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An integrated pathway system modeling of *Saccharomyces cerevisiae* HOG pathway: a Petri net based approach

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Abstract Biochemical networks comprise many diverse components and interactions between them. It has intracellular signaling, metabolic and gene regulatory pathways which are highly integrated and whose responses are elicited by extracellular actions. Previous modeling techniques mostly consider each pathway independently without focusing on the interrelation of these which actually functions as a single system. In this paper, we propose an approach of modeling an integrated pathway using an event-driven modeling tool, i.e., Petri nets (PNs). PNs have the ability to simulate the dynamics of the system with high levels of accuracy. The integrated set of signaling, regulatory and metabolic reactions involved in *Saccharomyces cerevisiae*'s HOG pathway has been collected from the literature. The kinetic parameter values have been used for transition firings. The dynamics of the system has been simulated and the concentrations of major biological species over time have been observed. The phenotypic characteristics of the integrated system have been investigated under two conditions, viz., under the absence and presence of osmotic pressure. The results have been validated favorably with the existing experimental results. We have also compared our study with the study of idFBA (Lee et al., PLoS Comput Biol 4:e1000–e1086, 2008) and pointed out the differences between both studies. We have simulated and monitored concentrations of multiple biological entities over time and also incorporated feedback inhibition by Ptp2 which has not been included in the idFBA study. We have concluded that our study is the first to the best of our knowledge to model signaling, metabolic and regulatory events in an integrated form through PN model framework. This study is useful in computational simulation of system dynamics for integrated pathways as there are growing evidences that the malfunctioning of the interplay among these pathways is associated with disease.

Keywords Metabolic pathway · Signal transduction pathway · Transcriptional regulatory network · Feedback inhibition · idFBA

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Introduction

Gene regulation, metabolic reactions and cellular signaling are some key primary processes essential for sustaining life. There exist several investigations [43, 16, 23] on each of these processes individually. However, studies [12, 10] involving integration of two or more of these processes are sparse. The study of coupling between metabolic reactions and gene regulation is supported by many direct and indirect evidences. Some assays indicate substrates of metabolic reactions influence the activities of transcription factors or signal transduction pathways [21, 45]. Several

experimental analyses show that metabolic enzymes are differentially expressed under different nutrient conditions or enzyme knock-outs [46, 68]. The influence of environment and metabolites on enzyme/gene regulation needs to be considered to make a study on how an organism is responsive to environmental changes. Therefore, it is necessary to focus not only on individual processes but also on their integration. However, the study on integration of gene regulation, metabolism and signaling events (pathways) in an organism is still in nascent stage.

In order to develop such an integrated model, it requires the formulation of new methodology, that can integrate the models for different types of processes, the existing models for individual systems need to be modified. Some of the attempts in this regards are as follows. A comprehensive mathematical model of the cellular response of yeast under hyperosmotic shock has been presented in [12]. They have simulated and monitored different events under osmotic adaptation in wild type and mutants with delayed glycerol accumulation in parallel. It allows one to study systems' properties and to predict the effects of a perturbation. Covert et al. [10] have developed an integrated FBA system, called iFBA, which combines a flux-balance-based central carbon metabolic and transcriptional regulatory model with an ODE-based model of carbohydrate uptake control of *E. coli*. The approach has integrated dynamic flux balance analysis (idFBA) [34] assumes quasi-steady-state conditions for "fast" reactions and incorporates "slow" reactions in a time-delayed manner. Here the methodology considers modeling of regulated metabolic networks, which includes logical simulation of gene regulatory networks (GRNs) and the Petri net (PN) modeling of metabolic networks [69]. The authors have used this approach to qualitatively model the biosynthesis of tryptophan in *E. coli*, where they have included product feedback inhibition and transcriptional inhibition.

Osmoregulation is a homeostatic process which is acquired by high osmolarity glycerol (HOG) signaling system in yeast *S. cerevisiae* [28]. An integrated HOG pathway of *S. cerevisiae* is shown in Fig. 1. It monitors osmotic changes through the plasma membrane localized sensor histidine kinase Sln1. Upon loss of turgor pressure, Sln1 is inactivated resulting in activation of a mitogen-activated protein (MAP) kinase cascade and phosphorylation of the MAP kinase Hog1 [44]. Otherwise, it is active and inhibits signaling under ambient conditions. Active Hog1 accumulates in the nucleus and targets two genes encoding enzymes glycerol 3-phosphatase (Gpp2) and glycerol-3-phosphate dehydrogenase (Gpd1) for glycerol production, and the gene encoding sugar transporter like 1 (Stl1) that acts as a transporter protein. Increased concentration of glycerol acts as an osmolyte. Transient processes of Hog1 activation and Hog1-dependent transcriptional stimulation indicates feedback control that is applied by several protein phosphatases, e.g., Ptp2, Ptp3 and Ptc1 [76].

A PN is a formal description for modeling concurrent systems developed by Petri in 1962 [51]. PNs are graphical and mathematical modeling tool applicable to a wide range of technical systems. Although the use of the PN formalism has been mainly used for graphical simulation of industrial and technical systems [81], current research involves the utility of PNs in systems biology such as in modeling and simulation of metabolic pathways, signal transduction pathways and regulatory networks [25, 8, 35]. PNs involve the use of concurrent components, i.e., which may be independent and/or causally dependent. PNs with some extensions may be used in order to find both qualitative and quantitative values from a model of a real system and this is described later in this section. PNs have a few innate advantages, which make it a superlative tool for graph-based simulation of biochemical networks [35]. Firstly, PN models can be intuitively framed on the basis of signal/metabolite flow and reaction rates that make it efficient for analysis of complex networks like signal transduction/metabolic pathways. Secondly, we can use PNs to evaluate both the qualitative and quantitative parameters in a network. Quantitative analysis involves simulation and observations of dynamic behavior of based on the temporal variations of a molecule. On the other hand, qualitative analysis is based on structural characterization of the model. Thirdly, PN can be fired stochastically or deterministically; thereby offering an option for capturing randomness within the modeled system.

In recent years, standard PN methodology has been extended and modified so that it can be applied on different kinds of biological pathways efficiently. PN theory [23] has been used to demonstrate the Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) regulation network, and the results have shown temporal information about signal propagation and also characterized some signaling routes as regulation motifs. Heiner et al. [26] have used PN modeling approach to model and validate apoptosis signaling pathway. The difficulty in obtaining kinetic parameters is well recognized, thus one can use recent discoveries those show that network structure alone can determine many aspects of a network's dynamics. Based on this idea, a novel computational simulation technique has been developed [61] in which PN based model of cellular signaling network has been presented. A dynamic model of the terpenoid biosynthesis pathway has been developed based on the hybrid functional Petri net (HFPN) technique [24] in which the model have simulated dynamic behavior of metabolite concentration. Banks and Steggles [3] have developed a framework for modeling and analyzing multi-valued gene regulatory networks (GRNs) using high-level PNs [19] and logic minimization techniques, and demonstrated it through

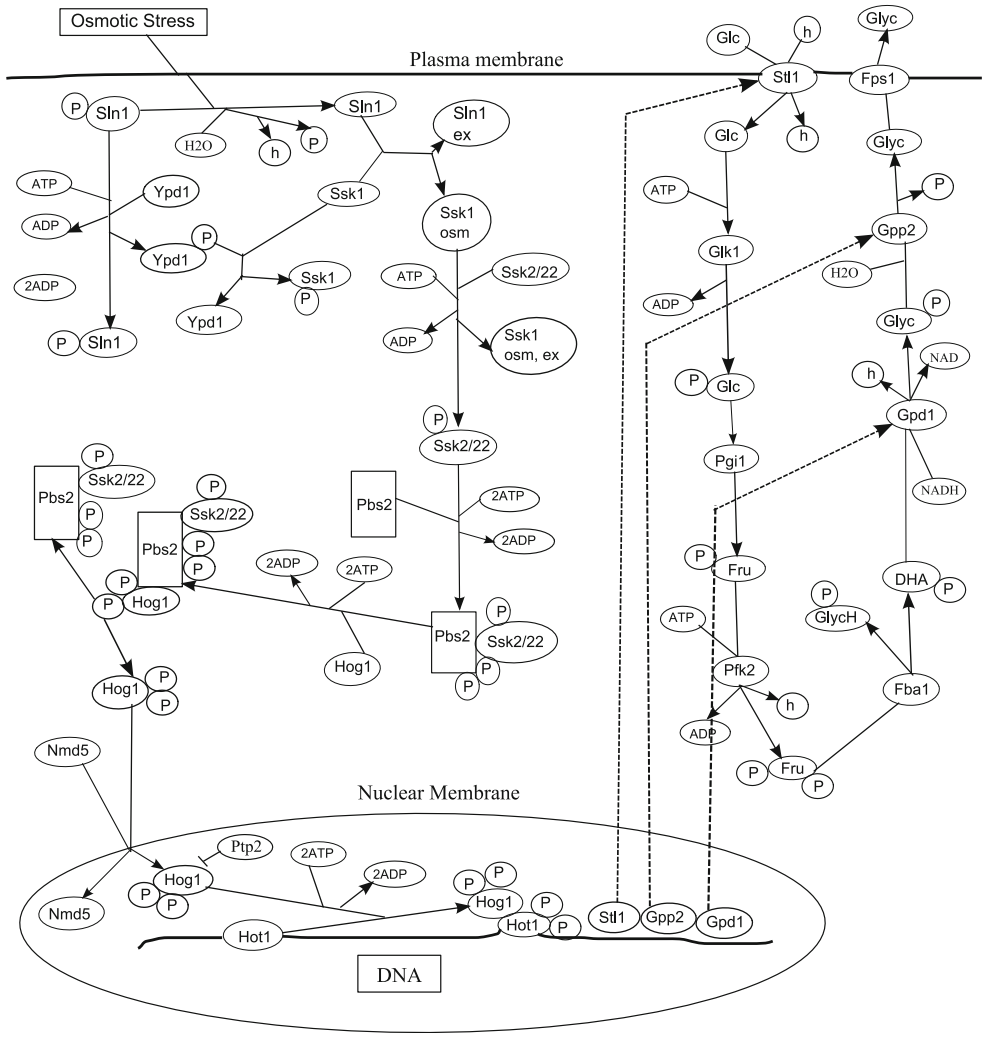


Fig. 1 An integrated system module of *S. cerevisiae* HOG pathway as adapted from [34] with addition of feedback inhibition

Table 1 Logical rules specifying the activation of the genes coding the proteins, Stl1, Gpd1 and Gpp2 involved in the TRN of the integrated *S. cerevisiae* HOG pathway

Osmotic stress	Hog1	2ATP	Hot1	Stl1	Gpp2	Gpd1
0	d	d	d	0	0	0
d	0	d	d	0	0	0
d	d	0	d	0	0	0
d	d	d	0	0	0	0
1	1	1	1	1	1	1

Here '*d*' stands for the case in which we do not know whether the entity is present (1) or absent (0)

a case study of the carbon starvation stress response in *E. coli*. Another attempt by Steggles et al. [72] also exists for qualitative modeling of GRNs in which they have used Boolean network approach and developed a PN model which uses logic minimization to automate the qualitative models. Their approach has been illustrated by a case study of the GRN which controls sporulation in *Bacillus subtilis*. Mura and Csikasz-Nagy [43] have developed a stochastic Petri Nets (SPNs) model from the deterministic ODE model of budding yeast cell cycle in a constructive way that it can be automated.

In the present study, we have used PN modeling approach for an integrated pathway which involves signaling and metabolic pathways, and TRN of *S. cerevisiae* HOG pathway. Here we have considered two conditions, viz., with and with no effect of osmoshock on the *S. cerevisiae* cell and monitored the behavior of multiple biological key species in the integrated HOG pathway through PN modeling. We have also included feedback loop by phosphatase in the integrated HOG pathway. We have modeled the integrated HOG pathway through implementing PN framework done by the software Cell Illustrator Online 5.0 (http://www.cionline.hgc.jp/cionlineserver/apps/usersman/main). The simulation results have been validated by comparing them

with existing experimental results. Our study has also been compared with the study using idFBA [34] and we have described the differences between two studies.

The article is organized as follows. Next Section briefly describes *S. cerevisiae* HOG pathway, while the results are analyzed in "Results" section and its subsections describe in details the validation of the results and comparison of the results of proposed method with that obtained by idFBA [34]. We have discussed an impact of study of an integrated pathway and the used methodology in "Discussion" section. The "Method" section provide the proposed methodology in details.

