–639.
562 30. Hassett KJK, et al. Stabilization of a recombinant ricin toxin A subunit vaccine through
563 lyophilization. *Eur. J. Pharm. Biopharm.* 2013;**85**:279–86.
564 31. Hill A, Kilgore C, McGlynn M et al. Improving global vaccine accessibility. *Current*
565 *Opinion in Biotechnology* 2016;**42**:67-73.
566 32. Klionsky DJ, Cuervo AM, Seglen PO. Methods for monitoring autophagy from yeast to
567 human. *Autophagy* 2007;**3**:181-206.
568 33. Kumar R, Kumar P. Yeast-based vaccines: New perspective in vaccine development and
569 application. *FEMS Yeast Research* 2019;**19**:1-22.
570 34. Kumar R, Rahman MA, Nazarko TY. Nitrogen starvation and stationary phase lipophagy
571 have distinct molecular mechanisms. *International Journal of Molecular Sciences*
572 2020;**21**:1-13
573 35. Kumar R. Investigating the long-term stability of protein immunogen(s) for whole
574 recombinant yeast-based vaccines. *FEMS Yeast Research* 2018;**18**:1-13.
575 36. Kumar R. Simplified protocol for faster transformation of (a large number of) *Pichia*
576 *pastoris* strains. *Yeast* 2019;**36**:399-410
577 37. Kumru OS, Joshi SB, Smith DE et al. Vaccine instability in the cold chain: mechanisms,
578 analysis and formulation strategies. *Biologicals* 2014;**42**:237-59
579 38. Kurtzman CP. Biotechnological strains of Komagataella (Pichia) pastoris are
580 Komagataella phaffii as determined from multigene sequence analysis. *Journal of*
581 *Industrial Microbiology & Biotechnology* 2009;**36**:1435–8.
582 39. Lee BY, Wedlock PT, Haidari LA et al. Economic impact of thermostable vaccines.
583 *Vaccine* 2017;**35**:3135-3142.
584 40. Leung V, Mapletoft J, Zhang A et al. Thermal Stabilization of Viral Vaccines in Low-Cost
585 Sugar Films. *Sci Rep* 2019;**9**:7631.
586 41. Liao YH, Brown MB, Martin GP. Investigation of the stabilisation of freeze-dried
587 lysozyme and the physical properties of the formulations. *Eur. J. Pharm. Biopharm.*
588 2004;**58**:15-24.
589 42. Lloyd J, Lydon P, Ouhichi R et al. Reducing the loss of vaccines from accidental freezing
590 in the cold chain: the experience of continuous temperature monitoring in Tunisia. *Vaccine*

