Supplementary Information (SI) for Biomaterials Science.
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Supplementary Data

Genetically Engineered Spirulina Plantensis Producing Human Insulin as a Potential Novel Oral Drug Delivery Carrier

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1. Plasmid Design

Transformation using Agrobacterium for genetic manipulation requires a binary vector. The binary vectors used for *Agrobacterium* transformation are derived from the tumour-inducing plasmid (Ti-plasmid). *Agrobacterium* transfers a part of the Ti-plasmid DNA (i.e., transfer DNA or T-DNA) into the plant genome [1]. The binary vector contains two T-DNA repeats flanking the DNA sequence (in this case, the human insulin (hINS) gene sequence), which will be transferred to the host plant. The following figure **(Fig. S1)** illustrates the components of the pCAMBIA-based plasmid containing the gene of interest, human insulin (hINS), used in the study. The pCAMBIA is a plant expression vector developed by the Cambia Foundation. A similar plasmid was reported to be used to transform *Dunaliella pseudosalina* [2], and *Arthrospira platensis* [3] .

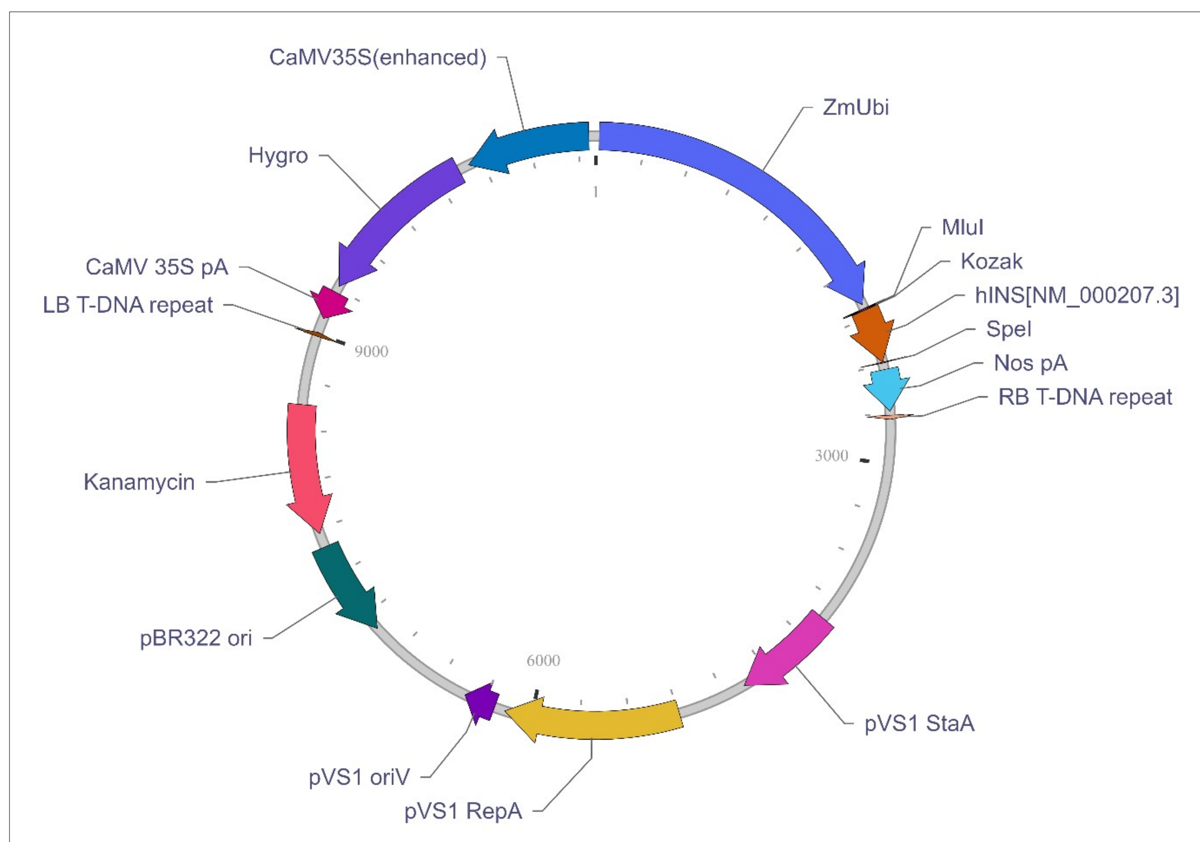

Figure S1: pCAMBIA–based plasmid with hINS gene designed using the VectorBuilder online tool.