HOG pathway of *S. cerevisiae*

Cellular signalling networks incorporate environmental stimuli with the information on cellular change status. Thus these networks must be robust against fluctuations in stimuli as well as some cellular parameters. Osmoregulation is an active process through which cell adjusts osmotic pressure, and controls its shape and relative water content. Each individual cell in a multi cellular organism has this kind of regulation and it is conserved from bacteria to human [71]. Yeast cells are immotile and thus they are not able to escape from a hostile environment. For this reason, they have an arrangement for internal adjustments to adapt to the increased external osmolarity. *Saccharomyces cerevisiae* possesses rapidly responding, highly complex signaling pathways which allow the cell to quickly adapt to the constantly changing environment. MAPK cascades are the prominent among yeast signaling pathways which can be activated by a variety of external stimuli. MAPK pathways convert these signals into appropriate metabolic responses. Five of these MAPK cascades have been characterized, which respond to such diverse environmental conditions, viz., as the presence of mating pheromones, changes under osmotic pressure, heat stress, and nutrient availability in the environment [27]. They synthesize and retain the compatible osmolytes like glycerol to increase the internal osmolarity, by modifying water efflux, and by adjusting cell cycle progression [64].

High Osmolarity Glycerol (HOG) pathway (Fig. 1) is a MAP kinase pathway through which *S. cerevisiae* adapts to high external osmolarity conditions. It is the most extensively studied eukaryotic signal-transduction cascade. It is activated by high osmolarity and is essential under this condition. The cells lacking this pathway cannot proliferate on media containing high levels of osmotically active molecules [6]. It responds to higher external osmolarity by increasing glycerol synthesis and decreasing glycerol permeability [4]. This helps in reducing the transmembrane difference of osmotic pressure and to prevent the loss of water.

HOG pathway monitors osmotic changes through plasma membrane localized sensor histidine kinase Sln1. Upon loss of turgor pressure, Sln1 inactivated [55]. It results in the activation of MAPK Hog1. Activated Hog1 enters the nucleus and activates High osmolarity induced transcription 1 (Hot1). Hot1 targets the genes encoding the enzymes for the glycerol accumulation (steps for integration of signal transduction and metabolism). These enzymes are Gpp2, Gpd1 and gene coding Stl1 which help in the conversion of glucose into glycerol (integration of transcriptional regulatory network and metabolism) and transport of glycerol outside the cell respectively. 6-phosphofructo-2-kinase (PFK2) is a key regulatory enzyme of glycolysis, which catalyzes the synthesis of fructose-2,6-bisphosphate (Fru-2,6-P2). Fru-2,6-P2 is the most powerful activator of 6-phosphofructo-2-kinase [16]. Glycerol accumulation is controlled by rapid closing of the osmolarity channel aquaglyceroporin Fps1. Hog1 activation and dependent transcriptional events indicate feedback control. Several protein phosphatases like, Ptp2, Ptp3 and Ptc1, are found to be negative regulator of the pathway.

Mechanisms which underlie the activation and deactivation of the signaling system, its feedback control and the relationship between different events under osmotic adaptation are important aspects of present study on HOG pathway. The integrated HOG signalling pathway of *S. cerevisiae* is shown in Fig. 1. The reactions included in the pathway are collected from literature and as given in [34]. The integrated HOG pathway comprises receptor stimulation, HOG signaling, activation of gene expression process, enzyme catalyzing cellular metabolism and glycerol accumulation. This integration of the biochemical reaction networks with the biophysical description of cellular volume control is important for understanding the activation and down regulation of signaling pathway. Using a combination of individual pathways, we describe mechanisms underlying feedback control of HOG pathway under osmotic adaptation. This mechanism ensures the important combination of osmoadaptation with effective feedback control.

We have explored the time course events occurring in a cell under osmoshock condition before implementing the methodology. Here is the brief description in which we have categorized time course events in various phases under osmoshock.

Phases of osmotic stress response in S. cerevisiae

The time course events of osmotic shock response depend on the severity of the shock. That is, the time window remains smaller after a mild shock and progressively becomes larger after a severe shock [80, 28]. The time

presented for each phase should be viewed as an estimation.

Phase I (≈1–2 min) After an increase in external osmolarity, the first mechanical response is the rapid loss of water that usually occurs in less than a minute [1]. It leads to a decrease in cell size and turgor pressure; and an increase in intra-cellular osmotic pressure. Moreover, after a sudden shift to high osmolarity, *S. cerevisiae* cells respond by transiently inducing the expression of stress-protective genes.

Phase II (≈2–60 min) Just after the osmoshock, a predominantly transient phase occurs as a result of rapid signaling events [17]. Rehydration starts and thus cell volume increases (though not completely restored) by the production and accumulation of compatible solutes, like glycerol [1, 58]. In wild-type cells, upon exposure to increased osmolarity, the level of phosphorylated Hog1 reaches a peak by 5 min and it translocates to nucleus. Nuclear accumulation of Hog1pp along with phosphorylated Hot1pp follow a distinct transcriptional response that can even last over a time period of about 90 min to several hours (depending on the severity of osmotic challenge). During this time, Hog1pp.Hot1pp complex transiently induces expression of genes encoding enzymes involved in glycerol synthesis, such as metabolic enzymes, viz., Gpd1 and Gpp2, and the gene encoding Stl1 [58]. It leads to glycerol synthesis and accumulation. Accumulation of intracellular glycerol has a rapid and reversible change in cytoskeleton organization, and proton motive force is restored [70].

Phase III (≈1 or more hours) Now, the yeast is fully adapted and in a state of equilibrated growth. Cell cycle is re-established as a result of a recently gained internal homeostasis. Cell wall and the nucleus are also remodeled as DNA/protein synthesis resume according to the expression of salt responsive genes [28].

Cell's response during lifetime It is important that signaling through this pathway be brought back to a basal level after Hog1 has performed its function under osmoshock. In this way, the cell is prepared for further osmotic challenge [37]. Moreover, constitutive activated HOG pathway causes inviability in the cell. Phosphatases are responsible for decreasing signaling in the HOG pathway by dephosphorylating Hog1 [76, 29]. This type of inhibition occurs during basal conditions and during adaptation to osmotic stress.

Method

The integrated PN framework facilitates the dynamic analysis of cellular metabolism on the genomic level which is affected by extracellular environmental stimuli. These stimuli are received by cellular receptors and its effects reaches the nucleus through a signal transduction pathway. We investigate the integrated system of *S. cerevisiae* HOG pathway which includes signaling, transcriptional regulation and metabolism. Figure 2 shows how these three pathways are combined to form the integrated system, following the idea provided in [34].

A cell responses to environmental stimuli through signalling events. It has an effect on cell's transcriptional regulatory network (TRN) in response to environmental cues. The sample integrated network (Fig. 2) includes a signaling pathway, a metabolic pathway and a transcriptional regulatory network. Signaling pathway comprises a set of reactions, like phosphorylation, which can be correlated with a typical biological signaling pathway. In this signaling pathway, ligands Lig1 and Lig2 bind to their

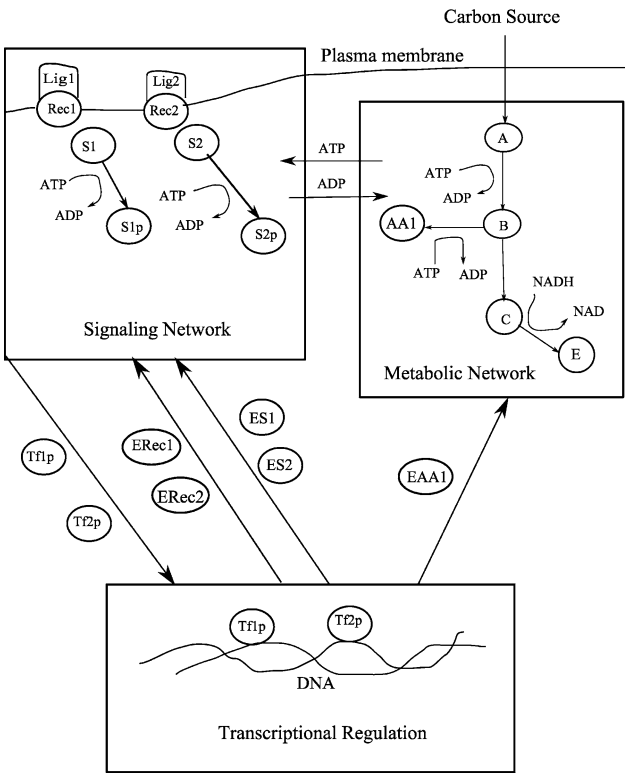


Fig. 2 The prototypic integrated system, adapted from [34], comprises three functional network modules. Rec1 denotes receptor 1; Rec2 denotes receptor 2; Lig1 represents ligand 1; Lig2 represents ligand 2; S1 shows signaling molecule 1; S2 shows signaling molecule 2; S1p stands for phosphorylated signaling molecule 1; S2p stands for phosphorylated signaling molecule 2; Tf1p denotes activated transcription factor 1; Tf2p denotes activated transcription factor 2; **a**, **b**, **c** and **e** are metabolites present in the metabolic pathway; AA1 stands for amino acid 1; EAA1 denotes the enzyme catalyzing the reaction synthesizing AA1; ERec1 and ERec2 stand for enzymes for the reactions synthesizing Rec1 and Rec2 respectively; ES1 and ES2 represent the enzymes catalyzing reactions synthesizing S1 and S2, respectively

respective receptors Rec1 and Rec2, and form the complexes. These complexes lead to the activation of signaling molecules which may further involve the activation of other signaling components. Subsequently, activated transcription factors (Tf1p and Tf2p) are formed as a part of downstream effector molecules. TRN comprises genes and corresponding transcription factors. Environmental stimuli, like availability of metabolites (amino acids), pH and activated transcription factors are inputs and the outputs are protein products of TRN. Metabolic processes within a cell produce energy and small molecules (like, metabolites) necessary for all cellular functions and growth. It involves several metabolites and enzymes, and produces biomass.

Petri net terminology

A PN is a directed, weighted, bipartite graph consisting of two major types of nodes, called as places and transitions. Graphically, places are drawn as circles and transitions are represented by boxes. The truth value of a place is called its token count. A place that has an outgoing arc towards a transition t is known as input place of t; a place that has an incoming arc from a transition t is known as output place of t. Generally, arcs are labeled with weights which represent the minimum tokens required by input places to enable the transition. When a transition is fired, it will take tokens from each input place equal to the weight of the corresponding input arc and create new tokens the weight of the corresponding output arc. Thus PN is defined as a 5-tuple, $(P,\ T,\ F,\ W,\ M_0)$, where $P = \{p_1, p_2, \ldots, p_m\}$ is the set of m places, $T = \{t_1, t_2, \ldots, t_n\}$ is the set of n transitions and $F \subseteq (P \times T) \bigcup (T \times P)$ is the set of arcs. $W : F \rightarrow N, N$ being the set of natural numbers, is called the weighting function and $M_0 : P \rightarrow \{0, 1, \ldots m\}$ stands for the number of tokens per place.

For biological networks, we may consider a token to be a unit of weight of a particular molecule in a given cell. A place-transition or transition-place connection is made by a weighted arc (directed edge), designating how much of the input places (reactants) are required to produce tokens in the output places (products) in a biochemical reaction. When the transitions are enabled, i.e., the token count of the input places are sufficient to fire the transition, then the transition fires (reaction occurs). Firing involves the removal of a number of tokens from each input place (as denoted by the weight of their respective input-transition arcs) and the subsequent creation of new tokens at each output place as per the weights of the output arcs. The rate of firing of a transition can be programmed either in discrete-mode or in continuous-mode for timed-Petri Nets in order to accurately reproduce the chemical kinetics following the Michaelis–Menten equations. The state of the system at every time instant is noted by the number of tokens in each of its places. This is called its marking and the initial marking of the PN is the state of the system at reference time zero. A representative chemical reaction is shown in Fig. 3, in order to explain the PN modeling paradigm. Figure 3(a) depicts that initially we have two molecules of hydrogen, 2 molecules of oxygen but no water. As the transition (Reaction 1) fires, the number of hydrogen, oxygen and water molecules become zero, one and two respectively.