591 2015;**33**:902-907.
592 43. Lovalenti PM, et al. Stabilization of live attenuated influenza vaccines by freeze drying,
593 spray drying, and foam drying. *Pharm. Res.* 2016;**33**:1144–1160.
594 44. Lu Y, Bellgrau D, Dwyer-Nield LD et al.. Mutation-selective tumor remission with Ras-
595 targeted, whole yeast-based immunotherapy. *Cancer Res* 2004;**64**:5084-8.
596 45. Maa YF, Ameri M, Shu C et al. Influenza vaccine powder formulation development:
597 Spray-freeze-drying and stability evaluation. *J. Pharm. Sci* 2004;**93**:1912-1923.
598 46. Milistien JB, Stanley ML, Peter FW. Development of a More Thermostable Poliovirus
599 Vaccine. *The Journal of Infectious Diseases* 1997;**175**(Suppl 1):S247-53
600 47. Mistilis MJ, et al. Long-term stability of influenza vaccine in a dissolving microneedle
601 patch. Drug Deliv. *Transl. Res.* 2017;**7**:195–205.
602 48. Nguyen P, Botyanszki Z, Tay P *et al*. Programmable biofilm-based materials from
603 engineered curli nanofibres. *Nat Commun* 2014;**5**:4945.
604 49. Norrby E, Uhnoo I, Brytting M et al. Polio närmar sig utrotning [Polio close to eradication].
605 *Lakartidningen*. 2017;**114**:EPDT.
606 50. Ohtake S, et al. Room temperature stabilization of oral, live attenuated Salmonella enterica
607 serovar Typhi-vectored vaccines. *Vaccine.* 2011;**29**:2761–71.
608 51. Ohtake S, et al. Heat-stable measles vaccine produced by spray drying. *Vaccine.*
609 2010;**28**:1275–84.
610 52. Patterson R, Eley T, Browne C et al. Oral application of freeze-dried yeast particles
611 expressing the PCV2b Cap protein on their surface induce protection to subsequent PCV2b
612 challenge in vivo. *Vaccine* 2015;**33**:6199-6205.
613 53. Pelliccia M, Andreozzi P, Paulose J et al. Additives for vaccine storage to improve thermal
614 stability of adenoviruses from hours to months. *Nat Commun* 2016;**7**:13520.
615 54. Ramchuran SO, Mateus B, Holst O et al.. The methylotrophic yeast Pichia pastoris as a
616 host for the expression and production of thermostable xylanase from the bacterium
617 Rhodothermus marinus. *FEMS Yeast Res*. 2005;**5**:839-50.
618 55. Schiller JT, Lowy DR. Raising expectations for subunit vaccine. *J Infect Dis*.
619 2015;**211**:1373-5.
620 56. Sears IB, O'Connor J, Rossanese OW et al. A versatile set of vectors for constitutive and
621 regulated gene expression in Pichia pastoris. *Yeast* 1998;**14**:783-90.
622 57. Song JG, Lee SH, Han H. The stabilization of biopharmaceuticals: Current understanding

623 and future perspectives. *J. Pharm. Investig* 2017;**47**:475–496.
624 58. Stubbs AC, Martin KS, Coeshott C et al. Whole recombinant yeast vaccine activates
625 dendritic cells and elicits protective cell-mediated immunity. *Nat Med* 2001;**7**:625-9.
626 59. Takeshige K, Baba M, Tsuboi S et al. Autophagy in yeast demonstrated with proteinase-
627 deficient mutants and conditions for its induction. *J Cell Biol* 1992;**119**:301-11.
628 60. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide
629 gels to nitrocellulose sheets: Procedure and some applications. *PNAS USA* 1979;**76**:4350–
630 4354.
631 61. Vartak A, Sucheck SJ. Recent Advances in Subunit Vaccine Carriers. *Vaccines (Basel)*.
632 2016;**4**:12.
633 62. Versteeg L, Almutairi MM, Hotez PJ et al. Enlisting the mRNA Vaccine Platform to
634 Combat Parasitic Infections. *Vaccines (Basel)* 2019;**7**:122.
635 63. Wang G, Cao RY, Chen R et al. Rational design of thermostable vaccines by engineered
636 peptide-induced virus self-biomineralization under physiological conditions. *PNAS USA*
637 2013;**110**:7619-7624.
638 64. Wang SH, Kirwan SM, Abraham SN et al Stable dry powder formulation for nasal delivery
639 of anthrax vaccine. *J. Pharm. Sci* 2012;**101**:31-47.
640 65. Xiang SD, Scholzen A, Minigo G et al. Pathogen recognition and development of
641 particulate vaccines: does size matter? *Methods* 2006;**40**:1-9.
642 66. Zhang W, Sack DA. Current Progress in Developing Subunit Vaccines against
643 Enterotoxigenic Escherichia coli-Associated Diarrhea. *Clin Vaccine Immunol*
644 2015;**22**:983-91.
645
646
647 **Legends**
648
649 **Tables**
650 **Table 1. Mass of lyophilized yeast powder stored at 30 °C for one year**
651 **Table 2. Mass of lyophilized yeast powder stored at 37 °C for six months**
652
653 **Figures**
654 **For Graphical abstract**
655 **Schematic showing the basic workflow of the entire study.**
656
657 **Figure 1. Expression of *E. coli* CSGA-GFP fusion protein in *P. pastoris***. (A) Map of the final
658 plasmid used for expression of the CSGA-GFP fusion protein. Expression of a CSGA-GFP fusion
659 protein in (B) PPY12h and (C) SMD1163 strain. Scale bar represents 5 uM. (D) Image of western