2. Antibiotic Sensitivity Testing

Antibiotic sensitivity testing is crucial for distinguishing between transformed and untransformed cells. In the present study, we used the kanamycin and hygromycin resistance

genes to design a pCAMBIA-based binary vector plasmid to screen for transformed Spirulina after transformation. Therefore, in the present study, we assessed *Spirulina's* sensitivity to various antibiotics, including penicillin, streptomycin, penicillin + streptomycin, hygromycin, rifampicin, cefotaxime, and kanamycin. The following figure (**Fig. S2) illustrates the results of antibiotic sensitivity testing of Spirulina against various** antibiotics.

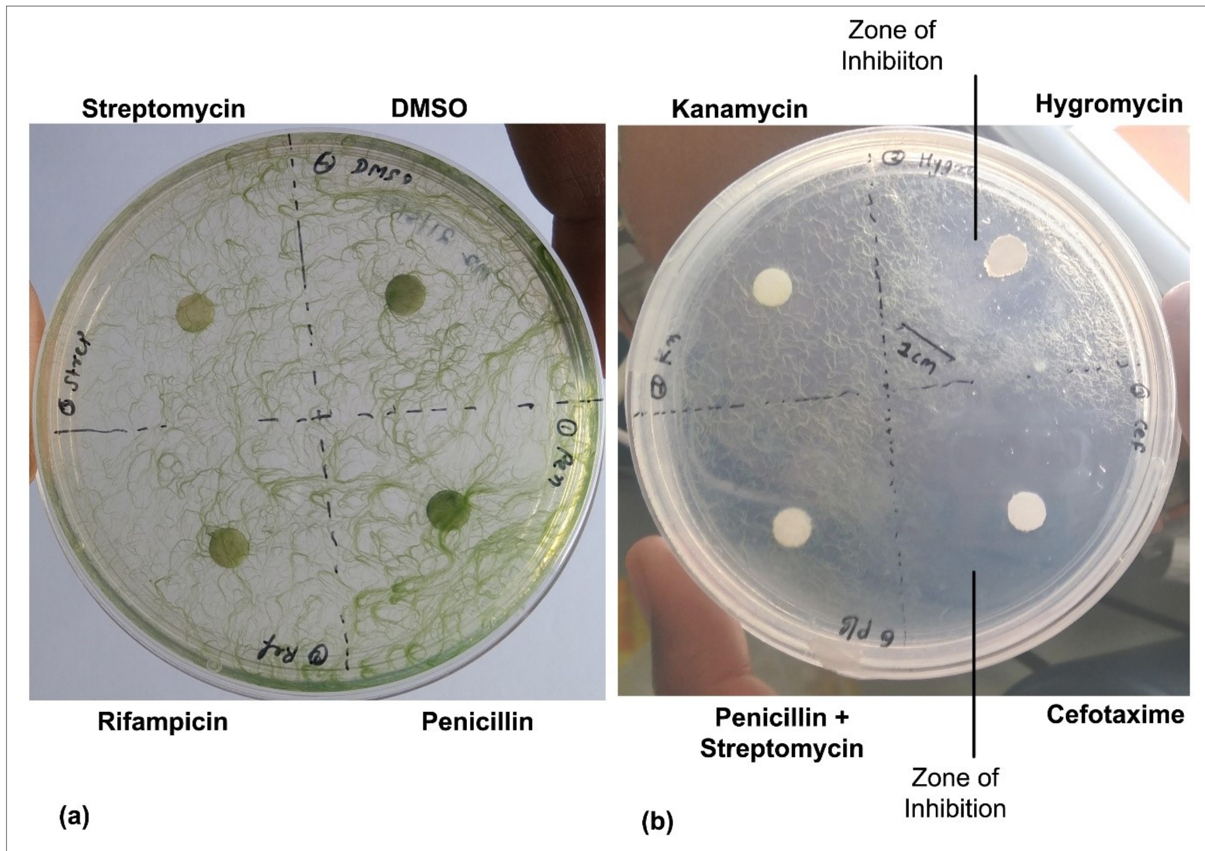

Figure S2: Antibiotic sensitivity of *Spirulina* grown on solid MS medium; **(a)** DMSO, penicillin, streptomycin, and rifampicin antibiotic discs (with no zone of inhibition); **(b)** Kanamycin, hygromycin, and cefotaxime antibiotic disc (with the zone of inhibition and lawn growth of *Spirulina*), penicillin + streptomycin antibiotic disc.

As illustrated in **Fig. S2**, *Spirulina* grew around the disc-shaped area of penicillin, streptomycin, penicillin + streptomycin, rifampicin, and kanamycin antibiotics, as well as DMSO (**Fig. S2a** and **Fig. S2 b),** indicating that *Spirulina* was resistant to these