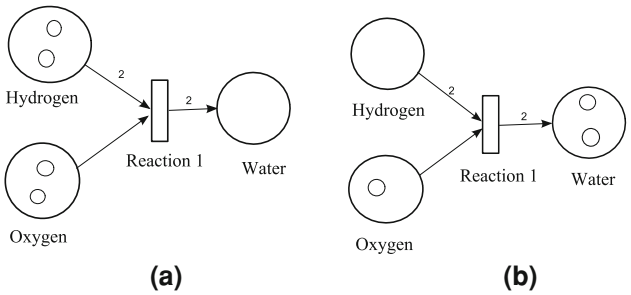


Fig. 3 The reaction $2H_2 + O_2 \rightarrow 2H_2O$ is shown using Petri Nets. (a) shows the initial marking before firing the enabled transition t; (b) shows the marking after transition labeled reaction 1 fires

Petri net modeling of the integrated HOG pathway of *S. cerevisiae*

The network diagram inferred from Fig. 1 shows the system input and measurable outputs as given in Fig. 4. PN can model the metabolic pathway, taking the stoichiometry of each reaction explicitly and the role of each enzymes. As the gene regulators are not consumed and the stoichiometry is not evident, we cannot use PN modeling directly for the case of TRN. Thus, we have adopted logical modeling approach, before applying systematic translation rules of logical regulatory schemes into standard PNs. Then we

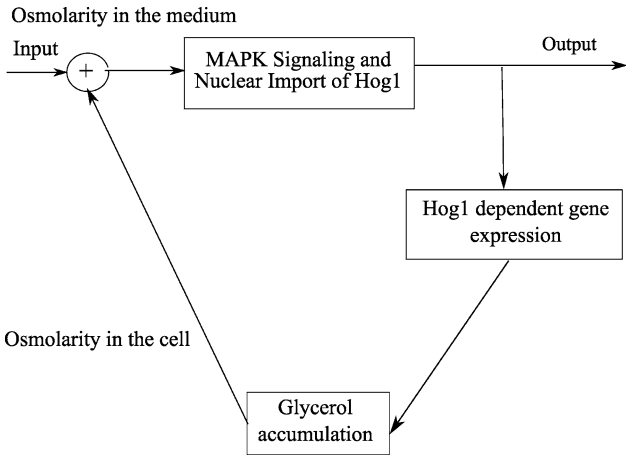


Fig. 4 Network model showing stimulation of *S. cerevisiae* HOG pathway

have proposed a methodology for integrated modeling of HOG system in terms of standard PNs. The implementation details are as follows.

Logical modeling of TRN of integrated HOG pathway of S. cerevisiae

Focusing on transcriptional regulation of gene expression which are responsible for formation of metabolic enzymes, we have formalized the logical rules as given in Table 1. We have considered the criteria of activation of each component of the TRN of *S. cerevisiae* HOG pathway. This involves the state of a gene, either transcribed or non-transcribed. Osmotic stress leads to the cascade of activation and deactivation of several signaling molecules. It activates the kinase Hog1 in a few steps. Phosphorylated Hog1 activates the transcription factor Hot1 in the nucleus. Activated Hot1 is involved in the synthesis of metabolic enzymes Gpp2 and Gpd1, and the protein Stl1 for sugar transport. These events are expressed as logical formalism as described in [69, 9].

Petri net modeling of TRN of S. cerevisiae HOG pathway

In Fig. 5, we have used PN in order to implement TRN in the integrated HOG pathway. From the truth table given in Table 1, we have found that these 3 genes, viz., Gpd1, Gpp2 and Stl1 are all expressed. The steps for designing the PN model for TRN are given below.

Step 1: We have considered the four inputs as continuous places as shown in Fig. 5 so that they can be connected directly from the remaining part of the integrated HOG PN model. Kinetic parameter values have been used to maintain realism.

Step 2: We have used associative connectors (indicated by dashed arrows) instead of process connectors as the former one transfer the token count of the input place to the transition without altering the token count, in the formalism of the TRN.

Integrated Petri net modeling of HOG pathway of S. cerevisiae

Here, we define all the ingredients required to systematically derive a PN representation of the integrated HOG pathway involves PN model implementation using kinetic equations followed by simulation of the integrated system for non-osmotic and osmotic conditions.

Table 2 The places of the PN model of *S. cerevisiae* integrated HOG pathway

Place names	Biological species
Sln1	Plasma membrane localized sensor histidine kinase
Sln1p	Phosphorylated histidine kinase
$Sln1_{ex}$	Extracellular histidine kinase
ATP	Adenine tri phosphate
ADP	Adenine di phosphate
H_2O	Water molecule
Ypd1	Histidine containing phosphotransfer (Hpt) osmoregulatory protein
Ypd1p	Phosphorylated osmoregulatory protein
Ssk1	Cytoplasmic response regulator
Ssk1p	Phosphorylated cytoplasmic response regulator
$Ssk1_{osm}$	Active dephosphorylated Ssk1
$Ssk1_{osm_e}x$	Extracellular osmotic stress on Ssk1
Ssk2/22	A MAPKKK
Pbs2	A MAPKK
Pbs2pp_Ssk2/22p	A phosphorylated complex
Pbs2pp_Ssk2/22p_Hog1pp	A phosphorylated complex
$Pbs2_{ex}ppSsk2/22p$	Hog1pp removed from the complex
$Hog1_{pp}$	Phosphorylated Hog1, a MAP kinase
Nmd5	Karyopherin
Hot1	High osmolarity induced transcription 1
Hot1pp_Hog1pp	A complex
Stl1	Sugar transporter like 1
Gpp2	Glycerol 3-phosphatase
Gpd1	Glycerol-3-phosphate dehydrogenase
Glc	Glucose
Glk1	Glucokinase 1
Glcp	Phosphorylated glucose
Pgi1	Phosphoglucose isomerase
Frup	Phosphorylated fructose
Pfk1	Phosphofructokinase
Frupp	Phosphorylated fructose
Fba1	Fructose 1,6-bisphosphate aldolase
GlycHp	D-glyceraldehyde-3-phosphate
DHAp	Di hydroxy acetone phosphate
Gpd1	Glycerol-3-phosphate dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
Glycp	Phosphorylated glycerol, L-a-glycerol phosphoric acid
Gpp2	Glycerol 3-phosphatase
Fps1	Aquaglyceroporin channel
Glyc	Glycerol
Ptp2	Protein tyrosine phospahtase2

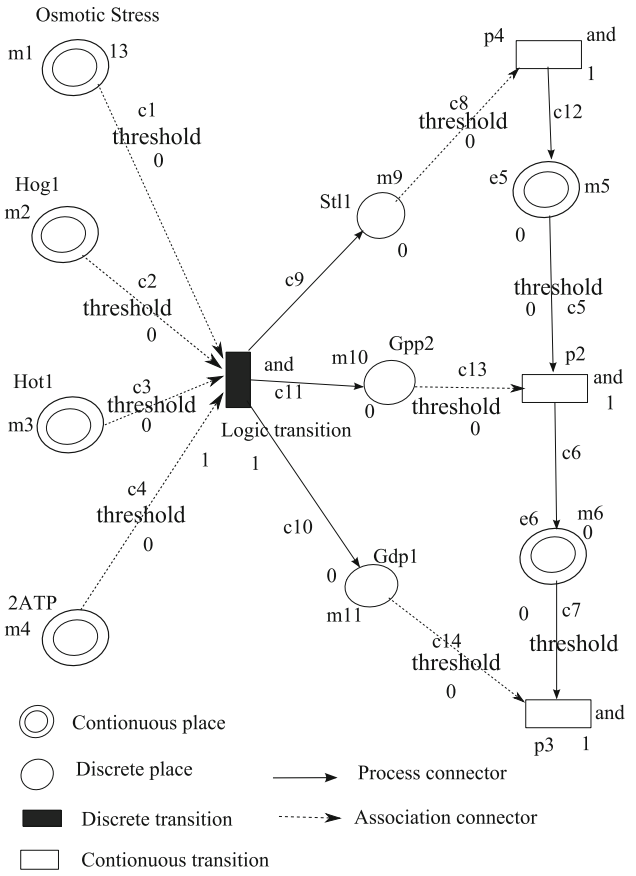


Fig. 5 PN model for TRN of *S. cerevisiae* integrated HOG pathway

For the integrated HOG pathway, a PN is modeled using the set of kinetic equations given in [12, 34, 22] and [71]. These kinetic equations and the corresponding references are given in Tables S1–S4 in Supporting Information. The places are constructed individually for all the molecules involved in the integrated system as well as for ATP and ADP molecules to allow uninterrupted use of ATP/ADP. However, intracellular and extracellular ATP molecules are left distinguishable in order to plot the variation of cellular ATP after simulation of the net. Interactions between the molecules are represented by transitions. The complete PN model is divided into 3 sub-networks, i.e., the signaling network on the left, the metabolic network on the right and the gene regulatory network at the bottom, as shown in Fig. 6. The names of places along with their biological significances and the names of transitions along with their counterpart biological reactions are given in Tables 2 and 3 respectively. After formulation of the PN using Cell Illustrator Online 5.0 (https://www.cionline.hgc.jp/cionlineserver/apps/usersman/main), simulation is performed as a continuous timed PN, and the variations of multiple biological species are plotted. We have assumed two environmental conditions in which a yeast cell is kept, i.e., non-osmotic and osmotic conditions as described below.

(i) Without the effect of extracellular osmotic stress all the places that are at the boundary of the PN are given a default initial marking and time instants are considered as absolute event-driven time instead of real-time.
(ii) With the effect of extracellular osmotic stress in which initial marking of osmotic stress place is set.

The resulting simulated concentrations of the studied molecules for both non-osmotic and osmotic stress conditions after simulation of PN model are shown as graphs in Figs. 7 and 8 (non-osmotic condition) and Figs. 9 and 10 (osmotic condition), respectively.

Inhibition of kinase by phosphatase

Till now, we have described the way of modeling the integrated system without incorporating Ptp2 and monitoring the concentrations of HOG pathway molecules against time. Now, we have incorporated feedback inhibition by adding Ptp2 and monitor the change in concentration of Hog1pp in the nucleus. For this purpose, we use the concentration of cytoplasmic glycerol in the cell as a threshold. Ptp2 is present in nucleus, thus we insert a place for Ptp2 where it can inhibit phosphorylated Hog1pp and study the concentration of Hog1pp without and with the inhibitory effect of Ptp2.

Results

We have modeled the integrated HOG pathway of *S. cerevisiae* for two different conditions, viz., non-osmotic and osmotic conditions. The methodology is based on the implementation of two established formalisms, (i) logical Boolean modeling and (ii) PN modeling. The PN model for the integrated HOG pathway has been given in Fig. 6. It consists of signaling events, metabolic pathway and transcriptional regulation in metabolic genes, i.e., genes coding enzymes catalyzing reactions in a metabolic pathway. Signaling molecules, genes, transcription factors, ATP molecules and metabolites have been considered as places, while complex formation, activation/deactivation and other reactions have been represented as transitions.