660 blot showing expression of the CSGA-GFP fusion protein in PPY12h. Bands of interest are pointed
661 by arrows towards them, while free GFP is pointed by an Asterisk mark. (E) Proper loading and
662 protein transfer are shown, by Ponceau S stained blot image. Well, 1 (pre-stained protein marker),
663 well 2 (empty vector as a negative), well 3-8 (transformants).

664 **Figure 2. Heat-inactivation and freeze-drying do not affect protein stability.** (A) Control
665 untreated viable cells. (B) Complete loss of viability on heat-inactivation. (C) Western blot image
666 showing the effect of heat-inactivation on protein stability. Well, 1, 2 (untreated cycling cells);
667 well 3 4 (heat-inactivated cells). (D) Proper loading and protein transfer is shown by Ponceau S
668 stain blot image. Western blot image showing the effect of freeze-drying on protein stability (E)
669 well 1, 2 (control cycling cells); well 3,4 (lyophilized yeast cells). (F) Proper loading and protein
670 transfer is shown by Ponceau S stain blot image. Bottom free GFP bands are due to vacuolar
671 protease (G) well 1 (PPY12 strain); well 2-3 (SMD1163, protease deficient strain, lack Prb1
672 cytosolic and Pep4 vacuolar protease). (H) Proper loading and protein transfer is shown by
673 Ponceau S stain blot image. Bands of interest are pointed by arrows towards them, while free GFP
674 is pointed by an Asterisk mark.

675 **Figure 3. Cells in lyophilized powder remain intact for more than a year.** Image of lyophilized
676 yeast powder stored at 30 °C for more than a year (A) cells was lyophilized without heat-
677 inactivation and (B) cells were heat-inactivated before lyophilization. SEM images showing
678 external morphology of lyophilized cells after one year (C) lyophilized cells without heat-
679 inactivation and (D) lyophilized cells after heat-inactivated.

680 **Figure 4. Expressed protein remains stable in powdered yeast.** (A) Stability of CSGA-GFP in
681 freeze-dried yeast powder stored at 30 °C after one year. (B) Stability of CSGA-GFP in heat-
682 inactivated lyophilized yeast powder stored at 30 °C after one year. (C) Stability of heat-inactivated
683 lyophilized yeast powder stored at 37 °C after six months. In each case, proper loading is shown,
684 by Ponceau S stained blot image. Note, the protein amount was normalized, based on yeast powder
685 used for protein extraction. Bands of interest are pointed by arrows towards them, while free GFP
686 is pointed by an Asterisk mark. Note in figure Y stand for yes and N for no for condition mentioned
687 in the figure.

688 **Figure 5. Freeze and thaws do not affect the stability of the expressed protein.** (A) Effect of
689 freeze and thaw at -20 °C. Control (lyophilized powder stored at 30 °C), well 1,2 (once the cycle
690 of freeze and thaw), well 3,4 (two-cycle of freeze and thaw). (B) Effect of freeze and thaw at -80
691 °C. Same common control as above, well 1,2 (one cycle of freeze and thaw), well 3,4 (two-cycle
692 of freeze and thaw). Note, in all case the protein amount was normalized based on the weight of
693 powder and, each sample was taken in duplex. Loading and proper transfer are shown through
694 Ponceau S stained of same blots. Also, a control sample is common in panel A and B.