antibiotics and to DMSO. In contrast, *Spirulina* filaments were not detected around the disc containing the antibiotics hygromycin and cefotaxime (**Fig. S2b), indicating that Spirulina is sensitive to hygromycin and cefotaxime**. Moreover, it is clear from **Fig. S2b** that *Spirulina* is more sensitive to cefotaxime than hygromycin. Based on these results, we designed our transfection

plasmid and added a hygromycin resistance gene, which can be exploited to screen out transformed *Spirulina* from untransformed *Spirulina*.

3. Plasmid Isolation

The *E. Coli* DH5α used for plasmid isolation was checked for contamination by streaking on an antibiotic-containing LB plate using a four-sector streaking technique. A well-grown and isolated colony was then selected for plasmid extraction. The purity and integrity of the isolated plasmid were verified by 1% agarose gel electrophoresis using both undiluted and 1:10-diluted plasmid extracts. The results are depicted in **Fig. S3** below.

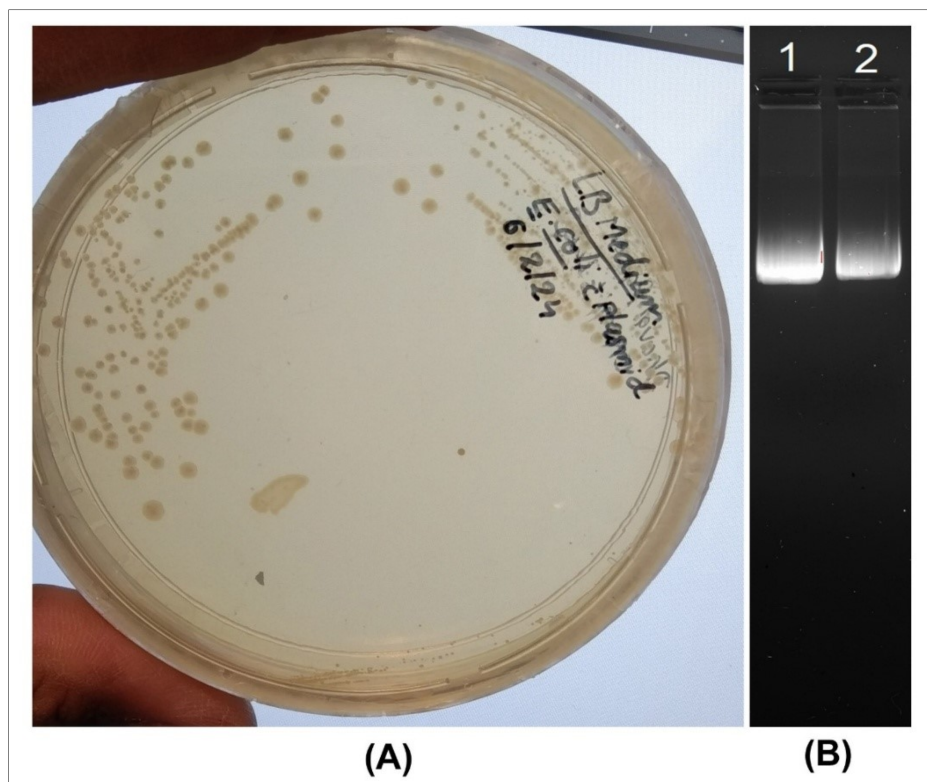

Figure S3: (A) Isolated colonies of *E. coli* DH5α grown on kanamycin-containing LB plate at 37 °C for 24 h, **(B)** Assessing plasmid purity on 1% agarose gel electrophoresis (**Lane 1** → plasmid sample from direct extract, **Lane 2** → 1:10 diluted plasmid sample from direct extract).