The integrated HOG pathway without feedback inhibition

Yeast *S. cerevisiae* synthesizes proteins to convert glucose into glycerol in response to the exposure of osmotic pressure. We have simulated the entire integrated yeast HOG pathway PN model, and observed the dynamics of the many key species, in both the presence and absence of

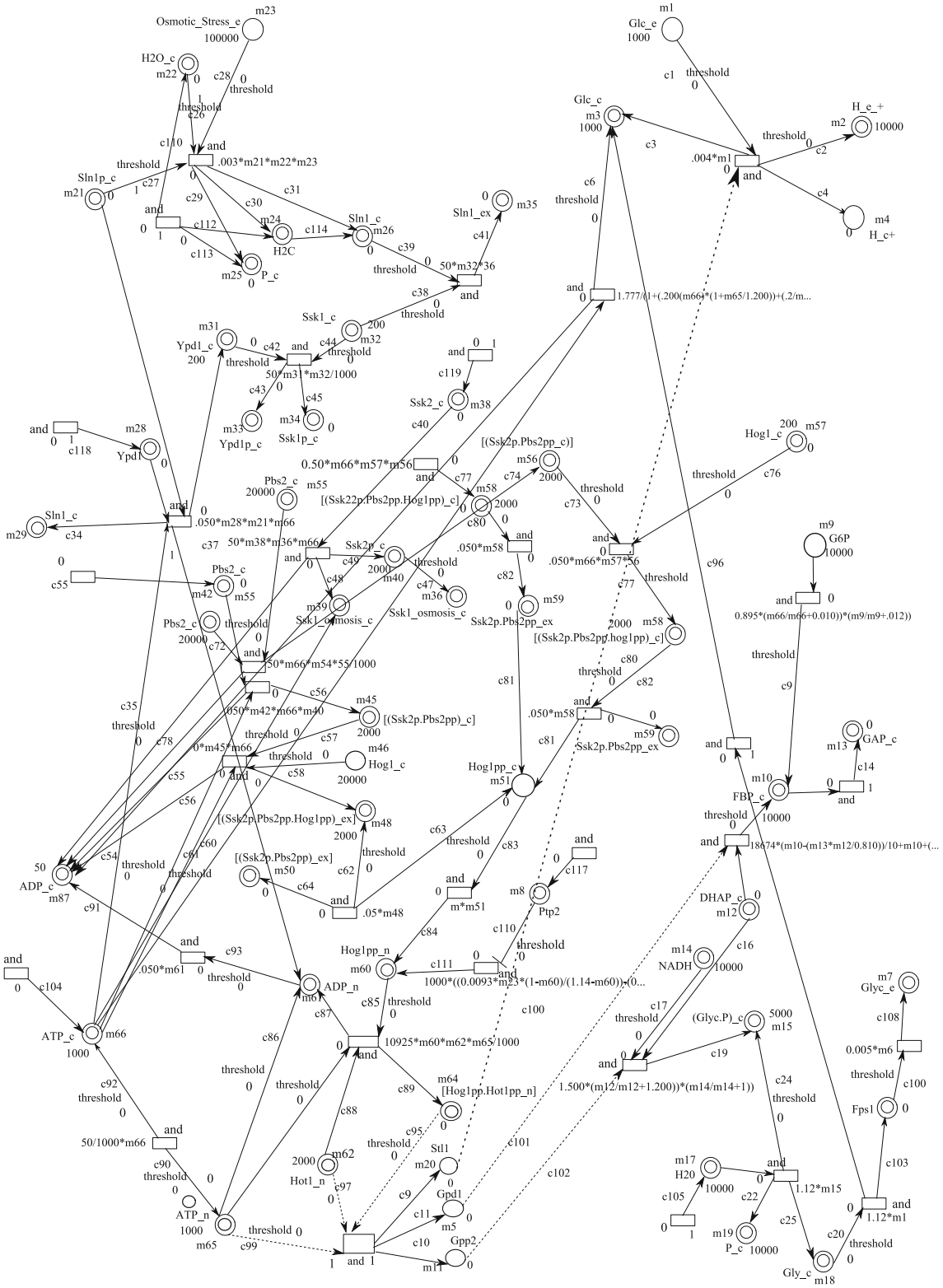


Fig. 6 PN model representation of the integrated *S. cerevisiae* HOG pathway using the software Cell Illustrator Online 5.0. Here c1, c2, c3, ... denote reactions and m1, m2, m3, ... stand for molecules

Table 3 The transition names of the PN model of *S. cerevisiae* integrated HOG pathway

Transitions' names	Biological events
v1_S	Dephosphorylation of Sln1p due to osmotic stress
v2_S	Sln1p transfers a phosphate group to an intermediate protein Ypd1
v3_S	Sln1 frees
v4_S	Ypd1p transfers the phosphate to a response regulator protein Ssk1
v5_S	Ssk1 binds to and phosphorylates the MAPK kinase kinases Ssk2
v6_S	Ssk2p binds and phosphorylates Pbs2, a complex is formed
v7_S	Pbs2pp-Ssk2p phosphorylates and activates the MAPK Hog1, a complex is formed
v8_S	Activated and phosphorylated Hog1pp frees from the Pbs2pp-Ssk2p-Hog1pp complex
v9_S	Phosphorylation of Ssk22
v10_S	Phosphorylation of Pbs2 by Ssk22p, a complex Pbs2pp-Ssk22p is formed
v11_S	Phosphorylation of Hog1 by phosphorylated Pbs2pp-Ssk22p, a complex is formed
v12_S	Activated and phosphorylated Hog1pp frees from the Pbs2pp-Ssk22p complex
v13_S	Hog1pp migrated to nucleus
v14_S	Activation of transcription factor Hot1 by activated Hog1pp, 2 ATPs are required
v15_S	ATP comes inside the nucleus
v16_S	ADP goes to cytoplasm
v17_S	Dephosphorylation of Hog1pp in nucleus by Ptp2
v1_m	Glucose uptake in the cytoplasm
v2_m	Phosphorylation of the glucose by Glk1 in cytoplasm, ATP is used
p27	ATP inside the cytoplasm
v3_m	Formation of Fructose 1,6-bis phosphate, ATP is used
v4_m	Formation of DHAP and GAP from Fructose 1,6-bis phosphate
v5_m	Formation of Glycp, i.e., L-α-glycerol phosphoric acid from DHAP, NADH is used
v6_m	Formation of glycerol in cytoplasm by enzyme Gpp2
v7_m	Cytoplasmic glycerol transferring to Fps1 channel
v8_m	Cytoplasmic glycerol moves outside the plasma membrane through Fps1 channel
p28	H_2O molecule in the cytoplasm
vreg_Stl1	Genes expression, *viz.*, Gpd1, Gpp2 and Stl1

osmotic pressure. Here all the concentrations and time are expressed in μM and units respectively. The results for both non-osmotic and osmotic stress conditions are shown in Figs. 7, 8, 9 and 10.

HOG pathway is found to be stimulated as glucose is added to the cell's medium [11], which leads to an activation of 6-phosphofructo-2-kinase (Pfk2). In our study, the initial concentration of the extracellular glucose has been found to be higher, and then it has decreased under osmotic stress condition (Fig. 9a). This has resulted in increase in cytoplasmic glucose concentration (Fig. 9b). Increased extracellular glucose concentration has uptaken by the cell to make osmotic balance thus we have observed difference between the extracellular and cytoplasmic glucose levels under osmotic condition. While under non-osmotic stress condition, extracellular glucose concentration (Fig. 7a) has been kept constant similar to the concentration of cytosolic glucose (Fig. 7b).

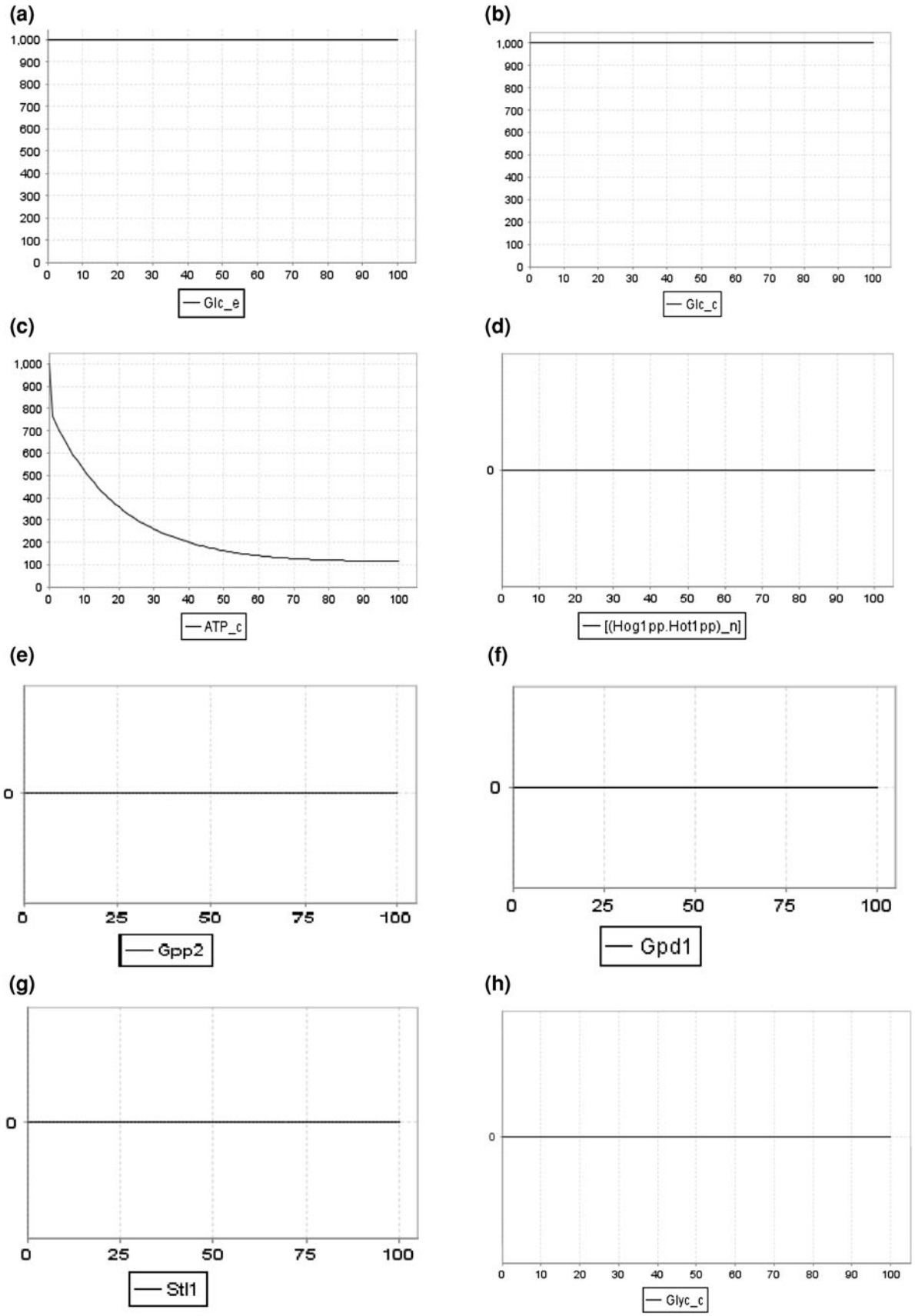
Fig. 7 Variation of concentrations of molecules with respect of time, of *S. cerevisiae* integrated HOG pathway under non-osmotic stress condition; *x*-axis shows time (units); *y*-axis denotes studied molecular concentration (μM); **a** extracellular glucose; **b** cytoplasmic glucose; **c** cytoplasmic ATP; **d** Hog1pp–Hot1pp in nucleus; **e** Gpp2; **f** Gpd1; **g** Stl1; and **h** cytoplasmic glycerol ▶