695 **Figure 6. The abundance of heterologous protein in a year old powdered and freshly**
696 **lyophilized cells are similar.** (A) The efficiency of protein extraction from different samples.
697 Cycling cells without lyophilized (well 2,3), an equivalent number of cells after lyophilized
698 powder (well 4,5), an equivalent amount of lyophilized powder stored at 30 °C for one year (well
699 6,7), well 1 for protein marker. (B) The abundance of expressed protein is similar in freshly

lyophilized cells and year-old powder lyophilized powder after one year at 30 C (well 2,3), freshly prepared powder (well 4) and protein marker (well 1). The same blot was Ponceau S stained before blocking in skimmed milk powder in TBST shown as a loading control (C).

Tables

Table 1. Mass of lyophilized yeast powder stored at 30 °C for one year

Month	*Heat inactivated*		*Without heat inactivated*	
	Tube 1	*Tube 2*	*Tube 1*	*Tube 2*
0	16.88	16.97	17.06	17.26
2	16.97	16.96	17.14	17.29
4	16.95	16.91	17.14	17.27
6	16.98	16.93	17.13	17.26
8	16.95	16.91	17.15	17.21
10	16.96	16.93	17.16	17.21
12	16.97	16.95	17.15	17.22

Note: Net mass is in grams. Reported mass is the combined mass of tube and powder.

Table 2. Mass of lyophilized yeast powder stored at 37 °C for six months

Month	*Tube 1*	*Tube 2*
0	1.02	0.98
1	1.02	1.01
2	1.05	1.01
3	1.05	1.00
4	1.04	1.00
5	1.05	1.00
6	1.05	1.00

Note: Net mass is in grams (reported are the values after deducting mass of empty tube)

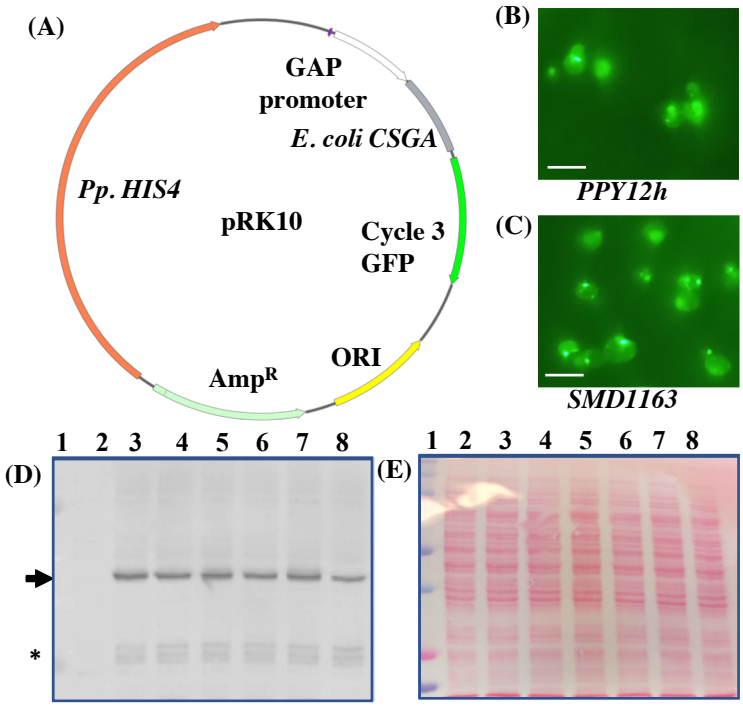

Figure 1. Expression of E. coli CSGA-GFP fusion protein in *P. pastoris.* (A) Map of the final plasmid used for expression of the CSGA-GFP fusion protein. Expression of a CSGA-GFP fusion protein in (B) PPY12h and (C) SMD1163 strain. Scale bar represents 5 uM. (D) Image of western blot showing expression of the CSGA-GFP fusion protein in PPY12h. Bands of interest are pointed by arrows towards them, while free GFP is pointed by an Asterisk mark. (E) Proper loading and protein transfer are shown, by Ponceau S stained blot image. Well, 1 (pre-stained protein marker), well 2 (empty vector as a negative), well 3-8 (transformants).