Figure S3a presents the four sectors streaking on a kanamycin-containing LB plate to obtain well-grown and isolated colonies of E. coli DH5α containing the plasmid construct. **Figure S3b** depicts a single band of plasmid extracted from a well-grown and isolated colony

of *E.* coli DH5α. A single band in lane 1 (i.e., undiluted plasmid extract) and lane 2 (i.e., 1:10 diluted plasmid extract) indicates the plasmid was pure and intact, which can be used for transformation. Although a DNA ladder is not included in this gel, a compact band corresponding to supercoiled and open circular plasmid, also confirmed by downstream PCR of hINS insert.

4. Standard Plate Count

The *Agrobacterium* culture was diluted to 10^{-5} and plated on an LB medium containing the appropriate antibiotics. The results of all dilutions are shown in **Fig. S4**.

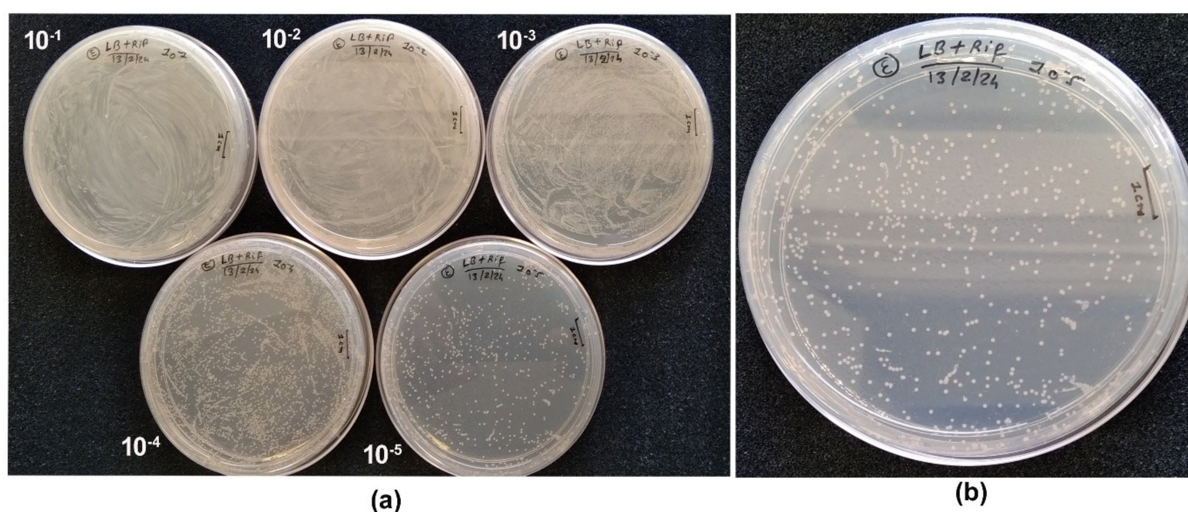

Figure S4: (a) Dilution plates (from 10^{-1} to 10^{-5}) of the standard plate count of *Agrobacterium* after transformation. **(b)** Colonies of *Agrobacterium* on a Rifampicin-containing medium.

As shown in **Fig. S4**, the plates of 10^{-1} to 10^{-4} dilution exhibited lawn growth of bacteria, and therefore, it isn't easy to pick up a single colony for colony PCR (**Fig. S4a**). The plate corresponding to the 10^{-5} dilution displayed well-isolated colonies (**Fig. S4b**) and was selected for colony PCR analysis. Colony PCR was performed to detect the presence of the hINS gene in the colony using hINS-specific primers, eliminating the need to isolate genomic DNA from the bacteria.