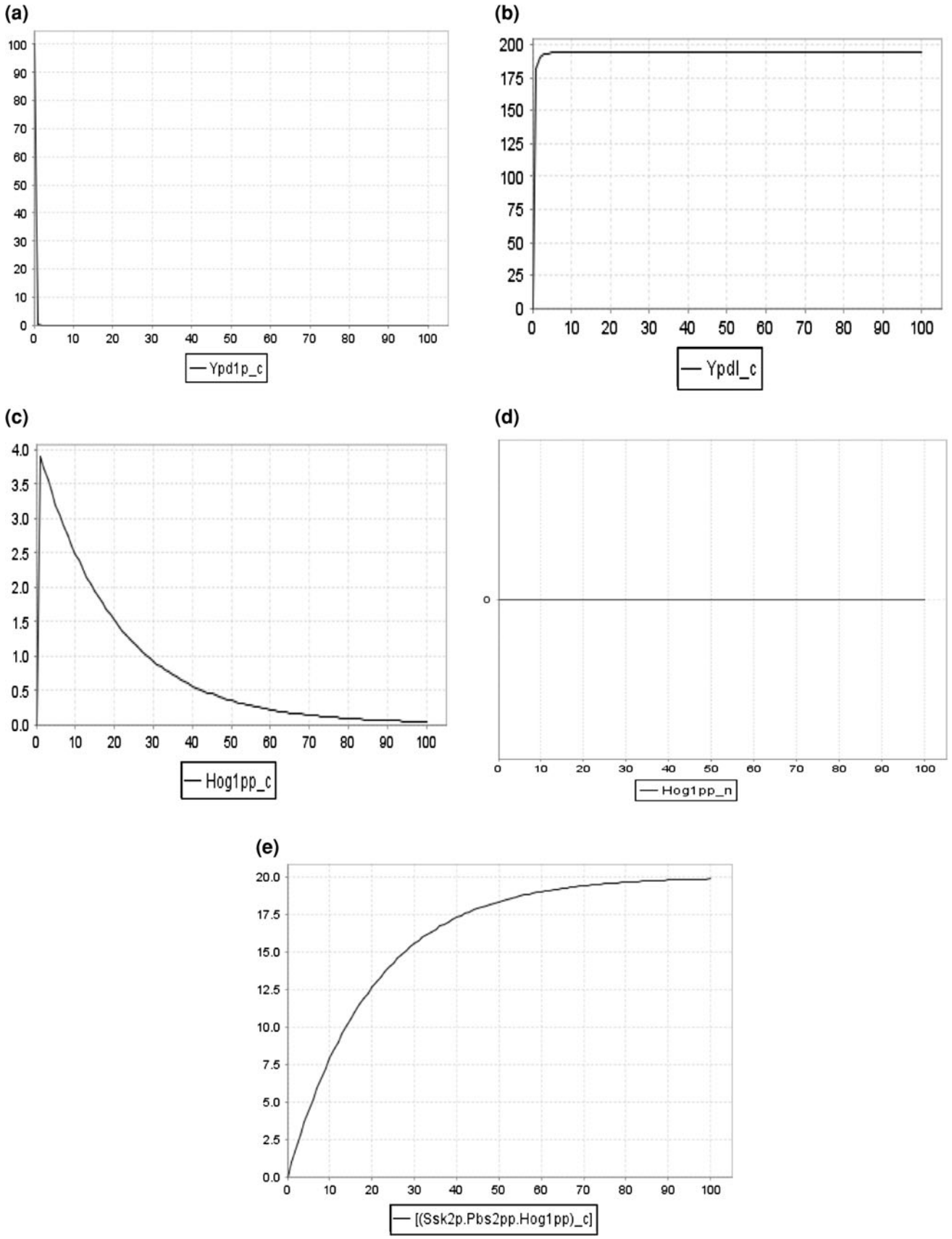
One of the major aspects of this simulation result is the observation of the concentration of the cytosolic glycerol. Under osmotic stress, cytosolic glycerol has increased and accumulated in the cell for a shorter time period to let the cell adapt to a newer environment, and after that, it has decreased (Fig. 9h). We have decreased the simulation time period to mark the glycerol accumulation in the cell. Increased cytosolic glucose is converted into glycerol by the enzymes Gpp2 and Gpd1. Thus we have got the increased surge of glycerol concentration in the cell. This result is closer to the results given in the study done by Lee et al. [34]. Then, intracellular glycerol has been exported out of the cell via Fps1 channel resulting in its lower concentration.

This result has been found to be different from that of the non-osmotic condition (Fig. 7h). This is due to the reason that the yeast cell does not need an extra amount of glycerol in non-osmotic environment and thus we have not observed glycerol formation.

Another important aspect of osmoadaptation in yeast is how the cell controls the biophysical system at the time of osmostress. Since, it is a PN model of an integrated pathway, thus, it has been done through adjusting glycerol production and water influx in the cell. We have not considered cell volume prior to osmoshock, thus Fig. 9i shows markedly dropped cell volume initially, which has increased in the time course during the process of adaptation. This shows that cell has regained its volume and now it has adapted to a new environment. It should be noted here that we have decreased the total simulation time period in order to mark the cell size recovery.

Energy is utilized in order to activate/deactivate the signaling molecules. Hog1 is a mitogen activated protein kinase and needs ATP for catalyzing a reaction. Therefore, we have considered the cytoplasmic ATP concentration in


(a)
1,000
900
800
700
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100
Glc_e
(b)
1,000
900
800
700
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100
Glc_c
(c)
1,000
900
800
700
600
500
400
300
200
100
0
0 10 20 30 40 50 60 70 80 90 100
ATP_c
(d)
0
0 10 20 30 40 50 60 70 80 90 100
[(Hog1pp.Hot1pp)_n]
(e)
0
0 25 50 75 100
Gpp2
(f)
0
0 25 50 75 100
Gpd1
(g)
0
0 25 50 75 100
Stl1
(h)
0
0 10 20 30 40 50 60 70 80 90 100
Glyc_c


(a)
Ypd1p_c
(b)
Ypdl_c
(c)
Hog1pp_c
(d)
Hog1pp_n
(e)
[(Ssk2p.Pbs2pp.Hog1pp)_c]

◀ **Fig. 8** Variation of concentrations of some more molecules with respect of time, of *S. cerevisiae* integrated HOG pathway under non-osmotic stress condition; *x*-axis shows time (units); *y*-axis denotes studied molecular concentration (μM); **a** cytoplasmic (phosphorylated) Ypd1p; **b** cytoplasmic Ypd1; **c** cytoplasmic Hog1pp; **d** Hog1pp in nucleus; and **e** Ssk2p–Pbs2pp–Hog1pp in cytoplasm

the present study. Initially, it has been found to be higher then it has decreased exponentially (Fig. 7c). This is the same for both non-osmotic and osmotic conditions (Figs. 7c, 9c).

Kinases are responsible for activating transcription factors in a signaling pathway. Now, we take the case of the activation of transcription factor Hot1 due to MAP kinase Hog1. We have simulated the concentration of Hog1pp–Hot1pp in nucleus over time for both the aforesaid conditions. The concentration of Hog1pp–Hot1pp complex in the nucleus has been found to be increased under osmotic stress condition (Fig. 9d). This is due to the reason that transcription factor Hot1 needs to be activated by kinase Hog1pp so that genes (Gpp2, Gpd1 and Stl1) can be expressed for glycerol conversion and transport under osmoshock condition. But its concentration have not been found to be increased from the basal value in non-osmotic condition (Fig. 7d) as the cell does not need these enzymes.

Activated Hot1 triggers the transcription of two metabolic genes, viz., Gpd1 and Gpp2. It also triggers the transcription of the gene Stl1 belonging to sugar transporter family. We have observed the concentration of the corresponding proteins in both the aforesaid conditions. It has been found that under osmoshock condition, the concentrations of these three genes have increased initially and then they become stable (Fig. 9e–g). Expression of the genes Stl1, Gpp2 and Gpd1 depends on the Hog1pp.Hot1pp complex in the nucleus. Thus we can relate their concentrations with the concentration of Hog1pp–Hot1pp complex (Fig. 9d–g). Stability of the expression levels of all these genes is also due to the fact that during the adaptation process, cell has accumulated enough glycerol and thus it does not need to have elevated expression of these genes. While under non-osmotic condition, the concentrations of these three proteins remain constant, i.e., at basal conditions (Fig. 9e–g) as there is no question of glycerol formation due to absence of cytosolic glucose.

We have done a comprehensive study of the integrated HOG pathway in yeast. We have included almost all the molecules and complexes present in cytoplasm and nucleus. They are Ypd1, phosphorylated Ypd1, Hog1pp in cytoplasm and nucleus, and Ssk2p.Pbs2pp.Hog1pp complex. Ssk2/Ssk22 (MAPKKK), Pbs2 (MAPKK) and Hog1 (MAPK) are the members of MAPK cascade components in the HOG pathway. Under normal environmental condition, the osmosensor Sln1 is autophosphorylated. Another protein, Ssk1 remains in an inactive phosphorylated state as histidine-containing phosphotransfer protein Ypd1 transfers phosphate to it [39]. Hyper-osmotic shock leads to a decrease in turgor pressure which inhibits the auto-phosphorylation of Sln1 and that increases the concentration of dephosphorylated Ssk1. Dephosphorylated Ssk1 activates Ssk2/22 [52], which then leads to the dual phosphorylation of Pbs2. It further phosphorylates Hog1 on Thr174 and Tyr176. Under normal condition, Hog1 is distributed throughout the cell but under hyperosmotic shock, the dually phosphorylated Hog1 translocates to nucleus [15], and its concentration depends on the severity of the osmotic shock [78]. This is the reason, we have included these molecules in the present study.

The resulting graphs for concentrations of these aforesaid molecules are given in Figs. 8 and 10 under non-osmotic and osmotic conditions respectively. We have got different variation pattern of the concentrations of dephosphorylated states of Ypd1 in cytoplasm under non-osmotic and osmotic conditions. Under osmotic condition, the concentration of phosphorylated Ypd1p has declined in cytoplasm and thus the concentration of dephosphorylated Ypd1 has found to be increased as Ypd1p has phosphorylated Ssk1, a cytoplasmic response regulator (Fig. 10a, b). The concentrations of phosphorylated Ypd1p have been observed to be similar for both the non-osmotic and osmotic conditions (Figs. 8a, 10a), respectively.

The concentration of Hog1pp in cytoplasm has increased initially, then decreased faster to the basal value under osmotic condition (Fig. 10c). Thus the case is opposite for the concentration of Hog1pp in nucleus. It has found to be gradually increased and then become steady (Fig. 10d). This is due to the fact that phosphorylated Hog1p has migrated to nucleus to activate transcription factor Hot1. We have found that the concentration for Hog1pp in cytoplasm has found to be similar both the aforesaid conditions (Figs. 8c, 10c). But its concentration in nucleus has found to be at basal levels (Fig. 8d) as kinase is not required to activate the transcription factor. The concentration of the complex Ssk2p-Pbs2pp-Hog1pp in cytoplasm, under osmotic condition, has decreased as Hog1pp has detached and migrated to the nucleus (Fig. 10e), while that under non-osmotic condition (Fig. 8e) its concentration has been found to be increased then gradually become constant. It is due to the fact that in non-osmotic condition, Hog1pp is not required to phosphorylate Hot1 in nucleus so the complex remains intact in cytoplasm.

Feedback inhibition of the integrated HOG pathway by phosphatase activity

The phosphorylation state of MAPK Hog1 is controlled by various protein phosphatases, such as the phospho-tyrosine phosphatases Ptp2 and Ptp3 [41, 76] as well as the phospho-