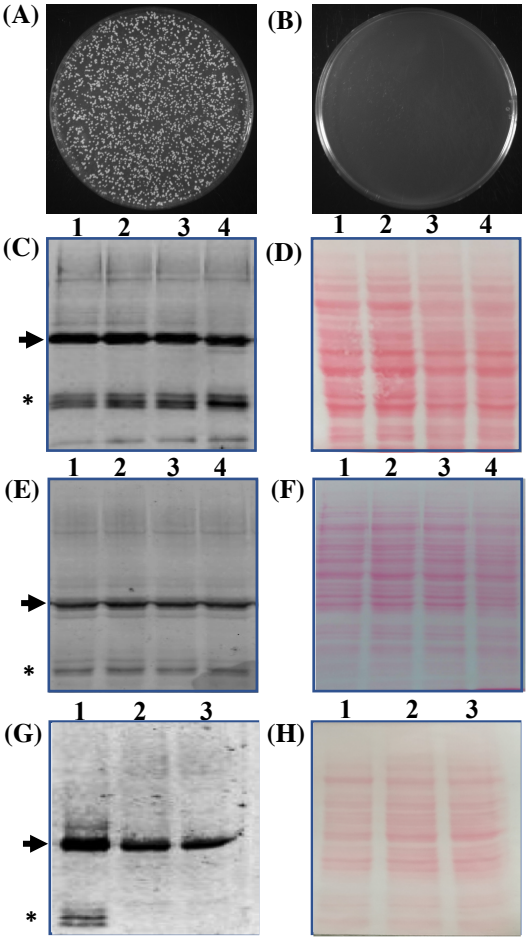

Figure 2. Heat-inactivation and freeze-drying do not affect protein stability. (A) Control untreated viable cells. (B) Complete loss of viability on heat-inactivation. (C) Western blot image showing the effect of heat-inactivation on protein stability. Well, 1, 2 (untreated cycling cells); well 3 4 (heat-inactivated cells). (D) Proper loading and protein transfer is shown by Ponceau S stain blot image. Western blot image showing the effect of freeze-drying on protein stability (E) well 1, 2 (control cycling cells); well 3,4 (lyophilized yeast cells). (F) Proper loading and protein transfer is shown by Ponceau S stain blot image. Bottom free GFP bands are due to vacuolar protease (G) well 1 (PPY12 strain); well 2-3 (SMD1163, protease deficient strain, lack Prb1 cytosolic and Pep4 vacuolar protease). (H) Proper loading and protein transfer is shown by Ponceau S stain blot image. Bands of interest are pointed by arrows towards them, while free GFP is pointed by an Asterisk mark.