Transformation efficiency measures a bacterium's ability to take up DNA. It depends on the competence of bacteria and the quality of plasmid DNA used. In the present study, the transformation efficiency of *Agrobacterium* was 2.86×10^{8} CFU/µg of DNA, indicating that the bacteria were healthy and had sufficient intact plasmid to transform the *Spirulina*. The successful transformation in *Agrobacterium* was verified using colony PCR (see **Fig. 2**; readers are advised to refer to **Section 3** in the main article).

5. Integrity of hINS

The integrity of the extracted hINS from the SIF exposure experiment was evaluated by Laemmli SDS PAGE followed by silver staining (**Figure S5**). Briefly, we incubated the GE *Spirulina* in SIF containing 1 mg/mL pancreatin for 3 h at 37°C with slight shaking. The hINS integrity was assessed from the suspension taken at the start (0 h) and after 3 h of exposure to SIF from the cell-free supernatant, followed by lysing the algae at both time points. The results are as follows.

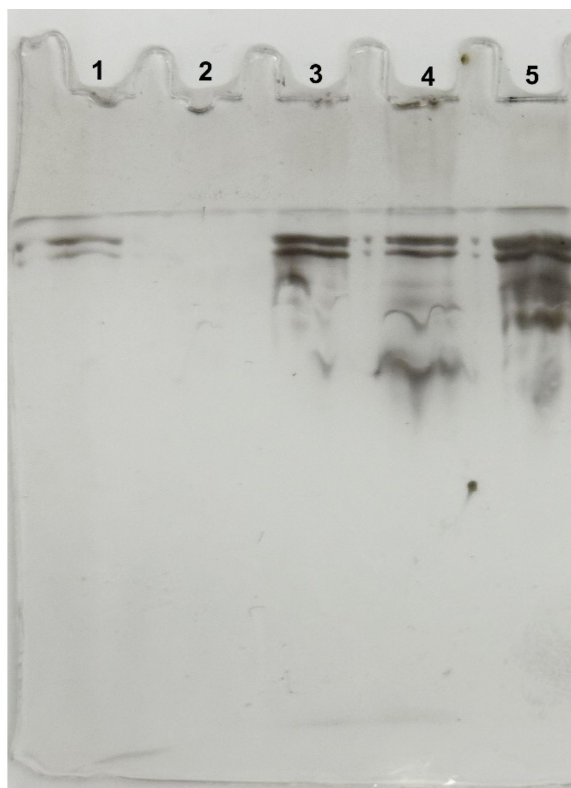

Figure S5: SDS PAGE (silver staining) of SIF exposure experiment. Lane 1→ standard/commercial hINS (200 µIU/mL), Lane 2 → cell-free broth at zero h, Lane 3→ cell-free broth at 3 h, Lane 4→ cell lysate at 3 h, Lane 5→ cell lysate at zero h.

As shown in **Figure S5**, the commercial hINS at a concentration of 200 µIU/mL was detected as two separate bands in Lane 1. Similar bands were also observed in Lanes 3 to 5, confirming that hINS remained intact inside GE *Spirulina* even after 3 h of incubation in SIF (Lane 4 compared to 5). Furthermore, we detected hINS in the SIF medium after 3 h of incubation, as evidenced by a band in Lane 3 that was absent before incubation (see Lane 2). These results indicate that the hINS in GE *Spirulina* is protected when exposed to SIF medium for 3 h.

References

1. L. Lee and S. B. Gelvin, *Plant Physiol.,* 2008, **146**, 325–332.

2. J. Dehghani, A. Movafeghi, A. Barzegari, and J. Barar, *Efficient and stable transformation of Dunaliella pseudosalina by 3 strains of Agrobacterium tumefaciens*, Maad Rayan Publishing Company, 2017.

3. J. Dehghani, K. Adibkia, A. Movafeghi, A. Barzegari, M. M. Pourseif, H. Maleki Kakelar, A. Golchin, and Y. Omidi, *Appl. Microbiol. Biotechnol.,* 2018, **102**, 9267–9278.