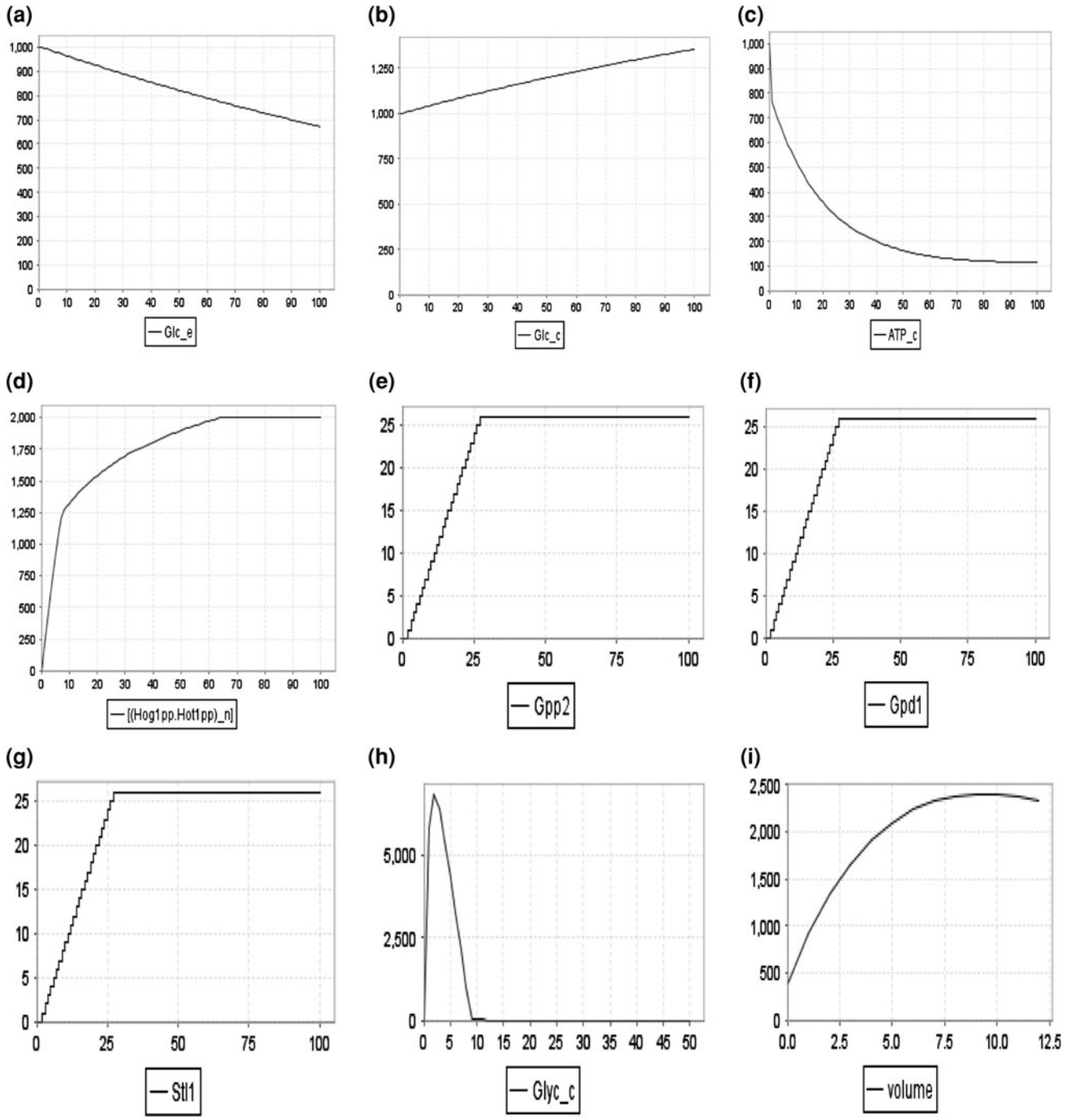


Fig. 9 Variation of concentrations of molecules with respect of time, of *S. cerevisiae* integrated HOG pathway under osmotic stress conditions, *x*-axis shows time (units); *y*-axis denotes studied molecular concentration (μM); **a** extracellular glucose; **b** cytoplasmic glucose; **c** cytosolic ATP; **d** Hog1pp–Hot1pp in nucleus; **e** Gpp2; **f** Gpd1; **g** Stl1; **h** cytoplasmic glycerol; and **i** cell volume

threonine phosphatase Ptc1 (Ptc2 and Ptc3 play a role, when over-expressed) [40]. We have simulated the integrated HOG pathway of *S. cerevisiae* with protein phosphatase Ptp2 to observe its effect on the concentration of phosphorylated Hog1 in the nucleus. We have found a difference between its concentration and time taken to be remained in that stage, in the presence and absence of Ptp2, under osmoshock. The concentration of the cytoplasmic glycerol has been used as a threshold. If glycerol is accumulated in the cell at a required concentration, it leads to the reswelling of the cell. It signals the phosphatase Ptp2 to inhibit Hog1pp in the nucleus. Feedback inhibition due to Ptp2 has resulted in the lower amplitude and faster decline in Hog1pp concentration in the nucleus. Simulation results in the absence

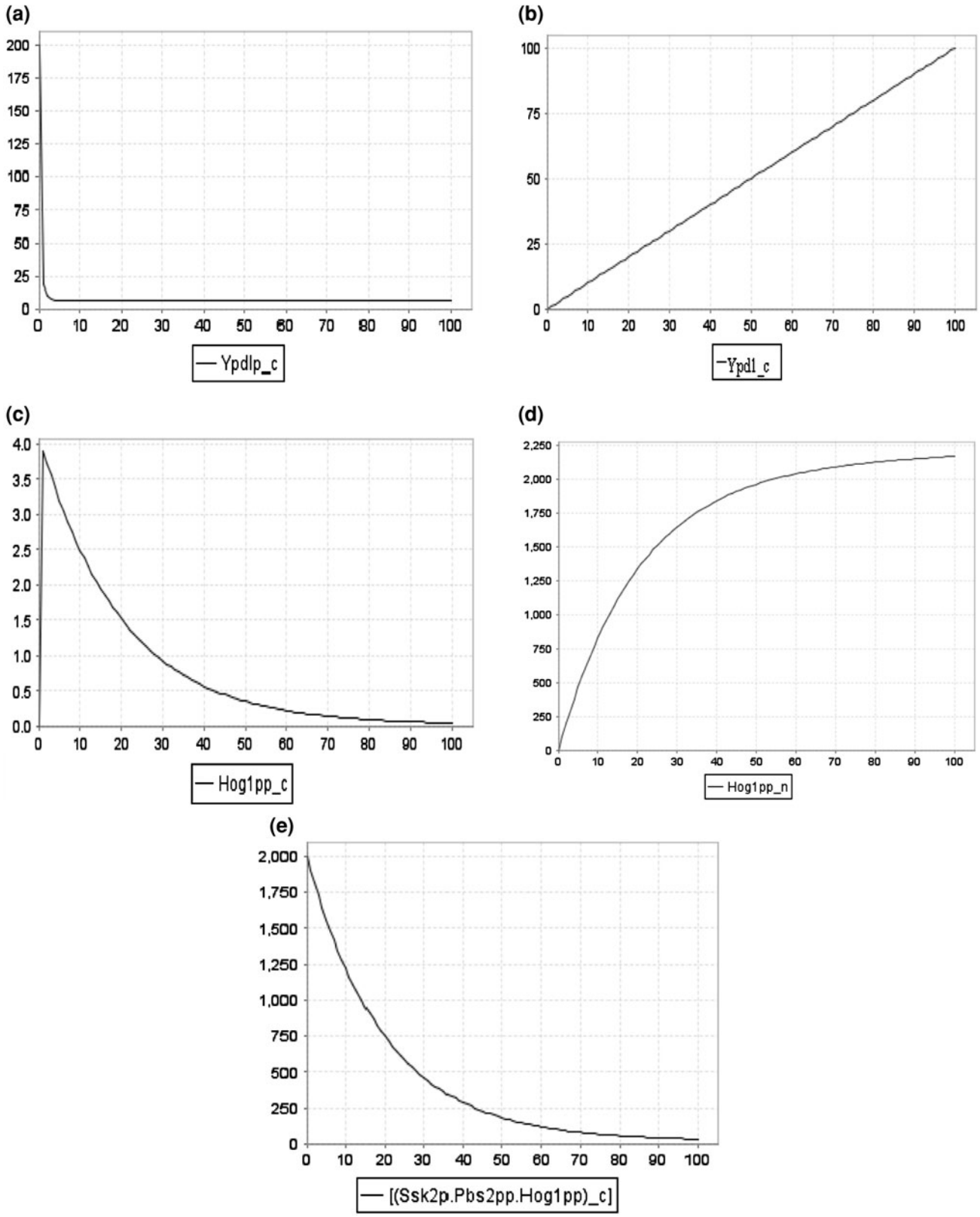


Fig. 10 Variation of concentrations of some more molecules with respect of time, of *S. cerevisiae* integrated HOG pathway under osmotic stress condition, *x*-axis shows time (units); *y*-axis denotes studied molecular concentration (μM); **a** cytoplasmic phosphorylated Ypd1p; **b** cytoplasmic Ypd1; **c** cytoplasmic Hog1pp; **d** Hog1pp in nucleus; and **e** Ssk2p-Pbs2pp-Hog1pp in cytoplasm

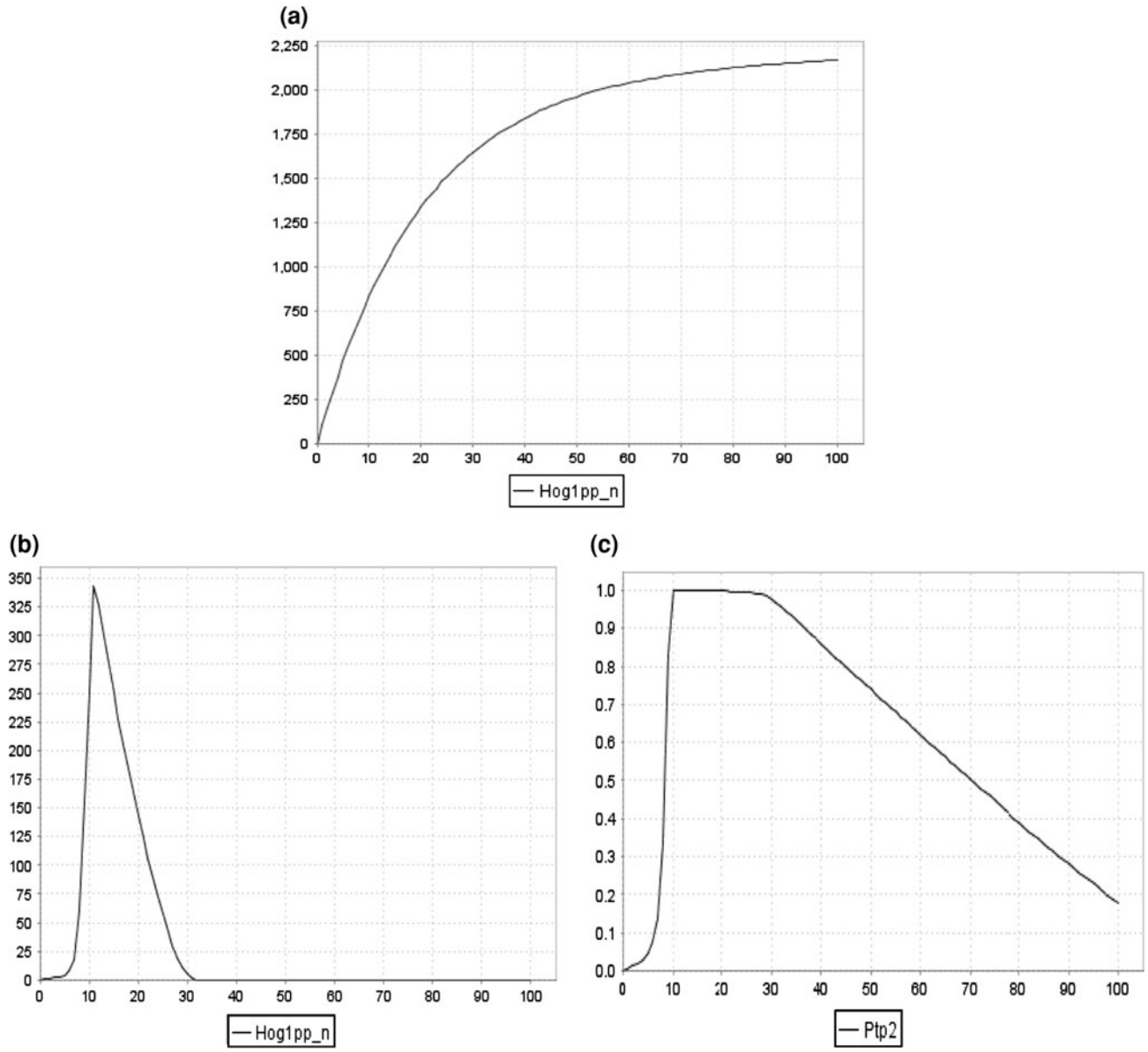


Fig. 11 Effect of enhanced protein phosphatase Ptp2 activity with respect of time, in *S. cerevisiae* cell; *x*-axis shows time (units); *y*-axis denotes studied molecular concentration (μM); **a** Hog1pp concentration in nucleus without the effect of Ptp2; **b** Hog1pp concentration in nucleus inhibited by Ptp2; and **c** Ptp2 concentration

of Ptp2, have shown enhanced and prolonged activation of Hog1pp. This can be observed in Fig. 11a, b. The concentration of Ptp2 has also been shown in Fig. 11c when it has inhibited Hog1pp. The concentration of Ptp2 has been found to be increased initially, but after a small period of time, it has started to decrease due to its consumption for the inhibition of Hog1pp in the nucleus.

Biological validation of the results

Saccharomyces cerevisiae adapts to growth under conditions of increased external osmolarity through stimulation of HOG MAPK pathway. The activation of this pathway ensures the accumulation of a high intracellular concentration of glycerol to reduce the transmembrane difference of osmotic pressure thus prevents the loss of water from the cell [5]. The stimulation of glycerol synthesis is achieved by activating transcription of genes required for glycerol synthesis [2].