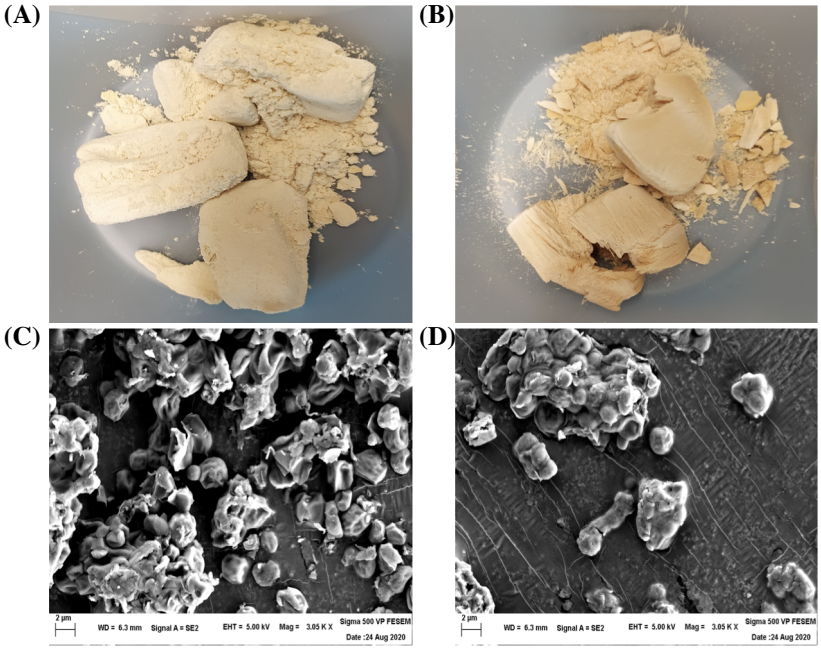

Figure 3. Cells in lyophilized powder remain intact for more than a year. Image of lyophilized yeast powder stored at 30 °C for more than a year (A) cells was lyophilized without heat-inactivation and (B) cells were heat-inactivated before lyophilization. SEM images showing external morphology of lyophilized cells after one year (C) lyophilized cells without heat-inactivation and (D) lyophilized cells after heat-inactivated.

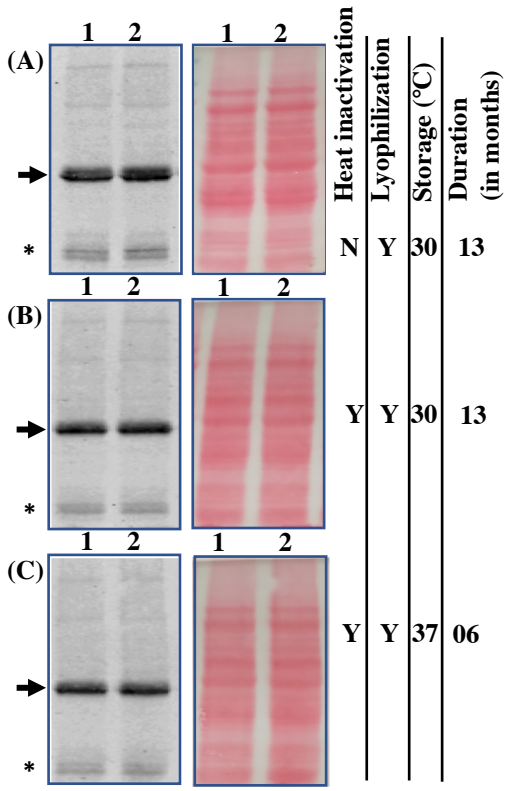

Figure 4. Expressed protein remains stable in powdered yeast. (A) Stability of CSGA-GFP in freeze-dried yeast powder stored at 30 °C after one year. (B) Stability of CSGA-GFP in heat-inactivated lyophilized yeast powder stored at 30 °C after one year. (C) Stability of heat-inactivated lyophilized yeast powder stored at 37 °C after six months. In each case, proper loading is shown, by Ponceau S stained blot image. Note, the protein amount was normalized, based on yeast powder used for protein extraction. Bands of interest are pointed by arrows towards them, while free GFP is pointed by an Asterisk mark. Note in figure Y stand for yes and N for no for condition mentioned in the figure.

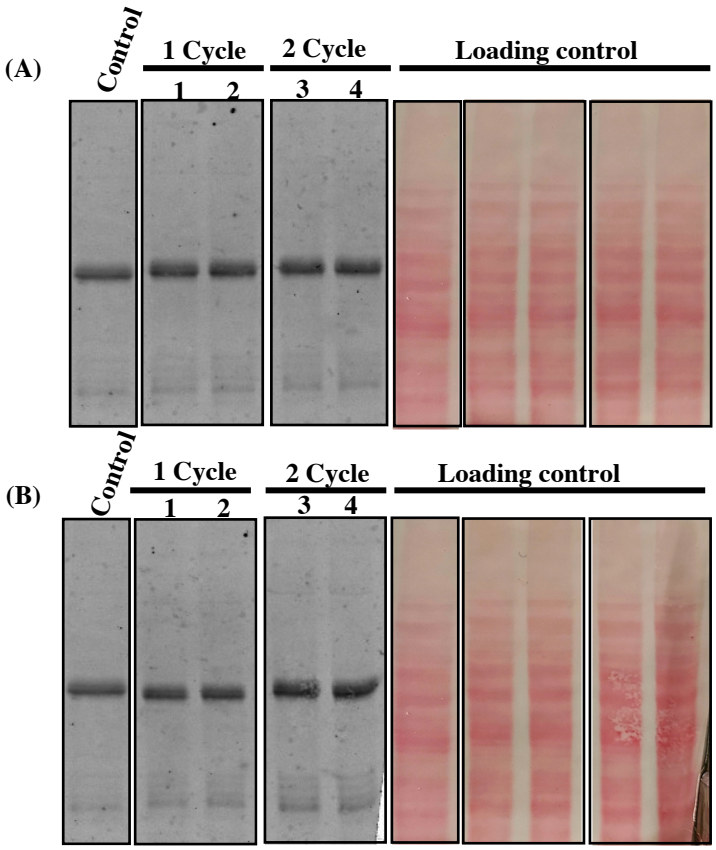

Figure 5. Freeze and thaws do not affect the stability of the expressed protein. (A) Effect of freeze and thaw at -20 °C. Control (lyophilized powder stored at 30 °C), well 1,2 (once the cycle of freeze and thaw), well 3,4 (two-cycle of freeze and thaw). (B) Effect of freeze and thaw at -80 °C. Same common control as above, well 1,2 (one cycle of freeze and thaw), well 3,4 (two-cycle of freeze and thaw). Note, in all case the protein amount was normalized based on the weight of powder and, each sample was taken in duplex. Loading and proper transfer are shown through Ponceau S stained of same blots. Also, a control sample is common in panel A and B.

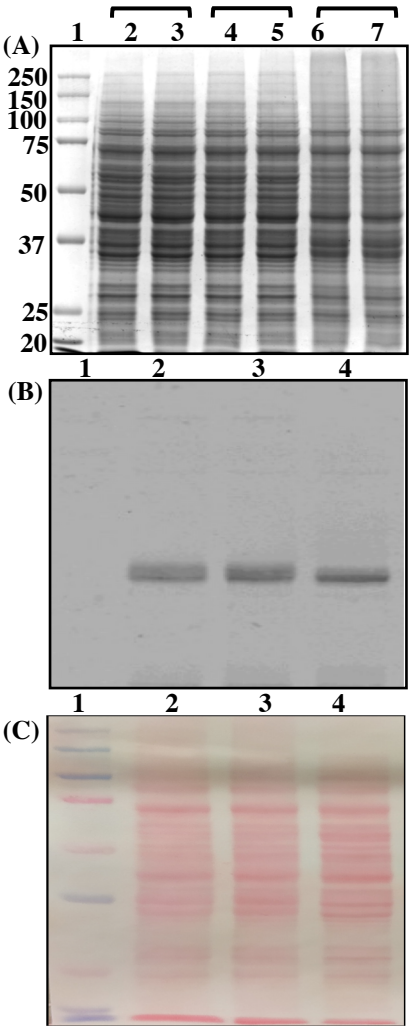

Figure 6. The abundance of heterologous protein in a year old powdered and freshly lyophilized cells are similar. (A) The efficiency of protein extraction from different samples. Cycling cells without lyophilized (well 2,3), an equivalent number of cells after lyophilized powder (well 4,5), an equivalent amount of lyophilized powder stored at 30 °C for one year (well 6,7), well 1 for protein marker. (B) The abundance of expressed protein is similar in freshly lyophilized cells and year-old powder lyophilized powder after one year at 30 C (well 2,3), freshly prepared powder (well 4) and protein marker (well 1). The same blot was Ponceau S stained before blocking in skimmed milk powder in TBST shown as a loading control (C).