Biological validation of the results for the integrated HOG pathway without feedback inhibition

Extracellular glucose concentration is kept constant in the environment in which the cell grows, thus there is no osmotic stress on the yeast cell. In non-osmotic

environmental conditions extracellular and cytosolic concentrations of the glucose have been found to be constant. This is shown in Fig. 7a, b. Under non-osmotic condition, cell does not produce glycerol in excess. This kind of behavior of the cell is due to the fact that metabolic genes, viz., Gpp1 and Gpd2, and the gene coding Stl1 are not expressed under the inactivation of the transcription factor Hot1 as shown in Figs. 7e–g. Thus, additional glycerol is not synthesized 8(H). On the other hand, when we increase the glucose concentration in the cell's environment, it uptakes the glucose. Then, there will be an osmotic disturbance between the cell and its environment. Higher extracellular glucose concentration stimulates HOG pathway [10].

There are two upstream transmembrane osmosensors branches of HOG pathway. They are referred to as Sln1 branch and Sho1 branch. Both branches can independently mediate activation of HOG pathway in response to increased external osmolarity. We have excluded Sho branch from the current study as the activation of Hog1 by Sln1 is more persistent than Sho1 branch. Sln1 transmembrane protein has a cytoplasmic Histidine kinase (HK) domain as well as a Receiver (Rec) domain. Under normal (relatively low) osmotic condition, Sln1 HK domain is kept in the active conformation and phosphorylates a histidine residue in HK domain. The phosphate is then transferred from Histidine to an Aspartate residue in the Rec domain near the C-terminus of Sln1. The phosphate is further transferred to an intermediary phospho-carrier protein Ypd1, and eventually to Ssk1 (an aspartate residue in another Rec domain protein) [54, 77, 63]. We have studied the concentration of Ypd1p (phosphorylated Ypd1) and dephosphorylated Ypd1 in the cytoplasm of osmoshocked cell. The concentration of Ypd1p has declined as it phosphorylates Ssk (Fig. 10a). Thus the concentration of dephosphorylated state of Ypd1 in cytoplasm has been found to be increased (Fig. 10b). However, under non-osmotic condition, Ypd1 follows a different pattern as shown in Fig. 8b but Ypd1p (phosphorylated state) has found to be decreased (Fig. 8a).

Under hyper-osmotic condition, Sln1 responds to changes in cellular turgor pressure, and Sln1 HK domain is altered to the inactive conformation, resulting in a cessation of the Sln1–Ypd1–Ssk1 phospho-relay reaction. Ssk1 is rapidly dephosphorylated by an unknown mechanism; the resulting dephosphorylated Ssk1 binds and activates the redundant Ssk2 and Ssk22 MAPKKKs, which then activates Pbs2 MAPKK [37]. The phospho-aspartate residue in Ssk1 is intrinsically unstable and prone to spontaneous hydrolysis, but it is stabilized when Ssk1 is bound to Ypd1 [31, 30]. Thus, the dissociation of the relatively stable Ssk1-Ypd1 complex may be the first step in the Ssk1-dependent activation of the downstream elements. Dephosphorylated Ssk1 is eventually degraded by the Ubc7-dependent ubiquitinproteasome system, which down-regulates HOG pathway after having the osmotic adaptation [65]. We have monitored the concentration of Ssk2p–Pbs2pp–Hog1pp in the cytoplasm for non-osmotic and osmotic conditions. Our PN model study has shown that the concentration of the Ssk2p.Pbs2pp.Hog1pp has found to be higher initially but gradually decreased under the exposure of osmotic stress in the environment as shown in Fig. 10e. Probably, it is due to the detachment of Hog1pp from this complex and migration to nucleus. While, under non-osmotic condition (Fig. 8e), it has found to be increased and become steady after some time as the complex remains intact in the cytoplasm.

The appearance of phosphorylated Hog1p is a transient event [73, 31]. The instant and period of the response depend on the severity of the shock. When lower dose of 0.4 M NaCl of an osmotic shock is given, Hog1p phosphorylation peaks within 1 min and disappears within about 30 min. With a more severe osmotic shock, e.g., 1.4 M NaCl, Hog1p phosphorylation peaks at about 30 minutes and remains high for several minutes before it declines [75, 29]. These observations illustrate that the pathway is controlled by specific feedback mechanisms. Fig. 10c shows that Hog1pp in cytoplasm peaks due to osmoshock in the cytoplasm, and Fig. 10d depicts the increased concentration of Hog1pp in the nucleus (after migration from cytoplasm to the nucleus). *Saccharomyces cerevisiae* Hog1p kinase is related to the p38 MAP kinase from mammalian cells. Sheikh-Hamad and Gustin [67] have used Immunoblotting technique and have been able to detect dual phosphorylation of Hog1 on T174 and Y176 in its activation loop by the MAPKK Pbs2.

We have described in "Method" section of this article that Hog1 is a kinase which migrates to the nucleus and phosphorylates the transcription factor by making a complex with it. In Fig. 9d, we have observed that the concentration of Hog1p–Hot1p complex has been higher as osmoshock was introduced in the environment. In a wild-type strain, Posas and Saito [53] has shown the influence of Hot1 (transcription factor) upon Hog1p kinase activity. Activation of Hog1p (a MAP kinase) and the inactivation (dephosphorylation) of the same in wild-type cells can be correlated with its translocation to the nucleus. When activated, Hog1pp enters the nucleus and modulates the activity of a transcription activator Hot1. This is the reason that we have observed increased concentration of Hog1pp in the nucleus while its concentration has been decreased in the cytoplasm as shown in Fig. 10c, d. Phosphorylation of Hog1 is necessary for its nuclear translocation, but not its kinase activity, as a catalytically inactive kinase (such as K52M mutant) which can translocate to the nucleus as efficiently as the wild-type, whereas a phosphorylation-defective mutant (T174A T176A) cannot. Moreover,

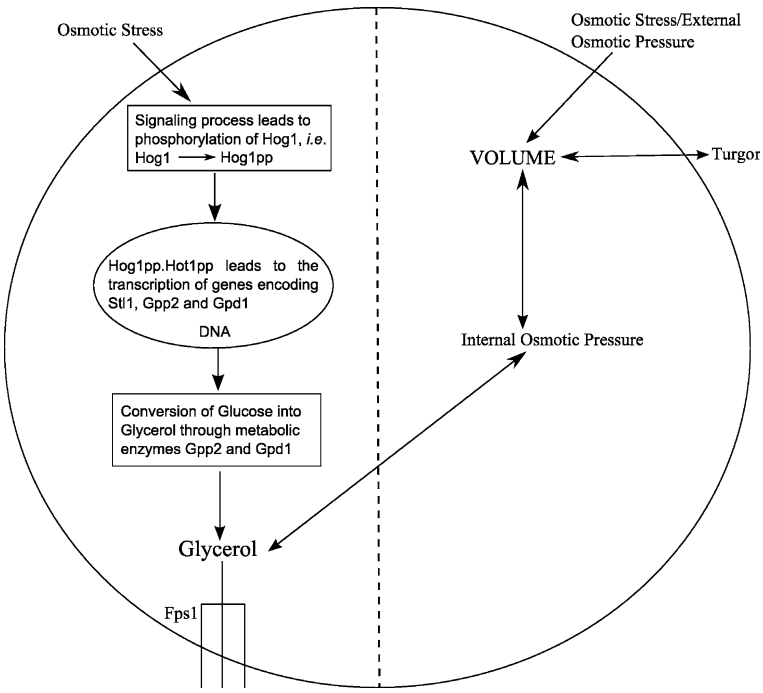


Fig. 12 Overview of osmoadaptation in *S. cerevisiae*. The biological control system is shown at the *left* and biophysical system is shown to the *right* of the *dotted line*. This figure is an adaptation of the figure given in [18] with a few changes

higher concentration of activated Hog1p in the nucleus has been observed if the cell is experiencing osmotic pressure in its medium/environment. This has also been observed in our study (Fig. 10d). In unstressed cells, Hog1 localizes evenly throughout the cell [15, 56]. We have found the similar graph patterns of Hog1pp concentration in the cytoplasm for the both non-osmotic and osmotic conditions (Fig. 8c), respectively. The concentration of Hog1pp in the nucleus has been found at basal value (Fig. 9d) under non-osmotic condition as it is not required in nucleus.

Glucose activates Pfk2 that leads to the activation of the upper part of glycolysis metabolic pathway. This activation is a precondition for glycerol accumulation in the yeast cell. Yeast cells containing Pfk2 accumulate three times more glycerol than those lacking Pfk2, which are not able to grow under hypertonic stress. This is the reason for observing the increasing concentration of cytosolic glucose with time, as cell has uptaken the glucose present in the its medium, as shown in Fig. 9b. According to the observations of Dihazi et al. [11], hyperosmolar extracellular glucose concentration of 1 M leads to the phosphorylation and activation of Pfk2. Pfk2 activation is accompanied by a 20-fold increase of fructose-2,6-bisphosphate concentration. This will lead to the formation of glycerol. Fig. 9h shows a rapid surge of glycerol production, which leads to its accumulation in a osmoshocked cell. It is closer to the graph given in [34]. We have assumed that when a sufficient amount of glycerol will accumulate in the cell and it will adapt to a new condition, glycerol can go out through Fps1 channel. A study [36] shows that a yeast cell has a mechanism to dispose/reduce their osmolytes, specifically their cellular glycerol content very rapidly, i.e., the process is completed within 2–3 min through the Fps1 channel.

Hog1pp–Hot1pp complex will lead to the expression of certain genes coding enzymes. The expressions of metabolic genes (Gpp2, Gpd1) and the gene Stl1 depend on the phosphorylation of transcription factor Hot1. The graphs for the concentration of (phosphorylated) Hog1pp–Hot1pp in the nucleus has shown different patterns under osmotic stress condition as compared to non-osmotic condition. This has been shown in Figs. 7d and 9d. Increased concentration of Hog1pp–Hot1pp in the nucleus leads to the higher expression of genes Gpp2, Gpd1 and Stl1 which can be observed in Figs. 9e–g. After certain period of time, these concentrations become constant, as cell has accumulated required glycerol for osmoadaptation.

Stl1 expression is strictly Hog1 dependent [48] and it has been found that its induction is completely absent in hot1Δ as well as hog1Δ mutants [59]. Ren et al. [57] have listed the values of some upregulated ORFs in saline stress response of *S. cerevisiae*, in which Stl1 has 496-fold induction value. Flow cytometry experiment has shown a Pbs2-dependent 20-fold increase in pStl1-qV reporter expression under osmostress condition. Deletion of either of the two transcription factors Sko1 or Hot1 has strongly reduced pStl1-qV expression [66].

Stl1 protein and the glycerol symporter activity are strongly but transiently induced when cells are exposed to osmotic shock. This expression is transient and dependent on Hog1pp.Hot1pp concentration in the nucleus [59]. The glycerol utilization phenotype study is the basis to analyze glycerol transport in stl1 mutants. Ferreira et al. [14] used

glucose-based complex medium containing 1 M NaCl and 15 mM glycerol to grow wild-type cells and stl1Δ cells. They have found that the deletion of Stl1 has diminished the active transport-driven glycerol accumulation in the cell. The experimental evidence validates the observation of increased concentration of Stl1 that can be related to glycerol accumulation in the osmostressed cell.

Expression of the genes, Gpp2 and Gpd1 also depend upon Hot1 as deletion of Hot1 reduces the mRNA levels of these two genes under osmotic stress by about 50% [7, 17]. The profiles of Gpd1 and Gpp2 mRNA levels after osmotic shock, have revealed that the hog1Δ single mutation has conferred a more severe reduction of the mRNA level and a delay in the response [58]. We can find the similarity between the graphs for genes Stl1, Gpp2 and Gpd1; and Hog1pp.Hot1pp complex to justify the expression of genes Stl1, Gpp2 and Gpd1. (Fig. 9d–g).

The genes encoding the isoforms of NAD-dependent Gpd1 and Gpp2 are differentially expressed and therefore have different physiological roles. Their expression is rapidly and transiently stimulated by an osmotic upshift to approx. 50-fold. The levels of Gpd1p and Gpp2p proteins as well as the specific enzyme activity for both Gpd1 and Gpp2 increase under osmotic stress about 3–10 fold, depending on the severity of the stress [2]. These observations can be compared with the results obtained for non-osmotic stress condition as given in Fig. 7e–g. Gene Gpd1 encodes an NADH-dependent glycerol-3-phosphate dehydrogenase (GPDH). Single gpp1Δand gpp2Δ mutants are not osmosensitive, while the double mutant is as sensitive as a gpd1Δ gpd2Δ mutant [49]. Hence, two isoforms can at least partially substitute each other. The single gpp1Δ mutant accumulates glycerol-3-phosphate but the double mutant accumulates even more; indicating that other phosphatases cannot dephosphorylate this compound. Effects on GPD1 transcript levels, the glycerol production rate and internal glycerol accumulation in the cell were found to be clearly decreased in hot1 mutants as compared to the wild type for a period of about 2.5 h when the osmolarity of the medium was suddenly increased (0.7 M NaCl) [60]. Another study by Albertyn et al. [2] has shown that hog1Δ mutants in osmostress-induced signal transduction has failed to increase GPDH activity and mRNA levels when osmotic stress was imposed. Thus, it is clear that increased concentration of Hog1pp–Hot1pp is necessary in the nucleus when osmolytes are present in the medium as observed in our study (Fig. 9d). But this is different under non-osmotic condition as it has been found at basal values (Fig. 7d).

Several studies have shown that the cell shrinks under osmotic shock and the accumulation of glycerol helps in adaptation to a new environment [50, 12, 42]. It results in an increase in internal osmolarity that leads to water influx and cell size recovery. We have also got the result for cell volume in an osmoshock condition (Fig. 9h), closer to these studies, where we have shown marked decrease in cell volume at the time of osmostress but later cell recovers its size as a part of adaptation process. We have justified this variation in cell volume through intracellular and extracellular glycerol concentration; and its relation with water influx and outflux. The cell has a control system responding to hyperosmotic environment by accumulating glycerol and thereby increasing the intra-cellular osmotic pressure. In this way, it regains its volume and turgor pressure [20, 44]. It has also been found that for a sufficiently large osmotic stress, the turgor pressure is abolished and the volume is markedly reduced [20, 28]. We have shown biological and biophysical changes in a cell during the process of osmoadaptation in Fig. 12, which is an adaptation of a figure given in [18] with a few changes.

We have not only compared our PN simulation results with the static experiments given in literature but also with time course experiments [12, 34]. Edda et al. [12] have monitored the response of a single osmoshock (0.5 NaCl at time 0 min) on the key molecules of HOG pathway. They have got increased concentration of gene Gpd1 and cell size recovery. We have got similar patterns of variation for Gpd1 (Fig. 9f) and volume of cell (Fig. 9i) with respect to time. Likewise, it has been observed from our simulation results that under non-osmotic condition, the variation of extracellular glucose (Fig. 7a), cytosolic ATP (Fig. 7c) and Hog1pp.Hot1pp in nucleus (Fig. 7d) with respect to time have followed that in [34], which is based on kinetic modeling. For osmotic condition, the varying concentration is found to be similar to [34] for cytosolic ATP (Fig. 9c), Hog1pp.Hot1pp in nucleus (Fig. 9d) and intracellular glycerol (Fig. 9h).

Glycerol and other polyols are used as osmoprotectants and taken up by yeast through proton symport. Glycerol can permeate the cell membrane of many organisms by specific members of the aquaporin family, the aquaglyceroporin channel proteins [13]. In *S. cerevisiae*, gene Fps1 encodes a channel-type protein belonging to the major intrinsic protein (MIP) family [36]. Fps1 has been shown to mediate the first-order kinetics of glycerol uptake [47], which is formerly considered as a passive diffusion process [33]. Fps1 regulates glycerol release under osmotic downshift [73] and it has been shown to be closed under osmotic up-shock, thus contributing to faster glycerol retention. Tao et al. [74] have found the elevated intracellular glycerol levels in the fps1 mutant. Mutants lacking Fps1 require more than an hour to achieve the same glycerol loss. Concentration of intracellular glycerol depends on the combination of closing the Fps1 channel (i.e., in prestress condition, k8_m $= 5 \times 10^{-3}\ s^{-1}$, as given in supplementary material of the present article) and glycerol

production. Fps1 closes and hence k8_m is assumed to decrease under osmotic stress [12]. Thus, it is clear that the accumulation of glycerol depends both on the glycerol production capacity and on the ability to close Fps1.

Biological validation of the results for the integrated HOG pathway with feedback inhibition

Activation of Hog1 MAPK is essential for yeast viability under high osmolarity conditions, yet constitutive Hog1 activation is proved to be lethal. It can be caused by sln1Δ mutation or expression of hyperactive Ssk2ΔN, which induces growth arrest and even lethality [77, 79]. The lethality of Hog1 hyperactivation can be suppressed by overexpression of Ptp2/Ptp3/Ptc1/Ptc2/Ptc3, suggesting that these Ptps can dephosphorylate Hog1. Ptp2p is predominantly localized in the nucleus, Ptp3p in the cytoplasm, while the protein phosphatases types 2C are located both in the cytoplasm and in the nucleus. The importance of phosphatases in HOG pathway can also be seen from the observation that disruption of both Ptp2 and Ptc1 results in lethal Hog1 hyperactivation [38]. Overexpression of Ptp2 and Ptp3 suppresses inappropriate activation of HOG pathway which can be conferred by the deletion of Sln1 or constitutive activation of Sln1p, Ssk2p, or Pbs2p. This evidence suggests that these phosphatases target the MAP kinase [29].

For the sake of simplicity, we have not included feedback inhibition by phosphatase Ptp3, Ptc1, Ptc2 and Ptc3. The effect of Ptp2 on the concentration of Hog1pp in the nucleus has been shown in Fig. 11b. We have observed that the effect of Ptp2 has changed the amplitude of Hog1p concentration and can be differentiated by referring to Fig. 11a, b. This result has been found to be in accordance with the simulation result found in supplementary material of Ref. [12]. The expression of Ptp2 after osmotic shock has been observed in a HOG-dependent manner which has been shown in Fig. 11c. This evidence can be used to show that phosphatase Ptp2 is a part of the feedback loop. Active Hog1 enhances the Ptp2 activity [29, 64]. Disruption of gene coding Ptp2 results in constitutive Hog1p tyrosine phosphorylation even in the absence of increased osmolarity. Thus, Ptp2p is a major phosphatase responsible for the tyrosine dephosphorylation of Hog1p [39]. It has been observed that when catalytically inactive $Hog1^{K/N}p$ is expressed in hog1Δ cells, it is constitutively tyrosine phosphorylated. Under osmotic shock, the cell increases its capacity to downregulate the pathway [77]. These observations suggest that the control of glycerol production is an essential event under osmoadaptation and partially controlled by HOG pathway. However, some evidences are also present, which suggests that probably, cell reswelling provides the signal that reverses activation of HOG pathway. This concept is supported by some recent experimental data given in [32] in which authors have shown that cells adapt at different rates depending on the rate of uptake of external osmolytes. Thus, we have used the concentration of cytoplasmic glycerol as a threshold after which phosphatase Ptp2 will start to inhibit Hog1pp in the nucleus.

Taking all these evidences into consideration, we can conclude that the main role of the phosphatases is to constantly counteract HOG pathway stimulation. However, slow pathway downregulation following osmotic shock is found due to slightly enhanced phosphatase expression in mutants which are not able to produce glycerol. Therefore, it appears that downregulation of the HOG pathway is mediated by the successful execution of an adaptation program.

Comparison of the results with the study using idFBA

We have compared our simulation results with the study of [34] using integrated dynamic flux balance analysis (idFBA). In their study, Lee et al. [34] proposed that dynamic and quantitative simulation of cellular behaviors of an integrated systems can be performed using an idFBA algorithm. It counteracts the difficulties faced by implementing FBA directly to an integrated system. Three types of pathways, viz., signaling, metabolic and transcriptional regulatory networks have been integrated and analyzed by idFBA. For signaling networks the objective function is modeled by introducing a binary matrix which incorporates only those reactions which optimize an underlying network objective. For a given reaction, at a specified time instant, the coefficient of the binary matrix I multiplied by the upper bound of the flux yields the rate of that reaction. Time delays can also be incorporated to exclude the reaction for future time instants to maintain the necessary optimized conditions. Owing to variable time-scales, slow reactions can be efficiently handled by the use of two parameters. Considering a long, continuous period of time the parameter indicates the delay time after which the slow reaction is considered to be active, and the parameter indicates how long the reaction continues. They have given more emphasis on the methodology, and thus they have observed and shown the behavior of a smaller number of HOG pathway participating molecules such as glucose, glycerol, Hog1pp–Hot1pp in the nucleus and ATP.

In contrast to this, we have included nine more molecules in our study. The important aspects of our study are the consideration of feedback inhibition by Ptp2, cell volume and Fps1 aquaglyceroporin channel, which have not been included by Lee et al. [34]. Decrease in cell size and its recovery are important features in the study of biophysical quantities, and they relate to elevated intracellular

glycerol level. Fps1 is one of the important molecules of HOG pathway for controlling the glycerol accumulation. Apart from them, the important aspect of our simulation study is the feedback regulation of the HOG pathway. It has been suggested that enhanced expression of genes encoding phosphatases accounts for feedback control. Thus, we have studied the regulation of HOG pathway by incorporating feedback inhibition by phosphatase Ptp2.

We have simulated the integrated HOG pathway of *S. cerevisiae* through PN modeling. We have proposed a stepwise model development via the translation of the biological interactions into logical terms for transcriptional regulatory network (TRN), which in turn has been transferred into net components. We have described a systematic approach to model and analyze the integrated system. PN provides useful unique visualization techniques with possibilities for hierarchical modeling and animation which is important for experimentalists, and for communicating between experimentalists and theoreticians. We have used the Michaelis–Menten kinetic equations to determine the continuous rates of transition firings. PN has been designed, and the dynamics of the system has been simulated and plotted according to time-variation. We have monitored the concentration of the multiple system quantities against a certain time period, many of which have not been included in [34]. We have given a sufficient amount of experimental results which provide the evidence of accuracy of the model.

Discussion

As reconstructions of large-scale signaling and metabolic networks are being done, there is a growing need for the development of a framework to study these networks from an integrated perspective with addition of regulatory molecules responsible for regulating these pathways. Our objective is to model the connection between specific outputs of one network with the inputs of another network in an "ordered" fashion. The complete set of interactions among the biochemical networks, expressed as an integrated system in our study.

We have evaluated the representative integrated yeast HOG pathway using the PN model framework. Through PN model implementation, the phenotypic characteristics of the integrated system are investigated under two conditions, the presence and absence of osmotic pressure present in the cell's-environment. We have aimed to generate a mathematical description of *S. cerevisiae* osmoregulation that can reproduce the experimental observations reasonably well.

We have integrated signaling, metabolic pathways and TRN of *S. cerevisiae* for the present study. Signaling pathways exhibit some special properties, in contrast to metabolic pathways. Signal flow is found in signalling pathways, for example, by phosphorylation and dephosphorylation of signaling molecules, but there is no substance flow like those found in the metabolic pathways. In order to model signalling pathways, one has to work on another abstraction level which can be possible by PN. PN modeling provides a generic description principle which can be applicable to any level of abstraction. These are some of the advantages PN modeling [62].

In this study, we have presented a generic qualitative modeling approach based on the logical expression of the TRN combined with PN representation of the integrated system of the eukaryotic model organism *S. cerevisiae*. Simulations have been taken into account at given time courses for osmotic treatment and non-osmotic treatment conditions. We have observed the changes in the concentrations of multiple biological species for both osmotic and non-osmotic stress conditions. We have also incorporated the regulation of HOG pathway through feedback by phosphatase. In turn, we have studied the concentration of Hog1p in the nucleus both in the presence and absence of Ptp2. We have correlated the simulations results well with experimental data. We have found that the biological information, the model assumptions, and the parameters used to match experimental data appear to be sufficiently accurate for a reliable simulation description. We have also compared our simulation study with a idFBA study given in Ref. [34]. We have monitored the change in the concentrations of multiple biological species over time and also studied the effect of feedback inhibition which has not been considered in Ref. [34].

Studies of the *S. cerevisiae* response to elevated osmolarity have revealed both the simplicity and the complexity of signaling in eukaryotic cells. The progress in the development of the modeling techniques helps in understanding of yeast HOG MAPK pathway might provide insights that are useful to researchers studying even more complex metazoan signaling pathways. As a concluding remark, we can say that this study is a first of its kind in terms of integrating signaling, transcriptional regulation and metabolic processes through implementing PN modeling approach.

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